ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY

Volume 568

HOT TOPICS IN INFECTION AND IMMUNITY IN CHILDREN II

Edited by
Andrew J. Pollard
and
Adam Finn

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Preface

This book is based on the course "Infection and Immunity in Children 2004" which was held at Keble College Oxford, UK in June 2004. This is the second book in this series covering topics in infection and immunity during childhood. These courses, and their companion books, have been put together to provide opportunities for paediatricians in Europe to keep abreast of the latest developments in the field. At the time of writing a third course is already at an advanced stage of planning for June 2005 with a completely new programme once again.

As with the first book, the contributions to this text are brilliant and written by an inspiring group of individuals. We hope this text will direct all readers to strive for excellence in the management of children with infectious diseases.

Andrew J. Pollard and Adam Finn
January 2005

Acknowledgments

We are indebted to all of the contributors to this text who have generously provided their carefully written manuscripts in good time for editing and publication. We are also grateful to the staff of Keble College, Oxford, UK who hosted *Infection* and *Immunity in Children 2004* on which the text is based.

We are particularly grateful to Julia Bremble for administration of the Course, to Sue Sheaf who assisted with the preparation of the edited manuscripts and to our colleagues and families who supported our own activities in this venture. Our gratitude also for the support provided by the European Society for Paediatric Infectious Disease (ESPID), the British Paediatric Allergy Infection and Immunity Group (BPAIIG), and the Royal College of Paediatrics and Child Health (RCPCH). Lastly, we are pleased to acknowledge the generous and unrestricted financial support of Acambis, Aventis Pasteur, Baxter, Chiron Vaccines, GlaxoSmithKline vaccines, and Wyeth Vaccines who made the meeting possible.

The Editors are especially grateful to Nigel Curtis for providing the photographs of the contributors to this volume taken at the course "Infection and Immunity in Children 2004, Keble College, Oxford, UK".

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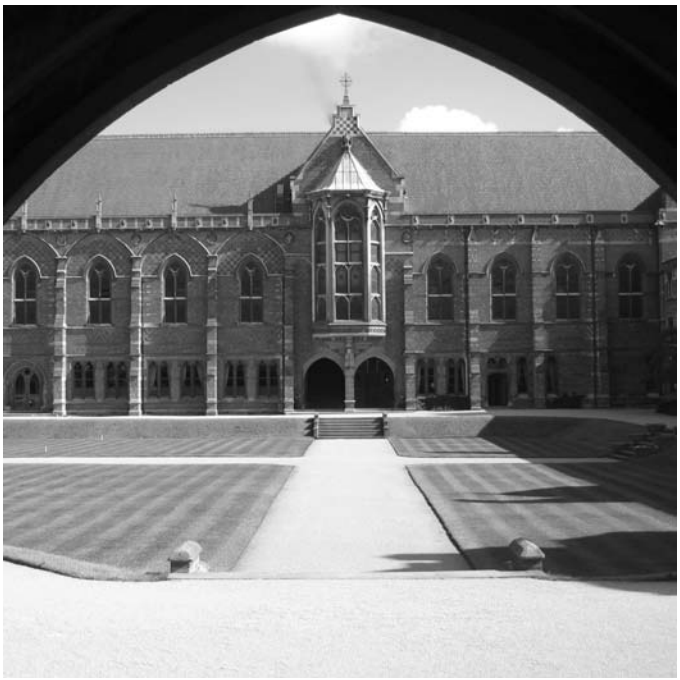

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The Paediatric Infectious Diseases Panel (From Left to Right: David Speert, Sarah Long, Ulrich Heininger, Karina Butler, Ronald De Groot.

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1

Emerging Infections and Children: Influenza and Acute Necrotizing Encephalopathy

Sarah S. Long

1. Introduction

Children can play a variety of roles in emerging infectious diseases. They can be victims, as in the vertical transmission of human immunodeficiency virus infection. They can be affected in a minor way compared with adults, as in West Nile virus infection, legionellosis, and coronavirus infection leading to severe acute respiratory syndrome (SARS). Sometimes, however, they play predominant roles—sources for other age groups—as do toddlers in out-of-the-home child care for transmission of penicillin-resistant pneumococci and cytomegalovirus to family members. Children have a uniquely central role in transmitting the influenza viruses that cause annual seasonal outbreaks and epidemic disease and could cause global epidemic (or pandemic) disease. Children themselves suffer excessive morbidity and mortality from influenza. Continuous minor changes in virus neuraminidase and haemagglutinin components (antigenic drift) or a major change in either (antigenic shift) render naïve children especially vulnerable. Anticipating that pandemic influenza could occur at any time because of a major change in a human strain of influenza virus or through acquisition of human transmissibility of reassortant avian influenza strain(s) (Nicholson et al., 2003), the international community of healthcare experts are collaborating increasingly, and countries are drafting influenza pandemic preparedness plans. A draft by the U.S. Department of Health and Human Services of "Pandemic Influenza Preparedness and Response Plan" was released for comment in August 2004 (http://www.hhs.gov/nvpo/pandemicplan). All plans highlight the critical importance of international surveillance, openness, and rapid response to investigate and contain emergent avian or human strains. In the midst of concern for novel influenza viruses, an apparently novel clinical manifestation of extant influenza virus has emerged recently. Acute necrotizing encephalopathy (ANE) is a new and severe manifestation of influenza in children.

Hot Topics in Infection and Immunity in Children, edited by Andrew J. Pollard, and Adam Finn. Springer, New York, 2005.

2. Epidemiology of Influenza

In prospective studies in the United States (Glezen and Couch, 1978; Neuzil et al., 2002) annual attack rates of influenza illness are between 15% and 42% in preschool- and school-aged children. Globally, it is estimated that 20% of children and 5% of adults have symptomatic influenza illness annually. Influenza is responsible for approximately one-third of excess outpatient visits to healthcare providers for children less than 3 years of age and for approximately one-third of excess prescriptions for antibiotics in the winter season for individuals less than 15 years of age (Neuzil et al., 2000; O'Brien et al., 2004). Antecedent influenza infection is associated with the development of invasive pneumococcal infection and staphylococcal pneumonia in children (O'Brien et al., 2000) as well as in adults.

Children have undue risk for complications of influenza and for hospitalization (Izurieta et al., 2000). Influenza-associated illnesses in children are not restricted to acute respiratory syndromes with systemic complaints or complications, and can involve a variety of organ systems (Table 1.1). Children under the age of 2 years have substantially higher risk of hospitalization than do older children. There are more than 10,000 children younger than 2 years of age hospitalized for influenza annually in the United States; rates of hospitalization range from approximately 200 to 500 per 100,000 population. The risk of hospitalization among children less than 4 years of age in one study was approximately 100 per 100,000 in healthy children and 500 per 100,000 in children with underlying conditions (Neuzil et al., 2000). The overall health impact of influenza in children, and the risk of hospitalization among children younger than 2 years of age, is similar to or greater than that among high-risk children and healthy 50- to 64-year-old adults (both groups for whom annual influenza immunization has been recommended in the United States for years). Recognizing this, universal influenza vaccine was recommended by the American Academy of Pediatrics (AAP) and the Centers for Disease Control and Prevention (CDC) in 2004 for healthy children 6 months to 24 months of age and for household contacts and out-of-home caregivers of all children younger than 24 months of age (Committee on Infectious Diseases, AAP, 2004; Table 1.2). Because of an unexpected shortage of vaccine doses in October 2004, recommendations

Table 1.1 Clinical Manifestations of Influenza Beyond Acute Respiratory Tract Illness

- Febrile illness with vomiting and diarrhea in the absence of respiratory tract symptoms
- Sepsis-like illness (especially in young infants) in the absence of respiratory tract or gastrointestinal symptoms
- Myocarditis
- Bilateral gastrocnemius myositis
- Rhabdomyolysis
- Invasive group A streptococcal, pneumococcal and meningococcal infection
- Toxic shock
- Post-influenza asthenia

Table 1.2. Recommendations by the American Academy of Pediatrics, 2004, for Influenza Immunization

I.	High-risk children and adolescents with the following conditions • Asthma or other chronic pulmonary disease • Hemodynamically significant cardiac disease • Immunosuppressive disorders or therapy • Human immunodeficiency virus infection • Sickle cell anemia and other hemoglobulopathies • Disorders requiring long-term aspirin therapy (e.g., rheumatoid arthritis and Kawasaki disease) • Chronic renal dysfunction • Chronic metabolic disease (e.g., diabetes mellitus)
II.	Women who will be in second or third trimester of pregnancy during influenza season
III.	Persons who are in close contact with high-risk children. These include: • All healthcare professionals in contact with children in hospitals and outpatient settings • Household contacts and out-of-home caregivers of high-risk individuals of any age
IV.	Young, healthy children 6 through 23 months of age (because of excessive risk of hospitalization, morbidity, and mortality)
V.	Household contacts and out-of-home caregivers of children younger than 24 months of age[a]
VI.	Any person older than 6 months of age who (or whose parent) wishes to be protected against influenza[a]

[a] Because of shortages of influenza vaccines that occurred in October 2004, the CDC and AAP have altered these recommendations temporarily. Regarding healthy contacts of healthy children (Recommendation V), only those children under 6 months of age (too young to be immunized) are recommended to receive vaccine. Other healthy individuals to whom these recommendations apply (Recommendation VI) were asked to defer immunization in order to channel available vaccine to high-risk individuals and their contacts (CDC, 2004a, b).

were modified temporarily to include healthy contacts only for infants under 6 months of age (who are themselves too young to be vaccinated) (CDC, 2004a, b).

3. Mortality from Influenza

Death from influenza has not been a reportable disease in the United States. However, more than 150 influenza-associated deaths among children under 18 years of age were reported to the CDC in the 2003–2004 influenza season. This voluntary reporting probably reflects a gross underestimate. Death due to influenza has been made a reportable disease for 2004–2005. Preliminary data from the 2003–2004 fatal cases show a median age of approximately 3 years; two-thirds were less than 5 years of age. Approximately one-half of the children were previously healthy, the others having a wide variety of underlying conditions, especially involving the central nervous system. Deaths did not occur in fully immunized children. In more than one-quarter of fatalities, death was rapid, occurring either at home or in a hospital emergency department. Causes included viral and bacterial pneumonia, invasive bacterial infection, cardiorespiratory deaths, and central nervous system syndromes. An emerging disease, influenza-associated ANE was one cause of mortality and significant morbidity.

4. Influenza-Associated Acute Necrotizing Encephalopathy

4.1. An Illustrative Case

A 3-year old US-born, previously healthy male of Indian descent was brought to medical attention in February 2004 because of lethargy and abnormal tongue movements. From 3 weeks until 1 week prior to admission, he was traveling in India with his family where he lived rurally with extended family members. He was exposed to multiple dogs, cows, and birds. He drank unpasteurized milk and had many insect bites. He took no malaria prophylaxis. Three days prior to admission he had sudden onset of fever to 39.0°C, vomiting, diarrhea, and abdominal pain. On the day of admission he was sleepy, unwilling to rise from his bed, and had unusual tongue movements.

Physical examination revealed a well-grown boy who was difficult to arouse. He appeared to understand some commands but was mute. Vital signs were normal as was the general physical examination. Function of cranial nerves were normal as far as could be assessed as was fundoscopic examination. He had generalized tremulousness (also affecting his tongue) and increased tone on the left side. Full blood count, electrolyte and metabolic screening tests, and chest radiograph were normal. Electroencephalogram showed continuous epileptiform discharges. Cerebrospinal fluid was acellular with protein concentration of 0.6 g/l and normal glucose concentration. Magnetic resonance imaging of the brain (see Figure 1.1) was abnormal.

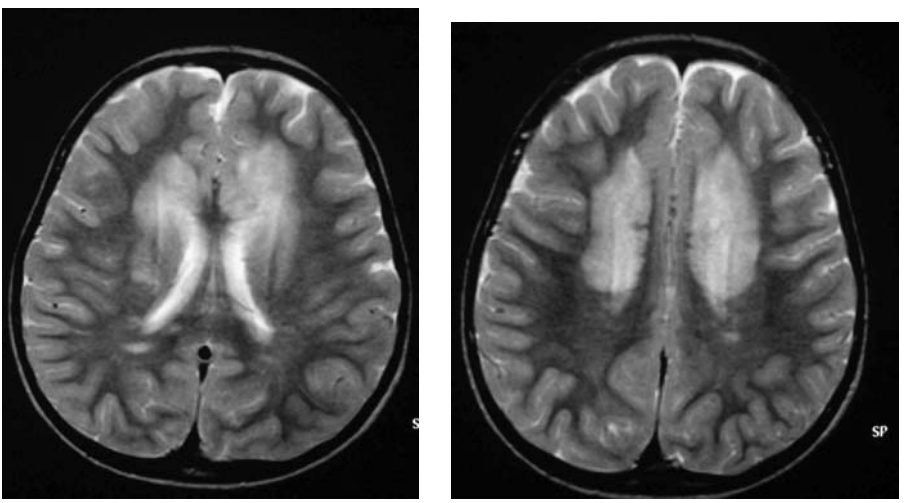

Figure 1.1. Appearance of T2-weighted images of the brain show increased signal intensity bilaterally and symmetrically in the periventricular and deep white matter (on the left) and the corona radiata (on the right). T1-weighted images were not remarkable. There was no enhancement after administration of gadolinium. Images are courtesy of Eric Faerber, M.D., Department of Radiology, St. Christopher's Hospital for Children, Philadelphia, USA

The differential diagnosis was lengthy, especially considering exposures. Multiple tests for possible aetiological infectious agents including bacterial cultures, antigen-detection tests, antibody tests, blood smears, and polymerase chain reaction assays were negative. Influenza B was isolated from the respiratory tract. The virus was subsequently identified by the CDC to be Sichuan group/Shanghai-like. Polymerase chain reaction testing for influenza was performed on the patient's cerebrospinal fluid at the CDC and was negative.

Despite all of this patient's exposures in India to what would be rather exotic infectious agents in the United States, he turned out to have an unusual manifestation of the most common infection (influenza) in the United States and Europe at that time. Incubation period would be compatible with acquisition during transcontinental travel or immediately upon return to the United States. More than 90% of influenza viruses isolated in the United States in the 2003–2004 season were type A. Type B accounted for the remainder; Sichuan group/Shanghai-like was the most common type B virus isolated.

Diagnosis was highly compatible with acute necrotizing encephalitis. Hospital course was protracted and complicated, with major problems of seizures, stupor, mutism, and left hemiparesis. Therapeutic manoeuvers included administration of oseltamivir, intravenous immunoglobulin, and solumedrol. None had immediate temporal benefit, but at the time of transfer to a rehabilitation hospital after 21 days of hospitalization under intensive care, he had increasing periods of alertness, appeared to recognize parents, demonstrated visual tracking, and made guttural sounds. Eight months after the illness, he has recovered remarkably, with mild residual hemiparesis and difficulty in speech and language.

4.2. History of Emergence of Acute Necrotizing Encephalopathy

In pandemics of influenza, as in 1918 and 1930, *Encephalitis lethargica* was a frequent cause of death. As gleaned from descriptions, the disease probably was in some cases influenza virus encephalitis and in others post-infectious encephalitis. We recognize both syndromes as occurring currently, sporadically during annual influenza seasons. In addition, rarely, hemorrhagic shock and encephalopathy, as well as Reye's syndrome can be associated with influenza, the latter especially but not always in conjunction with use of aspirin. The CNS disease represented by our case, ANE, was first described in Japanese children in 1995. By 1998, 148 cases of ANE were reported in Japan (Morishima et al., 2002) and it was estimated subsequently that more than 100 deaths were occurring annually in Japanese children from influenza-associated ANE (Kasai et al., 2000). Emergence of ANE in Japan occurred the year after cessation in 1994 of routine annual influenza immunization in children, a policy that had been in place since 1960s. Influenza A and B viruses were associated with direct proportion to causation of uncomplicated respiratory tract infection. In the United States, physicians were alerted to ANE in late 2003 and were encouraged to report cases to the CDC. More than 103 possible cases were reported. At this time, the reports of only a minority of cases meeting the screening case definition of having proven influenza plus more than 24 hr of altered mental status have been

Table 1.3. Clinical Characteristics of Influenza-Associated Necrotizing Encephalopathy

- Clinical
  - Age <5 years, rarely >10 years
  - Onset *during* the peak of the febrile illness (fever ± cough, vomiting)
  - Presentation with seizures
  - Presentation with altered mental status
  - Propensity for finding of akinetic mutism
  - Multiorgan failure that *follows* CNS symptoms
  - Disseminated intravascular coagulopathy that *follows* CNS symptoms
  - Unremarkable severity of influenza symptoms
  - Unremarkable complete blood count, serum chemical and hepatic enzyme tests
  - Normal cerebrospinal fluid (may have increased pressure, or protein elevation <100 mg/dl)
  - Negative polymerase chain reaction test for influenza on CSF
  - Autopsy → edema, apoptosis/necrosis but no inflammatory cells
- Cranial magnetic resonance imaging
  - Diffuse, bilateral, symmetric, high-intensity signal on T2-weighted images
  - Propensity for involvement of periventricular and deep white matter (thalamus, brain-stem tegmentum, cerebellum, or medulla)
  - No enhancement with gadolinium

investigated. The median age of cases is 4.5 years (1 month–17 years). States of children's residences have been broad. One-half have had seizures. One-half have made a full recovery while approximately one-quarter died and one-quarter have significant neurological sequelae.

4.3. Clinical Manifestations of ANE

As currently recognized, the course of ANE is characteristic (Table 1.3). Within 12–48 hr of onset of the febrile illness in an unimmunized child, the child has abrupt onset of seizures or mental status change or both. Findings range from obtundation to coma. Specific neurological abnormalities are not universal, but speech abnormalities and akinetic mutism occur frequently (Newland et al., 2003). Cerebrospinal fluid is acellular with normal glucose and protein concentration, or mildly elevated protein concentration. Multiorgan system failure can occur in some cases and *follows* rather than precedes onset of CNS symptoms and signs. Thrombocytopenia and severely elevated serum aspartate aminotransferase levels (AST > 1,000 IU/l) were associated with poor prognosis in Japanese children (Morishima et al., 2002). This disease is distinct from Reye's syndrome, which is encephalopathy with fatty degeneration of the liver and is associated with specific clinical and pathological abnormalities of the liver, hypoglycemia, and hyperammonemia.

Magnetic resonance imaging of the brain on T2-weighted images in ANE shows high-intensity signal diffusely in the periventricular and deep white matter bilaterally—characteristically affecting the thalamus, the brainstem, and the cerebellum. Autopsy of fatal cases has been remarkable, showing apoptosis of neurones with edema but no inflammatory infiltrate or vasculitis.

4.4. Pathogenesis of ANE

The pathogenesis of influenza-associated acute encephalopathy is unknown, but investigations in Japan have led to credible speculation. Influenza virus infection is necessary, but ANE is an uncommon event among infected individuals. While virus is present in the respiratory tract in ANE cases, viral RNA can be detected in cerebrospinal fluid of $<10\%$ of affected children, and the presence of virus in brain tissue in autopsied cases has not been proven despite testing by culture and gene amplification. This with the absence of inflammatory cells in the CSF or in samples of brain tissue suggests that direct viral invasion leading to tissue damage is unlikely to be the cause of CNS disease. On the other hand, in influenza-associated encephalopathy, elevated concentrations in the serum or CSF or both of several pro-inflammatory cytokines and cytokine receptors—interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF) alpha, soluble TNF receptor-1 (sTNF-R1)—have been documented, with elevations in proportion to the clinical severity of disease (Ichiyama et al., 1998; Togashi et al., 1999; Aiba et al., 2001). In 2003, Kawada et al. further demonstrated up-regulation of IL-6, IL-10, and TNF-alpha genes in patients with influenza-associated ANE, strengthening the postulation that glial cells in the CNS are cytokine releasing cells (similar to blood macrophages) that can overproduce pro-inflammatory cytokines in response to respiratory tract influenza virus, causing a "cytokine storm" in the CNS.

Mouse inoculation studies of human and avian recombinant influenza virus (human influenza not being pathogenic in mice) performed two decades ago may be pertinent (Reinacher et al., 1983). Intranasal inoculation causes viral spread to the olfactory bulbs, then to the trigeminal ganglion, and later to the brainstem, pons, cerebellum, reticular formation, and posterior colliculi. Virus subsequently spreads to the respiratory tract and bloodstream. Following intraperitoneal inoculation, virus spreads predominantly to the visceral organs, lungs, and bloodstream with no involvement of the midbrain or brainstem and only occasional evidence of virus presence in the CNS, in the lining of the ventricles. In a further experimental step, passively transferred antibody in high levels protected against both routes of inoculation.

The postulated route of influenza virus spread in children with ANE is the olfactory tract, which then could lead to access and stimulation of a CNS glial cytokine response. Occurrence of ANE in unimmunized children; the timing of encephalopathy early in the course of the influenza illness; the site, symmetry, and pattern of magnetic resonance imaging abnormalities; and inability to detect virus in serum or CSF of most affected patients, all support this pathogenetic sequence. It is further speculated that cytokine-induced disruption of the blood–brain barrier could lead additionally to "backward flow" of accumulated cytokines into the circulation to cause the systemic inflammatory response syndrome (SIRS) that appears to follow CNS malfunction in some patients (Morishima et al., 2002). At neuronal level, these aberrant responses lead to mitochondrial respiratory failure, edema, and apoptotic and necrotic cell death (Yokota et al., 2000).

If validated, what new virus-associated, host-associated, or environmental factor might be postulated to explain the emergence of influenza-associated cytokine deregulation that leads to ANE? No single influenza strain or unusual epidemic was

identified in association with ANE in Japan. Emergence of ANE after cessation of routine immunization may indicate importance of rapid spread or primary infections in children. First recognition in Japanese children suggested a possible genetic predisposition, which has not been discounted. However, description of cases in the United States and anecdotally in many European countries, notably in children of non-Asian ancestry, makes a racial genetic predisposition unlikely. It is likely that a changing environmental factor may be responsible. In Japan, use of non-steroidal anti-inflammatory drugs (NSAIDs) (especially diclofenate sodium and mephenamate) in febrile children was common by the mid-1990s, and NSAID use has recently become common in the United States. The NSAIDs belong to the class of medications known as cyclooxygenase (COX) inhibitors. They have antipyretic, analgesic, and anti-inflammatory effects. Antipyretic effect is attributable predominantly to inhibition of COX-derived prostaglandin E2. Direct anti-inflammatory effect of suppressing leukocyte–endothelial cells interactions, as well as causing fever-dependent and—independent dysregulation of inflammatory mediators could cause aberrant host–virus interactions. Does NSAID use in the context of early uninhibited virus replication in the nose precipitate or facilitate the CNS "cytokine storm"? Currently, a case–control study is ongoing in Japan to clarify the relationships between these drugs and the occurrence and severity of ANE. Meanwhile, pediatricians in Japan, highly attuned to influenza-associated ANE, now advise against the usage of NSAIDs in children with suspected influenza.

4.5. Suggestions

Collection of more clinical, epidemiological, and laboratory data, as well as performance of case–control studies of environmental exposures are urgently needed to attempt to understand this highly morbid, emerging disorder. If an environmental exposure such as NSAID use is identified, a remarkable opportunity for prevention exists. With the early imperfect evidence of possible associations of aspirin with Reye's syndrome, this clinician advised against aspirin use in children. Not fearful that failure to provide aspirin then or NSAIDs now would negatively impact upon children, it seems prudent to advise parents of children with possible influenza to (1) provide comfort, (2) avoid use of NSAIDs, and (3) give acetaminophen or paracetamol judiciously and carefully. There are decades of history of non-deleterious effect of these agents on the course of influenza in children. However, there are also reports of severe hepatic toxicity and fatality from inadvertent overdose of acetaminophen, especially in infants and during viral illnesses (Committee on Drugs, AAP, 2001). The most effective means of preventing influenza-associated morbidities is the widespread use of influenza vaccine as recommended in children and their caretakers in the healthcare system, at home, and in the community.

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2

Chickenpox Party or Varicella Vaccine?

Sophie Hambleton and Ann M. Arvin

1. Introduction

Chickenpox (varicella) is generally a much milder illness in children than in adults, with considerably lower rates of severe disease and death (Seward et al., 2000). Varicella is also virtually universal in many populations, meaning that very few individuals escape infection over a lifetime. Thus, a sound logic underlies the idea of chickenpox parties, at which susceptible children can acquire the contagious causative pathogen, varicella zoster virus (VZV), from their peers. However, chickenpox is not without risks, even for children of this age; severe, complicated, and occasionally fatal varicella occur in previously healthy children, as well as the immunocompromised (who are at very considerable risk) (Meyer et al., 2000; Galil et al., 2002a). There is an alternative in the form of a live attenuated vaccine that is both safe and effective in preventing varicella, particularly severe disease (Arvin and Gershon, 1996). In the United States, routine administration of this vaccine to young children has been advised since 1995 (Committee on Infectious Diseases, 1995; Centers for Disease Control, 1996). The European Working Group on Varicella recently advocated a similar strategy, provided a very high rate of coverage can be achieved (Rentier and Gershon, 2004). In the following article, we review the US experience of varicella vaccination, focusing on the consequent, profound changes in epidemiology and the challenges that may result over the coming years.

2. VZV Disease in Unvaccinated Populations

The human α-herpesviruses, herpes simplex viruses 1 and 2, VZV, and their precursors, have been coevolving with primates for over 70 million years, and represent supremely successful pathogens. The case of VZV illustrates the intriguing strategy of these viruses, since most individuals acquire lifelong VZV infection in childhood, with most viruses existing in latent form in the human population.

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Latent infection of sensory neurons in the dorsal root and trigeminal ganglia is established during the course of the brief and highly contagious primary illness, varicella (chickenpox) (Kennedy, 2002). Reactivation from latency can produce zoster (shingles), with the reemergence of infectious virus in skin vesicles and attendant possibility of spread to new hosts (Hope-Simpson, 1965; Gnann and Whitley, 2002). Thus, despite the tendency of VZV epidemics to exhaust the supply of naïve hosts, a large pool of virus is maintained with the potential to reinitiate the cycle of infection.

Varicella has long been known as one of the six exanthemata of childhood and this characterization has been amply confirmed in epidemiologic studies of unvaccinated populations living in temperate climates (Wharton, 1996; Seward et al., 2000). In contrast to the other human herpesviruses, primary VZV infection is almost always symptomatic if the exposed susceptible child is examined carefully at the end of the incubation period. In the landmark work of Hope-Simpson, almost 90% of children were noted to have experienced varicella by the age of 10 (Hope-Simpson, 1952). More recent studies have pointed, if anything, to a lowering of the average age at acquisition of chickenpox into the pre-school years, postulated to reflect the greater participation of these younger children in group care in the Western industrialized societies (Brisson et al., 2001). Currently, only 1% of US-born adults are believed to be varicella-susceptible. In contrast, varicella in tropical countries shows a lower incidence in children, with the result that many more adults remain susceptible (Seward et al., 2000).

Varicella is generally thought of as a mild illness, with its main symptoms being the hallmark pruritic vesicular rash, fever, and malaise. However, serious and occasionally fatal complications occur with significant frequency, particularly in neonates, adults, and the immunocompromised (Guess et al., 1986; LaRussa, 2000). These include secondary bacterial infections of skin and lung together with pneumonitis, encephalitis, and cerebellar ataxia. In the United States, these and other varicella-associated complications caused approximately 11,000 hospitalizations and 100 deaths annually prior to the introduction of varicella vaccination in 1995 (Meyer et al., 2000; Galil et al., 2002a). Despite the higher case-fatality in the above-mentioned risk groups, many deaths and severe complications occurred in previously healthy children due to their high incidence of varicella (Figure 2.1) (Peterson et al., 1996; Meyer et al., 2000).

Zoster is relatively uncommon in children but its incidence climbs steadily with age (Donahue et al., 1995); this is believed to reflect declining cell-mediated immunity (Berger et al., 1981; Levin et al., 2003b). Individuals with cellular, but not humoral, immunodeficiency are at increased risk for VZV reactivation and for dissemination (to skin beyond one or two contiguous dermatomes) and/or visceral disease (which is occasionally fatal) (Oxman, 2000; Gershon, 2001). Most zoster, however, consists of the classic vesicular rash in a unilateral dermatomal distribution and with accompanying pruritus and neuropathic pain (Gnann and Whitley, 2002). Children rarely experience troublesome pain, but the opposite is true in the elderly, many of whom go on to suffer debilitating postherpetic neuralgia (PHN) over the ensuing weeks and months (Gilden et al., 2000; Edmunds et al., 2001).

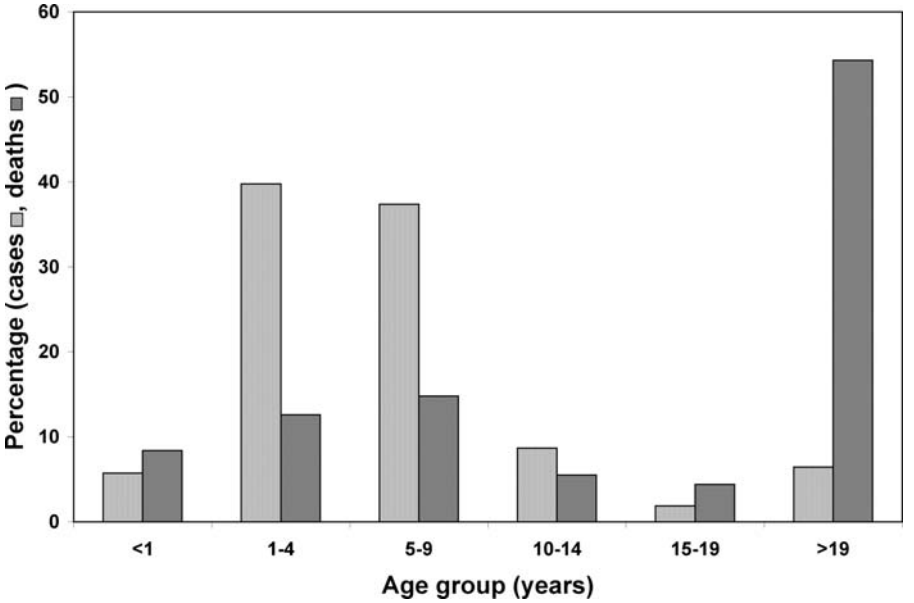

Figure 2.1. Proportions of varicella cases and deaths by age, 1990–1994 (pre-vaccine). Note that the majority of cases occurred in children, along with significant numbers of deaths despite a much lower case-fatality ratio than adults. Data are drawn from Meyer et al. (2000). *J. Infect. Dis.* 182, 383. (With permission from University of Chicago Press, © 2000 by the Infectious Diseases Society of America. All rights reserved.)

3. Varicella Vaccine

3.1. Safety, Immunogenicity, and Efficacy

Childhood deaths due to varicella were a major factor in stimulating the development of a live attenuated vaccine, which was accomplished in Japan in the 1970s. A clinical isolate ("Oka") was passaged through human and non-human cells in tissue culture, resulting in the accumulation of numerous mutations (Gershon et al., 2004). Collectively, these confer an attenuated phenotype as reflected by reduced replicative capacity in the SCID-hu mouse model and, under certain conditions, in tissue culture (Moffat et al., 1998; Arvin, 2001).

Ample evidence has now accumulated to suggest that the vaccine is also highly attenuated with respect to human hosts, less than 2% of whom develop rash following subcutaneous inoculation (White et al., 1991). Extensive post-licensure surveillance in the United States has disclosed that other adverse symptoms are rare, and complications typical of varicella (encephalitis, cerebellar ataxia) have not been linked to the vaccine (Wise et al., 2000; Sharrar et al., 2001). Immunocompromised individuals are at higher risk (40%) for vaccine-associated rash and, when present, this was associated with secondary cases in early vaccine trials among children in remission from leukemia (Tsolia et al., 1990). Hence, vaccination of the immunocompromised is contraindicated in the United States (with the exception of minimally

symptomatic HIV infection and pure immunoglobulin deficiencies) (Centers for Disease Control, 1996, 1999). However, experience to date suggests that dissemination of vaccine strain varicella occurs only in the context of profound cellular immunodeficiency (unsuspected at the time of vaccination) (Ghaffar et al., 2000; Kramer et al., 2001; Sharrar et al., 2001; Gershon, 2003; Levy et al., 2003; Levin et al., 2003a).

Pre-licensure trials in several thousand susceptible individuals showed that a single dose of vaccine was around 70–90% efficacious against varicella in healthy children (somewhat less so in adolescents and adults), over follow-up periods of 1–10 years (Weibel et al., 1984; Gershon et al., 1988; Kuter et al., 1991; White et al., 1991; Johnson et al., 1997; Kuter et al., 2004). Rates of breakthrough varicella varied considerably between studies. Breakthrough varicella is caused by wild-type VZV but is usually considerably milder than disease in the unvaccinated, indicating partial vaccine-induced immunity. Vaccine immunogenicity, generally measured by antibody response to viral glycoproteins, is rather loosely related to protection against varicella (Li et al., 2002); cellular immune responses can also be demonstrated (Kumagai et al., 1980; Arvin, 1996; Smith et al., 2003). Higher antibody titers and cellular immune responses are achieved with the use of more potent vaccine formulations and two-dose schedules, which are associated with improved protection against varicella (Watson et al., 1995; Arvin and Gershon, 1996; Varis and Vesikari, 1996; Kuter et al., 2004;). However, the vast majority of children show seroconversion after a single dose of vaccine (Weibel et al., 1984; White, 1996), which is obviously the most attractive schedule in terms of cost and compliance.

3.2. The US Varicella Vaccination Program: Design and Coverage

In view of the safety, immunogenicity, and efficacy of Oka vaccine among the major risk group for varicella-related morbidity and mortality, the US program was centered upon the vaccination of healthy pre-school children (Centers for Disease Control, 1996). The primary aims of this program were (1) the direct prevention of varicella-related morbidity and mortality among vaccinated individuals and (2) reduction of disease burden among remaining varicella-susceptibles (of all ages) as a result of effects on VZV transmission ("herd immunity"). By offsetting the cost of vaccinating against the reduction of direct (medical) and indirect (lost productivity) costs of varicella, the program was expected to be highly cost-effective (Lieu et al., 1994). US recommendations were issued in 1995 for the universal immunization of (VZV-naïve) healthy children aged 12–18 months and catch-up vaccination of all older varicella-susceptibles (using two doses rather than one from adolescence onward) (Committee on Infectious Diseases, 1995; Centers for Disease Control, 1996). As mentioned above, avoidance of varicella vaccine is counseled in most cases of the immunocompromised; these individuals are considered to be best protected by the immunization of any varicella-susceptible family members. Varicella-susceptible women should be vaccinated before and not during pregnancy.

National immunization statistics are estimated in the United States through an extensive telephone survey (the National Immunization Survey), making it possible to track varicella vaccine uptake over time (Centers for Disease Control, 2004).

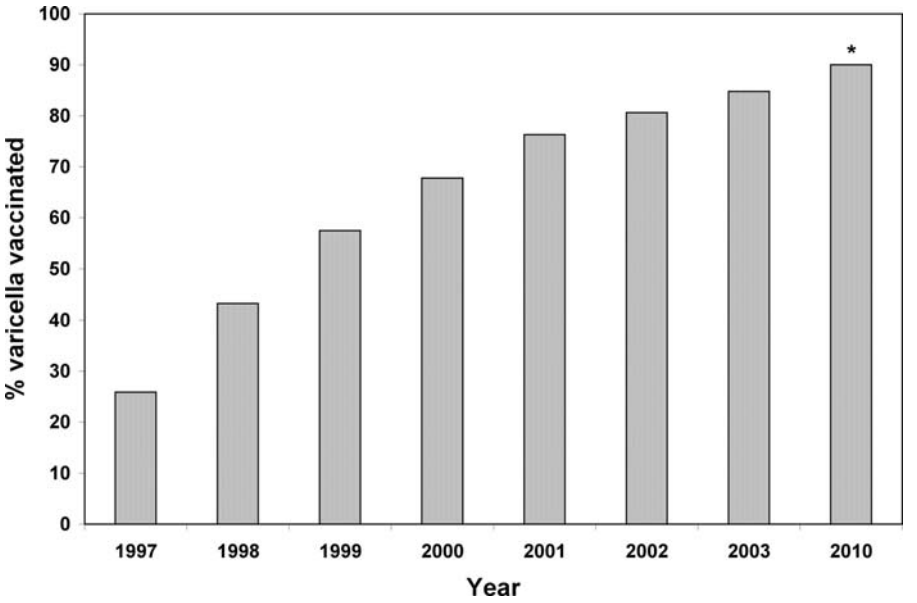

Figure 2.2. US national varicella vaccination coverage among children of ages 19–35 months. Data are drawn from the National Immunization Survey (NIS) (Centers for Disease Control, 2004). Asterisk indicates projected coverage level.

Latterly, many states have introduced a requirement for varicella vaccination (or positive disease history) among children entering daycare and/or elementary school. Such requirements, which are already commonplace in relation to other vaccines in the United States, have undoubtedly contributed to the increasing uptake of varicella vaccination. This is believed to have reached approximately 85% of 19–35-month olds across the United States by 2003 with a continuing upward trend (Centers for Disease Control, 2004) (Figure 2.2).

3.3. Impact of the US Varicella Vaccination Program

Within less than a decade, the epidemiology of varicella in the United States has been transformed from that of an almost universal childhood illness to a relative rarity. In the near future, we may expect varicella to become a notifiable disease nationwide, but clearly this would until recently have been difficult to achieve, considering the estimated 4 million cases of varicella every year. Therefore, documentation of the changing epidemiology of varicella has had to derive from other sources, detailed below. Collectively, these give a clear picture of declining varicella incidence and falling rates of varicella-related hospital admissions and deaths. Benefit extends from vaccinated to unvaccinated-susceptible groups (e.g., infants), confirming the existence of herd immunity.

3.3.1. Cases

Varicella incidence has been followed prospectively by the CDC since 1995 in sentinel counties of the United States (Figure 2.3). Following vaccine licensure in

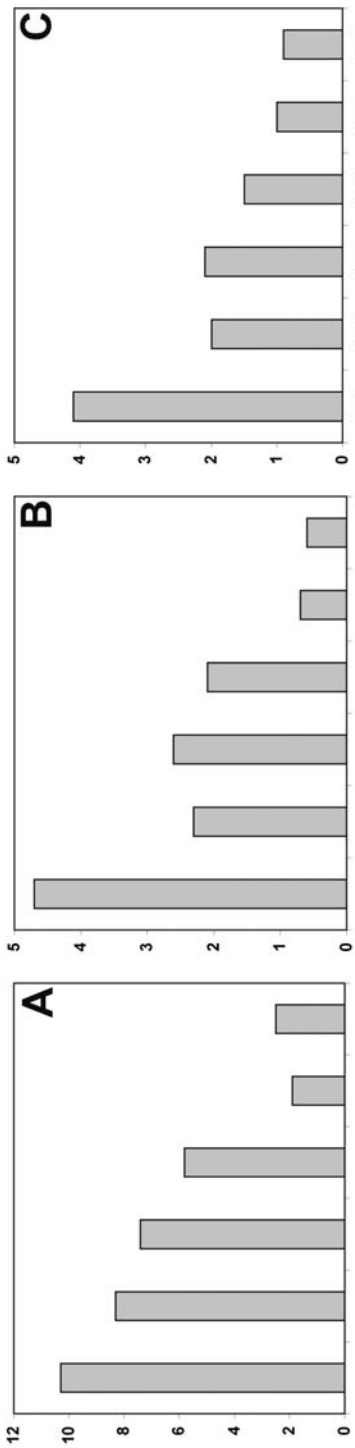

Figure 2.3. Varicella incidence in three sentinel counties of the United States following vaccine introduction in 1995. Data are drawn from Seward et al. (2002). *J. Am. Med. Assoc.* 287, 606 and represent annual rates of reported cases per 1,000 population in each county. (A) Antelope Valley, California; (B) Travis County, Texas; (C) West Philadelphia, Pennsylvania. (With permission, Copyright © 2002, American Medical Association. All rights reserved.)

1995, the proportion of immunized children rose steadily in these counties, while varicella incidence declined by between 71% and 84% by 2000 (Seward et al., 2002). An independent prospective cohort study elsewhere in the United States showed a similar trend as vaccine coverage within the study population rose to 63% over the period 1995–1999 (Clements et al., 2001). In both studies, varicella incidence declined markedly among both vaccinated and unvaccinated groups, strongly suggesting a herd immune effect despite only partial vaccine coverage. Further confirmation of the fall in varicella incidence comes from those few states that have had consistent (if incomplete) reporting of varicella over the last several years. Each of these four states has shown a steady decline in varicella incidence, with a 67–82% reduction by 2001 compared with the pre-vaccination period 1990–1994 (Centers for Disease Control, 2003).

3.3.2. Hospitalizations

Accompanying the falling incidence of varicella, one would expect a decline in related hospital admissions. Data are beginning to accrue that show this effect. A trend toward reduced hospital admissions was reported in the "sentinel counties" over the period 1995–2000 (Seward et al., 2002). Statistics from the National Hospital Discharge Survey, collated for the period 1988–1999, likewise showed a trend toward decreased hospitalizations which is anticipated will be confirmed over subsequent years (Galil et al., 2002a). A large health insurance claims database has been interrogated to disclose consultation and hospitalization rates for varicella and showed reductions of 59% and 80%, respectively, over the period 1994–2001 (as vaccination coverage rose to some 60%) (Jane Seward, CDC, personal communication). Of note, this was associated with a reduction of 75% in direct medical costs. Declining hospitalizations for varicella among military recruits have also been noted since the mid-nineties, part of which may be attributable to programs aimed specifically at the identification and vaccination of varicella-susceptibles in the armed forces (Ryan et al., 2003).

3.3.3. Deaths

Deaths from varicella are rare and fluctuated from year to year prior to the introduction of varicella vaccination (Meyer et al., 2000), so hasty conclusions could not be drawn regarding mortality rates. A recent statewide study, in California, is suggestive of the expected decline in varicella-associated mortality over the period 1988–2000 (McCoy et al., 2004). An analysis of varicella deaths nationally in the United States has yet to be published at the time of writing but is keenly awaited.*

4. Issues of Concern in the Varicella Vaccine Era

Following the introduction of new vaccines, it is naturally important to monitor disease activity particularly carefully as is the case for varicella. A number of problems need to be addressed.

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*An analysis of national death records, 1990-2001, has now been published and confirms a sharp decline in varicella-related deaths. See: Nguyen, H.Q., Jumaan, A.O., and Seward, J.F. (2005). Decline in mortality due to varicella after implementation of varicella vaccination in the United States. *N. Engl. J. Med.* 352, 450–458.

4.1. Identification and Vaccination of Varicella-Susceptibles

In the new era of low VZV circulation, there is a concern that significantly higher numbers of people may reach adulthood without developing immunity because they have neither encountered wild-type VZV nor received vaccine (or have responded poorly to vaccination, see next section). Disease modeling suggests that numerically, cases among adults (along with hospitalizations and deaths) will decline overall provided herd immunity is achieved by high rates of childhood vaccination (Halloran et al., 1994). However, should pockets of susceptibility occur there would be potential for nasty outbreaks of varicella in adults.

The US vaccination policy already calls for the immunization of varicella-susceptible adults (Centers for Disease Control, 1996, 1999), but there are difficulties in meeting this objective, first and foremost of which is the identification of such individuals. In the absence of specific outreach programs, this is very much an opportunistic affair, yet the groups at highest risk of being susceptible to varicella (e.g., immigrant workers) (Centers for Disease Control, 2000) are also among the least likely to participate in regular healthcare. As the first vaccinated cohorts reach adulthood, this problem may well be compounded by a number of factors. Firstly, the reliability of vaccination history is likely to be poor; secondly, there is no good laboratory correlate of immunity following vaccination (Gershon et al., 2004); thirdly, varicella is likely to be sufficiently rare so that public awareness of its risks will be limited. It will be important in public health terms to address each of these points, in order to avoid an excess of adult varicella cases (that will be attributed to failures of the vaccine program). Part of this preparedness should involve a strategy for the containment of outbreaks that would include screening (by history, vaccination records, and serology) and vaccination (including true post-exposure prophylaxis of history-negative individuals).

4.2. Breakthrough Varicella

As discussed above, both primary and secondary vaccine failures were demonstrated during pre-licensure trials. Since the introduction of routine varicella vaccination, active surveillance and studies during outbreaks have confirmed the occurrence of vaccine failure in the field, and offered the opportunity to assess risk factors for this (Table 2.1). At least two outbreaks appear to have been initiated by a case of breakthrough varicella, possibly reflecting primary vaccine failure in those children (Galil et al., 2002c; Lee et al., 2004). A recent study showed that, although in general breakthrough varicella is less transmissible than wild-type disease, this is not the case for those few children who develop more than 50 spots (Seward et al., 2004). It appears that children aged less than 15 months at the time of vaccination are more prone to breakthrough disease (Galil et al., 2002b). Some studies have suggested that individuals with asthma are at increased risk, particularly those requiring systemic steroid therapy (Izurieta et al., 1997; Verstraeten et al., 2003); further studies of this effect are ongoing. Administration of VZV vaccine within 28 days of MMR is also associated with vaccine failure.

All of the above-mentioned risk factors can be explained in terms of inadequate induction of adaptive immunity at the time of vaccination. However, the

Table 2.1. Representative Post-licensure Studies of Varicella Vaccine Effectiveness

Study	Vaccine effectiveness*		Study setting
	All disease	Moderate/severe disease	
Clements et al. (1999)	83% (67–90%)	100%	Prospective cohort
Galil et al. (2002c)	44% (−6–67%)	86%	Child care center outbreak
Izurieta et al. (1997)	86% (73–92%)	100%	Child care center outbreak
Lee et al. (2004)	56%	90%	School outbreak
Seward et al. (2004)	79% (70–85%)	92% (84–97%)	Secondary attack rate in households
Vazquez et al. (2004)	87% (81–91%)	98% (93–99%)	Case control, clinical practice

* 95% confidence intervals, where available, are shown in parentheses.

majority of individuals experiencing breakthrough varicella do not display these risk factors. Some primary vaccine failures may reflect problems in the preparation and storage of the vaccine, which is heat-labile. In addition, there are indications that the protection afforded by vaccination may wane over time, likely reflecting attrition of the immune response (so-called secondary vaccine failure). One study showed vaccine effectiveness declining after the first year before reaching a plateau (Vazquez et al., 2004), while other studies have suggested a slower effect (risk of breakthrough disease rising at 5 or more years after vaccination) (Lee et al., 2004). These findings at first appear to conflict with earlier data, in which immune responses appeared exceptionally robust (even showing a secondary rise some years after vaccination) (Johnson et al., 1997; Zerboni et al., 1998; Kuter et al., 2004).

What has changed between studies is the level of circulating VZV. There is good evidence of boosting of anti-VZV immune responses by both exposure to wild-type varicella and a second dose of vaccine (Arvin et al., 1983; Watson et al., 1995). Now that varicella is becoming rare, vaccinees may be exposed insufficiently to boost declining immunity to varicella, instead succumbing to breakthrough infection when an exposure eventually occurs (Figure 2.4). If this concept of an immune "threshold" is accurate, then it may be appropriate to consider giving booster doses of varicella vaccine later, as well as measures to maximize initial vaccine responses (e.g., a two-dose schedule or a higher titer vaccine).

4.3. Herpes Zoster

Little is yet known about zoster in vaccinees; in the immunocompromised, vaccine is significantly protective (Hardy et al., 1991) and it is reasonably assumed that zoster incidence will be reduced generally among vaccinated individuals. The mechanisms by which VZV immunity is maintained in the naturally infected host are not clear. Subclinical or abortive reactivations of the latent virus that do not result

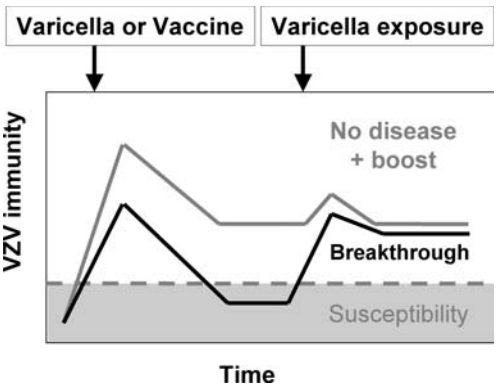

Figure 2.4. A model of VZV-specific immunity. Following vaccination or primary exposure to VZV, specific immunity (antibodies and T-cells) is generated. This wanes slowly over time. On re-exposure to VZV, subjects in whom immunity has fallen below a protective threshold become re-infected and experience breakthrough varicella (or, in the case of the unvaccinated, a second episode of chickenpox). Those with protective immune responses instead experience a boost to their immunity.

in the symptoms of herpes zoster have been demonstrated in the healthy and immunocompromised patients and have been shown to be associated with recovery of cellular immunity to VZV (Hata et al., 2002). Immunity to zoster may also be enhanced by periodic reexposure to VZV (Arvin, 1996; Thomas et al., 2002). There is thus a concern that, following the introduction of universal childhood vaccination, zoster may become more common among adults because of the loss of boosting exposures to varicella (Brisson et al., 2002). Active surveillance of zoster cases (among both vaccinated and unvaccinated) is underway in sentinel counties of the United States and will be important to guide future public health policy. While VZV IgG titers do not decrease with age, VZV-specific T cell responses decline. One possibility that is being pursued is to immunize aging individuals against zoster, using a higher potency, live attenuated VZV vaccine (Levin et al., 1998, 2003b); the results of a large trial are expected soon. Although current evidence suggests that the live attenuated varicella virus is less likely to reactivate than the wild-type viruses that circulate in the population, trends in zoster incidence over time may indicate that individuals who have been vaccinated against varicella would benefit from later immunization against zoster. An alternative strategy that might be particularly useful to protect immunocompromised individuals against zoster would be an inactivated VZV vaccine (Hata et al., 2002).

5. Summary

The most compelling rationale for introducing universal vaccination against varicella was the predicted benefits to healthy children. Current evidence from the US experience indicates that these benefits are being realized. As is true for any new vaccine, implementation of such a program necessitates disease surveillance and

modifications of the vaccine regimen as needed. In the case of VZV, the epidemiology of herpes zoster must be tracked as well as varicella disease trends. The prevention of herpes zoster may be another use of live attenuated or inactivated varicella vaccines.

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3

The ABC of Epstein-Barr Virus Infections

Upton D. Allen

1. Introduction

The Epstein-Barr virus (EBV) is a gamma herpesvirus that was first isolated in 1964 by Epstein, Achong, and Barr from Burkitt's lymphoma tissue (Epstein and Barr, 1964). It is of some historical interest that although the virus was reported to have been discovered by the above three scientists, Achong's name was not included in the naming of the EBV. The impetus for this discovery was the initial observation by Dr. Dennis Burkitt of endemic jaw tumors in equatorial Africa, which were believed to be due to an infectious agent. Humans and EBV have co-evolved over millions of years and consequently the virus has developed means of adaptation to the human host that enables a harmless host–virus relationship in the vast majority of immunocompetent individuals.

Like other herpesviruses, EBV has the ability to cause lytic infections and to remain latent within the host. The essence of the oncogenic potential of the virus relates to its ability to transform and immortalize B-lymphocytes, leading to the potential for uncontrolled proliferation of these transformed cells—a potential that is often realized in immunodeficient individuals (Henle et al., 1967; Pope et al., 1968; Petti and Kieff, 1988).

There are currently eight known human herpesviruses, with the EBV being human herpesvirus type 4.

- HHV-1 HSV type 1 HHV-5 CMV
- HHV-2 HSV type 2 HHV-6 (variants A and B)
- HHV-3 VZV HHV-7
- HHV-4 EBV HHV-8 KSHV

EBV shares the same subfamily with Human Herpesvirus 8 (γ herpesviruses). Herpes simplex virus and varicella-zoster virus occupy the alpha family, while the beta-herpesviruses include cytomegalovirus, human herpesvirus 6 (variants A and B), and human herpesvirus 7. These viruses all have a similar structure that consists of linear double-stranded DNA, an icosadeltahedral capsid, 162 capsomers, and an

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envelope. There are two known viral types of EBV (1 and 2 or A and B). While there is no known specific disease associations among these types, type 1 is more prevalent in western countries, while both types are equally prevalent in Africa and Papua New Guinea (Zimber et al., 1986).

2. Epidemiology and Model of Human Infection

2.1. Epidemiology

Humans are the only source of EBV. This virus has a worldwide distribution with seropositivity rates of 90% among adults worldwide. Peaks of infection occur in early childhood and young adulthood. As shown in Figure 3.1, seroconversion occurs at an earlier age in non-industrialized countries compared with industrialized countries (Epstein, 2001). In most non-industrialized communities, primary EBV infection is usually asymptomatic and occurs within the first 3 years of life (Rickinson and Kieff, 2001; Sumaya, 1996; Sumaya et al., 1975; Henle and Henle, 1980). In industrialized countries, infection is often delayed until the second decade of life or later (Rickinson and Kieff, 2001; Sumaya, 1996). Transmission of infection occurs as a result of close personal contact with oropharyngeal secretions. The existence of the virus within peripheral blood lymphocytes facilitates transmission via blood transfusions and donated organs. As will be discussed later in this chapter, the latter is of major concern in EBV-seronegative organ transplant recipients. Sexual transmission is possible, but is not well documented.

No seasonal patterns of EBV infections have been described. Excretion of virus in respiratory secretions may occur for several months after infection, and asymptomatic excretion of virus is common as outlined in the review of the model of human infection below. Indeed, the virus may be intermittently excreted for life.

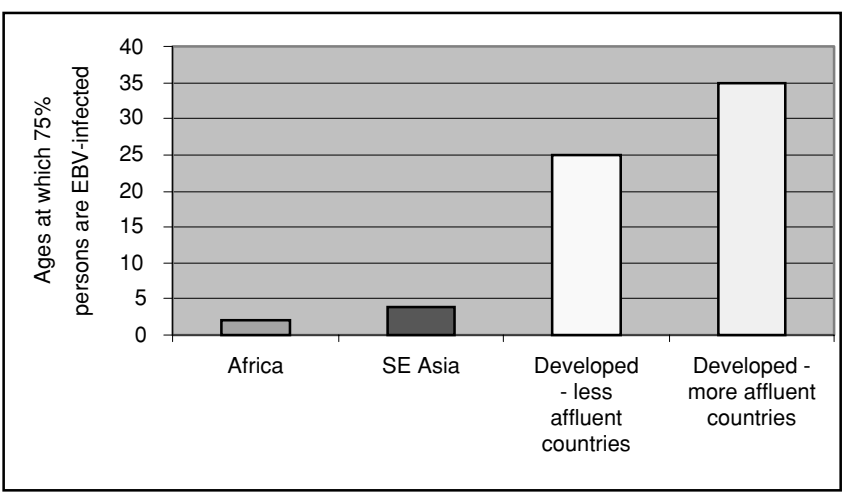

Figure 3.1. Comparison of the ages when different populations become infected with Epstein-Barr virus. The figure shows that in Africa and South East Asia, 75% of the population is EBV-infected before the age of 5 years, compared with 25–35 years of age in developed countries. Adapted with permission from Espstein (2001).

The period of communicability is indeterminate (American Academic of Pediatrics, 2003). The incubation period is estimated to be 30–50 days.

2.2. Model of Human Infection

As indicated above, infection usually results from contact with oropharyngeal secretions (above exceptions noted). In the oropharynx, the virus infects the resting B cells or the epithelial cells, which in turn infect the B cells (Cohen, 2000). As a result of primary infection, EBV-infected B cells undergo lysis with the production of virus. Alternatively, such cells may express the full complement of latent viral proteins. Latently infected cells are kept under control by cytotoxic T cells and NK cells. After recovery from primary infection, EBV remains in the peripheral blood in latently infected resting memory B cells. It is possible for these cells to undergo EBV reactivation and recognition and elimination by cytotoxic T cells. It is also possible for some latently infected cells to undergo lytic replication in the oropharynx, resulting in the production of viral particles that are shed in oropharyngeal secretions, thereby facilitating the continuation of the cycle of transmission of the virus.

3. EBV-Related Syndromes

The various syndromes that have been attributed to EBV can be categorized according to the cells of origin and the populations of risk. The major syndromes are summarized in Table 3.1. Among these syndromes, the association between disease and EBV infection is well established for Burkitt's lymphoma, infectious mononucleosis,

Table 3.1. Diseases Associated with Epstein-Barr Virus Infection

Diseases	EBV Association	Cell or Origin
Burkitt's lymphoma	Well established (endemic form)	Lymphocyte
Infectious mononucleosis	Well established	
XLPS	Well established	
B-Lymphoproliferative disease	Well established	
Chronic active EBV	Well established	
Hodgkin's disease	65–80%	
T/NK cell lymphoma	10–100%	
Primary effusion lymphoma	70–80% (100% have HHV8 DNA)	
Oral hairy leukoplakia	Well established—100%	Epithelial
Nasopharyngeal carcinoma Non-keratinized—100% Keratinized—30%	Well established	
Gastric carcinoma	Undifferentiated carcinoma of nasopharyngeal type—100% Adenocarcinoma 5–15%	
Leiomyosarcoma	Documented in HIV and transplant recipients	Other cells
Other possible tumors: salivary gland, breast carcinoma (ca), hepatocellular ca, thymoma	Not established	

Modified with permission from Macsween and Crawford (2003).
XLPS denotes X-linked lymphoproliferative syndrome.

X-linked lymphoproliferative syndrome, B-cell lymphoproliferative diseases, chronic active EBV infection, oral hairy leukoplakia, and nasopharyngeal carcinoma. Chronic fatigue syndrome is not specifically linked to EBV. A selection of these syndromes is discussed below.

3.1. Infectious Mononucleosis

Infectious mononucleosis is the prototype of primary EBV infection. This entity is typically characterized by fever, exudative pharyngitis (Figure 3.2), lymphadenopathy, hepatosplenomegaly, and atypical lymphocytosis (Figure 3.3). The circulating atypical lymphocytes are T cells that have been transformed (or activated) in response to EBV-infected B cells in infectious mononucleosis.

The spectrum of clinical features is wide, ranging from asymptomatic infection to fatal infections in individuals with congenital or acquired cellular immune deficiencies. In very young infants and children, infection is often asymptomatic. Infection with this virus is known to be associated with the development of a nonallergic morbilliform rash in about 90% of the individuals receiving ampicillin and other penicillins (Figure 3.4).

In most individuals, primary EBV infection is uncomplicated. However, the following complications may occur, based on the types of organ involved.

- Liver — Hepatitis
- Respiratory system — Upper airway obstruction, pneumonitis
- Neurological (may be isolated findings) — Encephalitis, aseptic meningitis, Guillain-Barré, transverse myelitis, others
- Spleen — Rupture

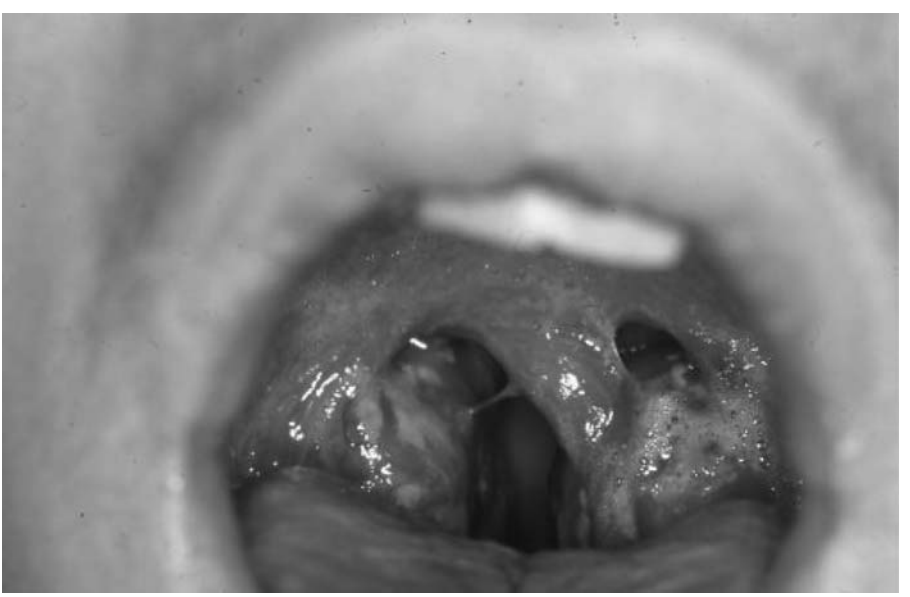

Figure 3.2. Exudative tonsillopharyngitis in infectious mononucleosis. Reproduced with permission from the Slide Library, Hospital for Sick Children, Toronto.

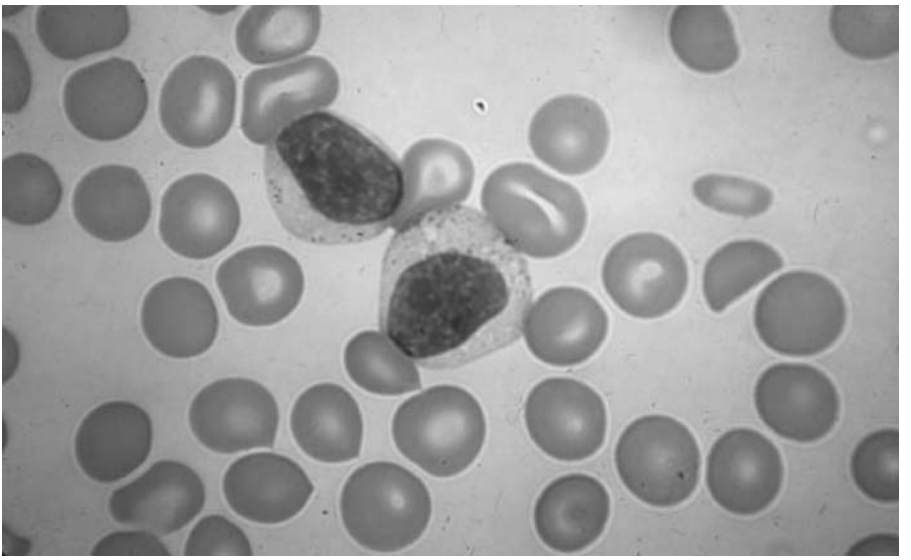

Figure 3.3. Atypical lymphocytes in a patient with infectious mononuleosis. Reproduced with permission from the Slide Library, Hospital for Sick Children, Toronto.

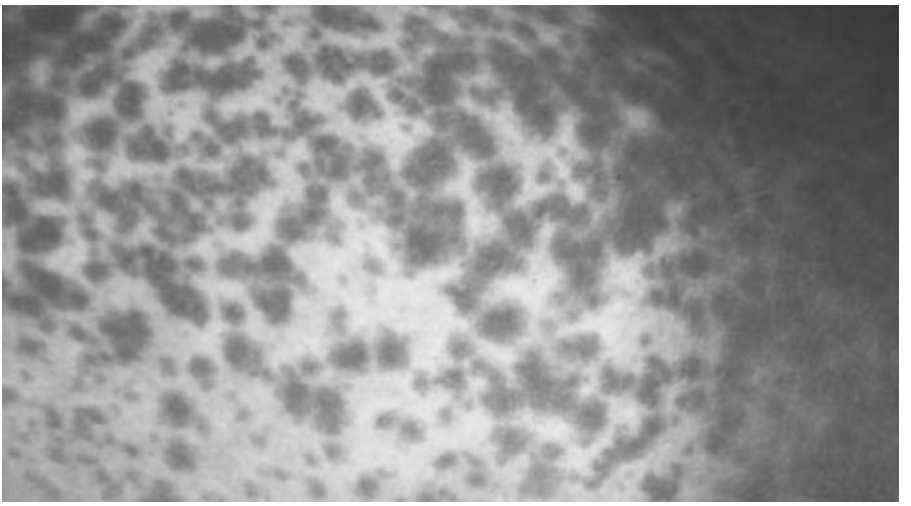

Figure 3.4. Beta-lactam induced rash in a patient with primary Epstein-Barr virus infection. Reproduced with permission from the Slide Library, Hospital for Sick Children, Toronto.

- Hematological — Decreased cellular elements, disseminated intravascular coagulation, hemophagocytic syndrome
- Others: bacterial superinfection, renal, cardiac, immunological, psychiatric

The treatment of patients with infectious mononucleosis is largely supportive. Antiviral agents are not usually employed, with the exception of severe IM in the immunocompromised host. In such situations, ganciclovir is more active against EBV

than acyclovir. Contact sports should be avoided until the patients have recovered and the spleen is no longer palpable. Steroids have been considered in some patients. However, their use should only be considered in patients with severe tonsillar inflammation with impending airway obstruction, massive splenomegaly, myocarditis, hemolytic anemia, or hemophagocytic syndrome (American Academy of Pediatrics, 2000).

3.2. X-Linked Lymphoproliferative Syndrome

The X-linked lymphoproliferative syndrome is inherited along maternal lines and affects boys (Bar et al., 1974; Purtillo et al., 1975). Usually, the boys are clinically well prior to the acquisition of primary EBV infection. These patients usually have normal immune function prior to EBV infection (although very mild immune deficits have been reported). It has been suggested that XLPS might be triggered by several viral infections. This condition has several phenotypic manifestations, including the occurrence of severe fatal infectious mononucleosis early in life and is associated with a poor prognosis. Most patients presenting with fulminant infectious mononucleosis die from this condition, often from fulminant hepatitis and a cytokine storm. Hemophagocytosis is often a major feature. Other manifestations of this condition include lymphoproliferative disorders, dysgammaglobulinemia and aplastic anemia.

3.3. B-Cell Lymphoproliferative Disorders

These entities occur among immunodeficient individuals. Such individuals have an inability to limit EBV replication due in part to the lack of EBV-directed cytotoxic T-lymphocyte surveillance activity. One of the groups affected by these lymphoproliferative disorders is organ transplant recipients, who develop primary EBV infection in the posttransplant period. Further details on this condition are provided in Section 6.

3.4. Chronic Active EBV Infection

This is a rare condition that typically affects individuals of East Asian origin (Okano and Gross, 2001; Okano, 2000). Patients present with recurrent infectious mononucleosis-like symptoms and have very high EBV titers. Epstein-Barr viral loads are usually elevated with lack of EBNA1. The key clinical features include intermittent fever, lymphadenopathy, and hepatosplenomegaly. Hematological manifestations include anemia, thrombocytopenia, lymphocytopenia or lymphocytosis, neutropenia, and polyclonal gammopathy. This condition is difficult to treat and has a poor prognosis. Diagnostic criteria have been proposed based on clinical, hemaotological, virological, and other parameters (reviewed in Okano, 2000).

4. Diagnostic Tests

4.1. Serologic Tests

The laboratory diagnosis of EBV infection depends primarily on serologic tests in the immunocompetent host. Nonspecific tests for heterophil antibody are

Table 3.2. Interpretation of Epstein-Barr Virus Serology

Infection	VCA IgG	VCA IgM	EA (D)	EBNA
No previous infection	−	−	−	−
Acute infection	+	+	±	−
Recent infection	+	±	±	±
Past infection	+	−	−	+

VCA IgG, immunoglobulin (Ig) G antibody to viral capsid antigen; VCA IgM, IgM to VCA; EA (D), early antigen diffuse staining; EBNA, EBV nuclear antigen. Adapted with permission from American academy of Pediatrics (2003).

readily available. Heterophil antibody is primarily immunoglobulin M and appears during the first 2 weeks of illness, disappearing over a period of 6 months. Heterophil antibody is often negative in children <4 years of age. During the second week of illness, there are usually elevated levels of atypical lymphocytes. The finding of $>10\%$ atypical lymphocytes along with a positive heterophil antibody is regarded as a diagnostic of acute EBV infection.

The patterns of results of specific EBV antibody tests provide an indication of whether a patient has acute, recent, or remote EBV infection (Table 3.2). The utility of single serologic tests is variable. In general, the patterns of results are more useful than the single results, with some notable exceptions. In this regard, antibody to EBNA usually does not occur until several months after the onset of infection. Thus, a positive anti-EBNA test rules out acute infection. A positive VCA IgM is consistent with acute or recent infection. These serologic tests are particularly helpful when investigating patients with heterophil negative infectious mononucleosis (IM). In such patients, it may be necessary to rule out other infections such as cytomegalovirus infection. Other infectious agents that may cause heterophil-negative IM-like syndromes include HHV-6, HHV-7, rubella, and *Toxoplasma gondii*.

4.2. Nucleic Acid Detection

In immunocompromised patients, serology is less useful as a diagnostic test. In such patients, nucleic acid detection methods are employed. In this regard, polymerase chain reaction (PCR) can be used to determine the presence of EBV DNA qualitatively. In addition, semi-quantitative PCR may be used to determine the circulating levels of EBV in different settings, including EBV-related lymphoproliferative disorders.

5. EBV and the Immune System

5.1. EBV-Encoded Genes in Different States of EBV Infection

The virus has developed clever mechanisms by which it evades the immune system by convincing the host that it is a friendly guest, thereby creating a host–virus symbiotic relationship. The virus encodes for several genes that are involved in various aspects of this symbiotic relationship.

5.1.1. Lytic Infection

In the lytic cycle, EBV encodes for the expression of genes that facilitate viral replication. The first of these genes to be activated is *Bam*HI-Z leftward reading frame-1 (BZLF-1). This gene expresses the transactivator function of BZLF1 protein or *Bam*HI-Z EB replication activator (ZEBRA) (Okano and Gross, 2001). Other genes such as early antigen (EA)-diffuse (D) and EA-restricted (R) are also expressed. Subsequently, viral DNA synthesis occurs and viral capsid antigen (VCA) is expressed.

5.1.2. Latent Infection

In latent infection, EBV DNA encodes for at least nine related proteins plus two non-polyadenylated RNAs. These proteins are EBNA-1, -2, -3A, -3B, and -3C, leader protein (LP), latent membrane proteins (LMP)-1, LMP-2A and LMP-2B, plus EBV-encoded RNAs (EBER)-1 and -2, respectively (Okano, 1998). EBNA appears in infected cells several hours after infection, with subsequent cellular DNA synthesis and proliferation of infected cells. In this process, LMPs also appear on the cell membrane together with activation markers such as CD23 and adhesion molecules, including leukocyte functional antigens (LFA) and intercellular adhesion molecules (ICAMs). Cells expressing EBNA-2, -3A, and -3C and LMP-1 are highly susceptible to the action of EBV-specific cytotoxic T-lymphocytes (EBV-CTL) that develop during the acute to convalescent phase of a primary infection. *Bam*H1-A rightward transcript (BART) is usually found in all infected cells (Brooks et al., 1993).

EBNA-1 is essential for the replication of the plasmid form of EBV, and EBNA-2 is highly associated with cell proliferation, as is LMP-1. Functions of other latent proteins and RNAs remain obscure, but are considered to be related to both cell proliferation and viral replication. The function of LMP-1 is similar to that of other members of the tumor necrosis factor (TNF)-receptor family and is similar but not identical to that of CD40. LMP-1 interacts with TNF-receptor-associated factors (TRAFs) and the TNF-receptor-associated death domain in infected cells, and activates nuclear factor kappa B and c-Jun N-terminal kinase pathways, resulting in B-cell activation and proliferation (Rowe, 1995, Eliopoulos and Young 1998, Izumi and Kieff, 1996). In addition to EBNA-1, -3A, and -3C, LMP1 is required for immortalization of B cells (Okano, 1998, Okano and gross, 2001, Farrel, 1995). LMP-1 expression induces bcl-2 expression that inhibits apoptosis of B cells (Rickinson and Kieff, 2001).

5.1.3. Healthy Persons

The sites of latency of EBV in healthy individuals are CD23-positive B cells. These resting B cells express LMP-2A, EBER-1, EBER-2, and BART (Okano and Gross, 2001; Chen et al. 1999).

5.1.4. Immunodeficient Patients

Despite the spectrum of genes potentially expressed in EBV infections, the actual expression of specific genes varies with the type of EBV-related diseases. For example, EBV-genome positive Burkitt's lymphoma cells express EBNA-1, EBER-1, and EBER-2. In immunodeficient patients with lymphoproliferative disorders,

infected cells express all nine of the EBV-related latent proteins, all adhesion molecules, co-stimulatory molecules, and activation markers (Okano and Gross, 2001).

5.2. Evasion of Immune System

The virus uses CD21 as a receptor. This is the same receptor for the C3d component of complement (Fingeroth et al., 1984). The CD21 ligand is the outer envelope Gp350 of EBV and there is homology between Gp350 and C3d. Thus, the host cannot tell the difference between EBV and complement (C3d) and welcomes the virus readily. This welcome is extended in several ways as EBV interacts with the immune system. The virus encodes for proteins that facilitate the upregulation of CD21 receptors, thereby creating more sites for binding to B lymphocytes. There is evidence that MHC class II molecules serve as cofactors for EBV infection of B cells. Antibodies that block the gp42-MHC class II interaction inhibit EBV infection. In addition, cells that lack MHC class II can be superinfected only if MHC class II expression is restored (Li et al., 1997).

EBV encodes for the IL-10-like gene, *BCRF-1* (*Bam*HI-C rightward reading frame-1). BCRF1 shares 70% of its amino acid sequence with IL-10 and thus it is able to mimic the activity of IL-10 by inhibiting interferon-gamma production. EBV also encodes for proteins that inhibit apoptosis (Hsu et al., 1990; Moore et al., 1993). Overall, EBV infection facilitates a Th2-type response profile that favors viral replication with decreased interferon-gamma production, and less CTL activity.

Mature B cells are necessary for EBV infection to occur. Evidence for this comes from the observation that patients with X-linked agammaglobulinemia are resistant to EBV infection (Faulkner et al., 1999). Such patients do not succumb to overwhelming EBV infections in contrast to the XLP patients. In the latter patients, there is a gene that is mutated on the X chromosome, signaling lymphocyte activation molecule (SLAM). Another molecule, SLAM associated protein (SAP) is not functional in these patients (Cohen, 2000). Lack of a functional SAP impairs T- and B-cell interaction, resulting in unregulated growth of EBV-infected B cells.

The resting memory B cells represent the sites of persistence of EBV. Thus, EBV may be “eradicated” in bone marrow transplant recipients if B cells are eliminated during the conditioning phase of bone marrow transplantation.

5.3. Acquired Disturbances in Immune Surveillance for EBV

In the normal host, EBV is kept under control within a range of 1–50 infected B cells per million in the circulation (Tosato et al., 1985). Acquired disturbances of immune surveillance may affect this control with the potential for lymphoproliferation. In the remainder of this chapter, we will review two examples of acquired immune dysregulation and the impact it has on EBV activity.

5.3.1. Immune Dysregulation due to Space Flight

It has been shown that as a result of space flight, several immune dysregulatory events occur. There are reduced blastogenic responses, decreased cell mediated immunity (skin test response), altered cytokine production, decreased NK cell

function, changes in circulating leukocyte population, variable changes in immunoglobulin levels, among other changes (Konstantinova, 1991; Cogoli, 1993; Sonnenfeld, 1999). Studies have shown that during space flight, the excretion of EBV in saliva increases several fold compared with before and after flight (Pierson, 2004). Others have shown increased shedding of EBV just prior to launch relative to during and after flight (Payne et al., 1999). This increase in salivary viral load correlates with increased lytic viral activity (Preiksaitis et al., 1992). It has also been shown that elevated anti-VCA antibodies occur just prior to launch compared with the annual medical examinations of astronauts. In addition, after flight anti-EA antibodies are elevated several fold (Stowe et al., 2000). There is no evidence that these individuals develop uncontrolled proliferation of EBV after they return to earth. When their immune system is no longer dysregulated, they are able to bring EBV back under control. Thus, the experience of astronauts has taught us that transient immune dysregulation can lead to upregulation of EBV activity, which is brought back under control when the immune function is restored.

5.3.2. Immune Dysregulation due to Organ Transplantation

Transplant recipients are not as fortunate as their astronaut counterparts. The former are profoundly immunosuppressed during the early stages after transplantation and their immune systems do not return to normal after transplantation. Their inability to control EBV activity after transplantation, although quite variable between organ transplant groups, creates the potential for EBV-related posttransplant lymphoproliferative disorders (PTLD).

6. Post-Transplant Lymphoproliferative Disorders

6.1. Clinical Manifestations and Diagnosis

The spectrum of clinical manifestations of EBV in transplant recipients includes non-specific viral syndrome, mononucleosis, lymphoproliferative disorders, and malignant lymphomas (e.g., Burkitt's lymphoma). In the transplant recipient, benign manifestations of EBV may evolve into more serious entities (Green and Webber, 2003).

The actual manifestations of PTLD will depend on the organs or tissues that are involved. Typically, the organs of the reticuloendotheial system as well as the transplanted organs are most often involved. Nonspecific symptoms include fever, malaise, lethargy, and weight loss. In addition, patients may present with swollen lymph nodes, abdominal pain, gastrointestinal bleeding, nausea, and vomiting. Symptoms of allograft dysfunction may occur. Patients with central nervous system involvement may have headaches and focal neurologic symptoms. Physical findings may include pallor, lymphadenopathy, subcutaneous nodules, tonsillar enlargement, hepatosplenomagely, and focal neurologic signs.

The diagnosis of PTLD is based on the clinical finding and laboratory investigations. The gold standard for confirming the diagnosis is pathologic examination of biopsy material. In addition to the morphologic classification, tissue samples are

usually examined to determine the cell of origin (B, T, null, and mixed), clonality (monoclonal, polyclonal, and mixed) and the EBV status of the lesions.

6.2. PTLD Burden and Associated Risk Factors

Risk factors for PTLD include primary EBV infection, the type of organ transplanted, the type and intensity of immune suppression and co-existent CMV infection (Ho et al., 1985; Walker et al., 1995; Basgoz and Preiksaitis, 1995; Reding et al., 1994; Shapiro et al., 1988; Ho, 1995; Cox et al., 1995; Swinnen et al., 1990; Cockfield et al., 1991). Children are particularly affected because they are most often EBV-seronegative pre-transplant (Ho, 1995) and experience primary EBV infection more often than secondary infection after transplantation. However, while primary infection is a major risk factor for PTLD, in some pediatric centers, approximately 25% of the PTLD cases occur among previously seropositive recipients (Allen et al., 2001a).

Most cases of PTLD are due to EBV infection and usually occur during the first 2 years after organ transplantation. At the Hospital for Sick Children, Toronto, 81% presented during the first 2 years posttransplantation (Dror et al., 1999). This condition affects 2–5% of all organ transplant recipients (Basgoz and Preiksaitis, 1995).

The incidence of PTLD among children is significantly higher than in adults, with rates as high as 14–22% for some categories of pediatric transplant recipients (Reding et al., 1994; Cox et al., 1995). An incidence of 15% was reported for heart transplant recipients, 10% for liver transplant recipients, and 1.2% for renal transplant recipients (Dror et al., 1999).

While the mortality and morbidity of PTLD are high in many centers, these vary across centers (Green and Michaels, 2001). EBV may lead to severe outcomes among transplant recipients even if PTLD does not occur. The experience in some centers has been that EBV represents more of a disease and management burden than CMV. The latter has long been considered as the most frequent and important viral infection in transplant patients (Patel et al., 1996).

6.3. Detecting EBV Lymphoproliferation

Since primary EBV infection is a risk factor for PTLD, it is necessary to identify patients at risk by performing EBV serology prior to transplantation (Carpentier, 2003). However, in the posttransplant period, serology is unreliable as these patients may have marked delays in their humoral response to EBV antigens and many do not develop IgM antibodies (Collins et al., 2001). In addition, transplant patients often receive blood or blood products with the passive transfer of donor antibodies, thereby affecting the interpretation of antibody tests.

6.4. EBV Viral Load as a Marker of EBV Lymphoproliferation

Using PCR, it is possible to detect EBV DNA in the peripheral blood lymphocytes of healthy seropositive adults. Semiquantitation of EBV load is regarded as a useful indicator of the changes in viral dynamics that precede the development of

PTLD. This test also enables the detection of increased viral replication that is associated with acute (non-malignant) EBV disease. Semiquantitative PCR is useful in predicting PTLD based on the fact that transplant recipients who are at risk of PTLD have high-circulating EBV genomic equivalents (Rickinson and Kieff, 2001; Riddler et al., 1994; Kenagy et al., 1995; Savoie et al., 1994; Martinez et al., 1995). Studies have evaluated the utility of EBV viral load testing in the surveillance and diagnosis of PTLD as well as when employed in strategies to prevent and treat PTLD (Allen et al., 2001b; McDiarmid et al., 1998; Green et al., 1997; Rowe et al., 1997; Green et al., 1998; Preiksaitis and Keay, 2001). EBV viral load measurements, as determined by EBV semiquantitative PCR, have high negative predictive values, but lower positive predictive values for diagnosing PTLD. However, sustained elevations of viral loads, while not necessarily predicting PTLD, suggest an environment in which EBV replication and/or proliferation of transformed lymphocytes are abnormal.

6.5. Treatment of PTLD

There is no firm consensus on the most appropriate way to treat patients with PTLD (Preiksaitis and Keay, 2001). The various therapeutic modalities are summarized in Table 3.3. The most important aspect of treatment is reduction of immunosuppression. This enables the restoration of some degree of CTL activity. Antiviral therapy is often used along with immunoglobulin in pediatric patients. However, the benefit of these agents is unproven. Chemotherapy is usually the first line management of patients with CNS lymphoma or overt malignancies such as Burkitt's lymphoma.

Table 3.3. Potential Treatment Strategies for Posttransplant Lymphoproliferative Disorder

Therapies	Comments
First-line therapies	
Reduced immunosuppression Antriviral therapy (ganciclovir) Chemotherapy	Reduced immunosuppression is effective to varying degrees in most patients. Antiviral therapy is unproven, but is widely used. Chemotherapy is first-line for overt malignancy (e.g., Burkitt's)
Second-line therapies	
Anti-B-cell monoclonal antibodies Intravenous immunoglobulin (containing anti-EBV antibodies) Chemotherapy Surgery (±) radiation therapy	Studies are currently being conducted using newer modalities such as anti-B-cell monoclonal antibodies. Second-line therapies show varying degrees of promise in different scenarios. Some are becoming less used in recent years due to concerns regarding adverse events. For example, interferon-alpha may be associated with severe rejection.
Other	
Cellular Immunotherapy Interferon-alpha	

7. Summary

The EBV has evolved mechanisms that allow it to take advantages of different aspects of the human immune system, resulting in evasion of the defense mechanisms of the host. The virus is capable of resting calmly in a "sea of tranquility". The virus has the unique ability among herpesviruses to transform infected B lymphocytes, thereby creating the potential for uncontrolled lymphoproliferation. Such lymphoproliferation occurs when the immune system is deregulated as a result of inborn defects or acquired defects (e.g., XLP syndrome and organ transplantation, respectively). Otherwise, EBV is kept in check by the surveillance activity of cytotoxic T lymphocytes and NK cells.

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4

The Immune Response to Viral Lower Respiratory Tract Infection

Caroline A. Lindemans and Jan L. L. Kimpen

1. Introduction

Viruses are responsible for the majority of respiratory infections in childhood, causing considerable morbidity and mortality. It is estimated that in the United States approximately $ 652 million per year is spent on medical costs for respiratory syncytial virus (RSV) related disease alone (Paramore et al., 2004). Viruses cause a variety of respiratory diseases in children from the common cold to life-threatening pneumonia and bronchiolitis. The host reacts to a viral infection with a combination of innate and adaptive immune mechanisms, usually resulting in the clearance of the virus and clinical recovery. However, there is an accumulating evidence for a number of viral infections that the host immune response actually enhances disease in the course of clearing virus from the infected organs. Interestingly, the effectiveness of the immune response seems to be dependent on the age and probably genetic background of the child. This has important implications for treatment as well as vaccine development.

Viral infections play an important role in both childhood and adult asthma. They might be instrumental in the inception of asthma and are associated with the majority of exacerbations in asthmatic individuals (Johnston et al., 1995; Bont et al., 2000).

In respect to the role of viruses in the pathogenesis of acute and chronic airway disease in children, it is of utmost importance that we gain a proper understanding of the underlying mechanisms involved in order to design effective therapeutic and preventive strategies.

Although viral respiratory tract infections are considered to be mainly pediatric diseases, there is an increasing acknowledgement of their pathogenic potential in the immunocompromised host of all age groups and in the elderly.

2. Epidemiology and Clinical Aspects

Viruses involved in respiratory tract disease in children induce quite similar respiratory illnesses. In many cases, and especially in the ambulatory setting, the

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causative agent is not identified. When an upper respiratory tract infection in an infant progresses to lower respiratory tract disease, bronchiolitis and pneumonia are most common. Both disease entities are hard to differentiate and no clinically relevant differences with regard to outcome have been identified (van Woensel et al., 2003).

2.1. Respiratory Syncytial Virus

2.1.1. The Virus

RSV belongs to the paramyxoviridae family and the genus of Pneumovirus. It is an enveloped unsegmented single-stranded RNA-virus of which two subtypes are known (A and B). A clear relationship between subtype and disease severity has not been established (Kneyber et al., 1996). Since RSV infection does not lead to complete immunity, reinfection is common. Immaturity of the immune system during initial infection seems to be the main cause of incomplete memory-response although an as yet undefined mechanism of partial immune evasion by RSV cannot be ruled out (Bont et al., 2002). Recently, it was suggested that RSV could cause persistent infection or latency (Dakhama et al., 1997; Schwarze et al., 2004), although the significance of this is not clear.

2.1.2. RSV Respiratory Disease

RSV affects 70% of infants in the first year of life, and by the age of two nearly all children have been infected. (Figure 4.1) It is likely that a specific balance and timeframe of changes in air temperature and humidity are responsible for the well-defined yearly winter outbreaks of RSV (Stensballe et al., 2003). In most infants as well as in older children and adults, RSV is the cause of upper respiratory tract infection with mild symptoms. However, in the very young, the infection spreads to the lower respiratory tract in approximately 40% of cases. One to three percent of infants develop bronchiolitis or pneumonia requiring hospitalization, with a considerable number requiring mechanical ventilatory support. Apart from the obvious respiratory symptoms, very young children frequently present with atypical symptoms such as impaired feeding, vomiting, lethargy, and apnea (Kneyber et al., 1998). Several risk factors for more severe disease have been identified, including age less than 6 weeks, prematurity, pre-existent cardiorespiratory disease and immunological impairment (Bont and Kimpen, 2002). Respiratory symptoms are directly related to airway pathology. Necrosis of the airway epithelium is a key phenomenon resulting in sloughing of the epithelial cells. Together with a dramatic influx of inflammatory cells into the airways and increased mucus production, this leads to the formation of copious secretions that block the small airways. Mucosal edema and bronchospasm through irritation of subepithelial nerve endings further compromise airway diameter.

Although antibiotics are prescribed for up to 50% of children with lower respiratory tract infections, proof of bacterial superinfection is only found in a minority of patients and the role of this event in the pathogenesis of severe disease remains controversial (Purcell and Fergie, 2002; Bloomfield et al., 2004). On the other hand,

it has been demonstrated that RSV infection of airway epithelial cells *in vitro* enhances adherence of *S. pneumoniae* (Hament et al., 2004).

2.2. Influenza

2.2.1. The Virus

The influenza virus belongs to the orthomyxoviridae family and is an enveloped segmented single-stranded RNA (ssRNA) virus (Table 4.1). The structural proteins of the virion are encoded by separate gene segments and include three viral RNA polymerases, nucleoprotein, matrix, and the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. On the basis of their nucleocapsid and matrix protein antigens, the influenza viruses are divided into three distinct immunological types (A, B, and C). Although all three influenza viruses cause respiratory disease in humans, only A and B are known to cause epidemics.

Induction of a memory-response results in long-lasting immunity and it is the antigenic variation that is responsible for frequent reinfection with the virus. The most antigenic variation is seen in the virus that infects both animals and humans, influenza A. Fourteen subtypes of hemagglutinin (H1–H14) and nine types of neuraminidase (N1–N9) are circulating in nature. The segmentation of the genome makes exchange of genetic material between subtypes possible, resulting in the structural changes observed in influenza, which has caused pandemics in the past. When two different subtypes of influenza A virus infect the same cell, major changes (antigenic shifts) can occur through rearrangement of genetic segments from both infecting viruses. Minor changes (antigenic drifts) in NA and/or HA proteins occur through accumulation of point mutations, and provide a mechanism for the virus to escape protective antibodies and cause respiratory symptoms every year.

Table 4.1. Overview of Respiratory Viruses, Their Family, and the Proteins Used for Infection of Respiratory Epithelium

Virus species	Family	Genus	Viral attachment protein	Host cell receptor (respiratory epithelium)
RSV	Paramyxoviridae	Pneumovirus	G-protein F-protein	CX3CR1 (fractalkine), TLR
Influenza	Orthomyxoviridae	Influenzavirus A, Influenzavirus B, Influenzavirus C	HA-glycoprotein	Neuraminic acid
Parainfluenza	Paramyxoviridae	Paramyxovirus	HN-glycoprotein	Sialic Acid
Adenovirus	Adenoviridae	Mastadenovirus	Fiber-protein	Coxsackie and adenovirus receptor (CAR)
Rhinovirus	Picornaviridae	Major group Minor group		ICAM-1 LDL-R
hMPV	Paramyxoviridae	Pneumovirus	G-protein	
Sars-CoV	Coronaviridae	Coronavirus	Spike (S-) glycoprotein	ACE2

2.2.2. Influenza Respiratory Disease

Influenza virus epidemics are difficult to separate in time from RSV epidemics, and the diseases caused by both viruses can also be difficult to differentiate (Zambon et al., 2001). (Figure 4.1) Compared to other viruses, morbidity caused by influenza is high in all age groups. Children with influenza infection of the respiratory tract are more likely to present with fever. Infants aged less than 6 months and older children with an impaired immune system, or other serious health problems, have a higher risk of hospitalization and mortality. Subclinical infections with influenza in children are common, suggesting children can be an important reservoir and source of transmission.

Yearly updated vaccines are available and effective, and recently it has been proposed to extend the current recommendations to children less than 2 years of age, children with recurrent acute otitis media or respiratory tract infections, and healthy children attending day-care centers or elementary schools (Principi and Esposito, 2004).

2.3. Adenovirus

2.3.1. The Virus

Adenoviruses, belonging to the adenoviridae family and the genus Mastadenovirus, are a group of DNA-viruses of which at least 47 serotypes are known. For lower respiratory disease, subtypes 1, 2, 3, 4, 5, 7, and 21 are most important. It is an icosahedral capsid virus with extruding fiber proteins, which are required for viral entry to epithelial cells (Howitt et al., 2003).

2.3.2. Adenovirus-Induced Respiratory Disease

Although human adenoviruses are ubiquitous, and cause primary infection in the first year of life, there is geographical variation in the distribution of serotypes and in the association of serotypes with different age groups. In Europe, adenovirus is the cause of infection in approximately 5% of hospitalized patients with viral lower respiratory tract disease. However, in some South American and Asian countries, adenovirus is the second most prevalent pathogen for acute lower respiratory tract infection in children after RSV (Carballal et al., 2002). Although adenovirus infections in general occur the whole year round, respiratory adenovirus infections are most common during late winter, spring, and early summer. Adenovirus type 7, acquired by inhalation, has been associated with more severe lower respiratory tract disease (Larranaga et al., 2000). Subtype-specific immunity occurs. However, some types are capable of establishing persistent asymptomatic infections in tonsils, adenoids, and intestines of infected hosts, and shedding can occur for months or years.

2.4. Parainfluenza Virus

Parainfluenza virus (PIV), another pathogen like RSV belonging to the paramyxoviridae, causes a spectrum of disease varying from common cold, croup, and bronchiolitis to pneumonia. Clinically relevant infections are mainly seen in

children under 6-years old. Though it is the causative agent of similar disease entities, hospitalizations occur four times less frequently than for RSV infections (Hall, 2001). Two subtypes are clinically important respiratory pathogens in children, PIV-1 and PIV-3. PIV-1 is the main cause of croup in 2–6-year olds, while PIV-3 is responsible for parainfluenza bronchiolitis in children under 6-months old. PIV-4 only causes mild upper respiratory tract infections. Because of acute narrowing of the subglottic region of the larynx, moderate to severe croup may require emergency management with systemic or inhaled corticosteroids, which are effective in improving stridor in a few hours (Cetinkaya et al., 2004). The hallmark cytopathic effect of acute infection with PIV-3 is comparable to that of RSV with extensive cell fusion resulting in syncytium formation. For fusion to occur, two PIV glycoproteins are required, including the hemagglutinin–neuraminidase (HN) glycoprotein interacting with host cell sialic acid receptor and the viral fusion (F) glycoprotein.

2.5. Rhinovirus

Rhinovirus infections account for the largest number of respiratory tract infections in children. However, rhinovirus infections produce mild symptoms compared to RSV, PIV, and influenzavirus. Most of the symptoms caused by rhinovirus are confined to the upper respiratory tract. Although present in the community the whole year round, rhinovirus infections peak at the onset of fall, which is probably related to schools starting after summer break. Rhinoviruses can cause severe lower respiratory tract infection (Guittet et al., 2003; Papadopoulos, 2004) and in immunocompromised patients, life-threatening pneumonia. Rhinovirus is a positive-stranded RNA-virus belonging to the Picornavirus family and over 100 serotypes exist, making it difficult to develop an effective vaccine. Rhinovirus subtypes have been divided into a major and minor group with respect to the receptor used for cell entry (Table 4.1). Major group rhinoviruses use epithelial intracellular adhesion molecule l (ICAM-1) for cell entry, while minor group viruses bind to the low-density lipoprotein (LDL) receptor. During rhinovirus infection, a predominant granulocyte and monocyte recruitments are observed. While specific antibody production occurs, it is probably not required for viral clearance, although neutralizing antibodies can provide some temporary protection against rhinovirus reinfection (van Kempen et al., 1999). ICAM-1 blocking antibodies have also been utilized and have been shown to decrease inflammation *in vitro*. There are, however, indications that rhinovirus can adapt to this with changes in receptor usage (Reischl et al., 2001).

2.6. Human Metapneumovirus

In 2001, a new respiratory virus was identified in the Netherlands causing infections similar to RSV in children (van den Hoogen et al., 2001). The reported incidence rate of human metapneumovirus (hMPV) infection in children with acute respiratory symptoms varies between 4% and 16%, of which three-quarters occur in children less than 1-year old (Williams et al., 2004). By the age of 5 years, approximately 70% of children have developed antibodies to hMPV. HMPV very much resembles RSV in its clinical spectrum, varying from coryza to bronchiolitis and

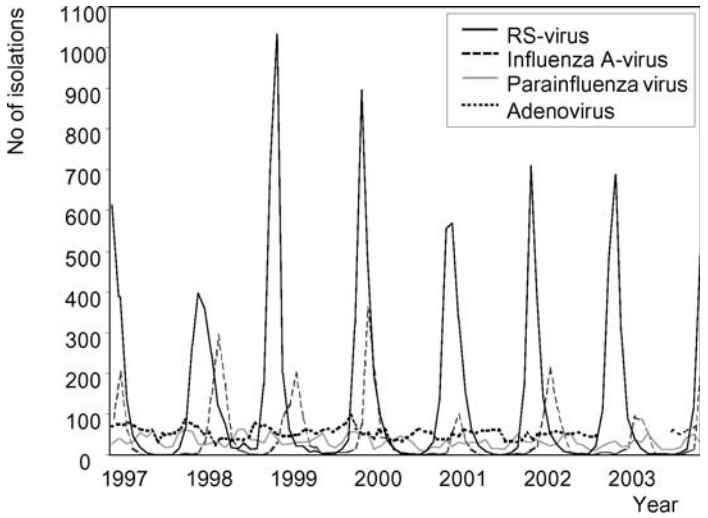

Figure 4.1. Epidemiology of RSV, influenza A, PIV, and adenovirus in the Netherlands. Data derived from the weekly reports of the sentinel system of the Dutch Working Group on Clinical Virology.

pneumonia. However, hMPV is less likely to cause pneumonia than RSV and influenza virus. Children with hMPV infection present less frequently with atypical symptoms such as vomiting, and on physical examination, rales and wheezing are found less often. Co-infection with RSV and hMPV does occur and has been suggested to result in more severe disease (Greensill et al., 2003). There is some evidence that secondary hMPV infection occurs frequently in childhood, probably accompanied only by mild symptoms (Ebihara et al., 2004).

Several investigators have found chemokine profiles during acute infection to be different in children with hMPV infections compared to those with RSV infections, with higher interleukin-8 (IL-8) and lower RANTES concentrations in hMPV patients. However in another study, inflammatory cytokine (IL-8, TNF-α, IL-1β) levels in respiratory secretions were 6-fold lower than in children infected with RSV (Jartti et al., 2002; Laham et al., 2004). Tthe physiological relevance of these observations remains unclear.

2.7. SARS Coronavirus

The outbreak of Severe Acute Respiratory Distress Syndrome (SARS), which started in late 2002 in East Asia, and spread throughout the world during that winter, was found to affect mainly health-care workers and close contacts of diseased individuals. SARS, which was proved to be caused by a new coronavirus, induces an atypical pneumonia with fever, dry cough, and shortness of breath. Many adults also suffer from myalgia, dizziness, chills, and rigors. It was concluded from postmortem examinations that SARS-pathology is primarily caused by immunological damage

to the lungs. The interstitial space of the lungs was mainly filled with mononuclear infiltrates and there was diffuse hemorrhage on the lung surface.

SARS coronavirus (SARS-CoV) spreads mainly via the respiratory route, the epithelial cell being its primary target cell. As for other coronaviruses, the spike proteins, S1 for cell entry and S2 for fusion, also seem to be important for SARS entry of host cells, despite the fact that SARS is only 20–30% homologous to other coronaviruses. Very recently, angiotensine converting enzyme (ACE2) was identified as the host cell receptor for SARS-CoV (Li et al., 2003) and surface expression of ACE2 on alveolar epithelial cells was demonstrated (Hamming et al., 2004).

Transmission of SARS-CoV occurs by droplets and most cases have occurred through close contact exposure. However, recently evidence of airborne transmission has emerged (Yu et al., 2004). Although many individuals were infected in the initial 2 weeks of the epidemic and the disease spread rapidly over several countries, the numbers of infected children stayed relatively low in all regions ($<5\%$). Furthermore, children tend to develop less severe disease. After an incubation period of 5–10 days, similar to adults, infected children developed symptoms of a mild upper respiratory tract infection, clinically indistinguishable from other common colds. None of the 100 pediatric SARS cases in Hong Kong turned out to be fatal and only one adolescent required mechanical ventilation (Leung et al., 2003). Adolescents are more likely to develop severe disease, as observed in adult SARS patients (Leung et al., 2004). A sore throat and a high initial and peak peripheral blood neutrophil count were found to be independent risk factors for severe disease in children with a laboratory-confirmed SARS infection. Furthermore, children seemed to spread the disease less easily to others and there have appeared no reports in the literature demonstrating transmission from children to other individuals. Many children with laboratory-confirmed SARS do not meet the WHO criteria for diagnosis of SARS. As children seem to have a much milder clinical course, the term "SARS" may not represent the disease in children very well.

3. Immunology of Viral Lower Respiratory Tract Infections

3.1. Innate Immunity

The role of the innate immune system in viral lower respiratory tract infection has not been studied intensively until recently. Studies have focused on the adaptive immunity with the goal of developing a vaccine, for example, for RSV. Understanding the mechanisms underlying primary and recurrent viral infection has attracted increased attention. The immunological response against viral invasion of the lower respiratory tract comprises both adaptive and innate immune response mechanisms with both beneficial as well as detrimental characteristics. The innate response occurs in the early phase of the infection and increasing evidence suggests that these early events determine disease course and possibly even long-term outcome (Garofalo and Haeberle, 2000; Tasker et al., 2000). For the rest of the discussion on immunological phenomena, focus will be on RSV as a prototype.

Epithelial cells are key regulators of the innate immune response against viral infections (Garofalo and Haeberle, 2000), producing a number of inflammatory

mediators in response to RSV infection. These include cytokines (interleukin-6, -1, tumor necrosis factor (TNF)-α), several chemokines (IL-8, macrophage inflammatory protein (MIP)-1α, monocyte chemotactic protein (MCP-1), RANTES), type-l interferon (IFN-α/β), and growth factors (GM-CSF, G-CSF). Epithelial-derived levels of chemokines correlate with disease severity (Bont et al., 1999; Smyth et al., 2002). Surfactant proteins produced by epithelial cells (SP-A and SP-D) may also play a role as opsonins for viruses and bacteria. Thus, epithelial cells provide a potential mechanism for serum-independent phagocytosis. Many of these mediators are induced both at the level of secretion and transcription. Interestingly, some mediators (e.g., IL-8 and RANTES) are also upregulated by inactive forms of the virus (Harrison et al., 1999).

RSV uptake by immune and non-immune cells is a receptor-mediated process. Experiments with blocking antibodies against G-protein revealed inhibition of binding of RSV to epithelial cells. The fractalkine receptor, also known as the CX3CR1 chemokine receptor, is involved in G-protein-mediated uptake by epithelial cells (Tripp et al., 2001). Other receptors may very well be involved in uptake by dendritic cells, macrophages, and other cells of the innate immune system (Harris and Werling, 2003), and Toll-like receptors (TLRs), especially TLR-4, are being investigated as possible candidates for mediating viral uptake (Haeberle et al., 2002a, b; Monick et al., 2003).

Several groups have demonstrated activation of the transcription factor NF-kB in RSV-infected epithelial cells (Tian et al., 2002). Many of the exhibited effects observed in epithelial cells can be explained by activation of NF-kB. Several cytokines associated with RSV infection have NF-kB binding sites in their promoter or enhancer regions (Bitko et al., 1997). Epithelial NF-kB activation has also been observed in other viral infections, including parainfluenza, influenza A, and rhinovirus (Pahl and Baeuerle, 1995; Kim et al., 2000; Bose et al., 2003). NF-kB could be an exciting target for therapy development and experiments in which BALB/c mice were treated with perflubron have confirmed this concept. Perflubron has already been shown to be effective in clinical trials of patients with respiratory distress syndrome because of its physical characteristics. Besides the beneficial physical effect in improvement of gas exchange and of lung compliance, this agent was found to have anti-inflammatory effects. RSV-infected BALB/c mice treated with perflubron intranasally showed a reduction in cellular inflammatory infiltrates and decreased chemokine expression in the lung tissue. Both the anti-inflammatory effects were directly linked to interference of perflubron with NF-kB-mediated transcription (Haeberle et al., 2002a, b).

The chemokines produced by epithelial cells attract T-cells, neutrophils, monocytes, and possibly eosinophils to the respiratory tract. Besides induction of secreted products, epithelial cells upregulate expression of adhesion molecules for neutrophils on their surface, allowing neutrophils to adhere firmly to infected cells (Wang and Forsyth, 2000). Furthermore, neutrophils are the dominant cell type found in bronchoalveolar lavage (BAL) fluid of RSV patients (Everard et al., 1994, McNamara et al., 2003). However, their role in fighting viral infection is not as well established as in bacterial infections. Pathological studies of lungs of RSV-infected calves have shown a major influx of neutrophils in the infected airway mucosa,

observed earlier than any other cell type involved. Furthermore, neutrophils are the dominant cell type found in bronchoalveolar lavage (BAL) fluid of RSV patients (Everard et al., 1994, McNamara et al., 2003)"

Several chemokines and cytokines involved in neutrophil activation have been associated with RSV lower respiratory tract infections. Recently, local neutrophil IL-9 production has been linked to RSV bronchiolitis (McNamara et al., 2004). As shown by Wang et al., a major increase in epithelial damage occurs, when RSV-infected epithelial cells are co-cultured with neutrophils (Wang and Forsyth, 2000). This is suggestive of a detrimental role for neutrophil-induced immunopathology in lower respiratory tract infections.

RANTES and MIP-1α are produced by the epithelium in response to RSV infection and these chemoattractants recruit eosinophils to the inflammatory site. The analogy between clinical features of virus-induced wheezing illnesses and asthma has made eosinophils an attractive subject for studies aimed at improving understanding of RSV pathogenesis. However, mainly because of their absence in BAL of RSV patients, their involvement remains controversial. However, eosinophil-derived cationic protein (ECP) has been linked to bronchiolitis and post-bronchiolitic wheezing (Garofalo et al., 1992; Pifferi et al., 2001; Dimova-Yaneva et al., 2004). *In vitro*, eosinophils have also been shown to be susceptible to RSV. Eosinophil priming, superoxide production, and degranulation were induced by incubation with RSV (Garofalo et al., 1992; Kimpen et al., 1992; Olszewska-Pazdrak et al., 1998; Tachibana et al., 2002). Rosenberg and Domachowske (2001) have suggested a beneficial role for eosinophils in RSV bronchiolitis. They identified antiviral properties for the eosinophil based on ribonuclease activity of eosinophil-derived neurotoxin (EDN) and ECP. This enzymatic activity leads to destruction of extracellular ssRNA virions and delayed replication both *in vitro* and *in vivo*.

Recently, there has also been great interest in the involvement of macrophages and dendritic cells in RSV pathogenesis. These cells were already appreciated for their role in antigen presentation, at which dendritic cells are by far superior. Macrophages express phagocyte activity, which may be of importance in clearance of infected epithelial cellular debris. Fascinating new players in host defense against viruses are pattern recognition receptors. Toll-like receptor-4 (TLR-4) and CD14, both present in a complex on these cells, have been found to interact with RSV and receptor-binding results in triggering of the innate immune system. TLR-4 has been shown to activate NF-kB in macrophages of RSV-infected mice (Haeberle et al., 2002a, b) and TLR-4-deficient mice have impaired NK-cell and CD14+ cell trafficking and delayed viral clearance (Haynes et al., 2001). Furthermore, intracellular pattern recognition receptors TLR-3 and -7, may be involved in recognizing double- and single-stranded RNA (dsRNA/ssRNA), respectively (Akira and Hemmi, 2003; Lund et al., 2004). dsRNA is produced during replication of RNA-viruses and is a potent inducer of IFN-α/β. All human cells can produce IFN-α/β in response to viral infection, while only T-cells and NK-cells produce IFN-γ. dsRNA also activates dsRNA-dependent protein kinase R (PKR) and NF-kB via distinct pathways. Transcription of PKR is under control of IFN-α/β. PKR controls enzymes directly involved in protein synthesis, thereby inhibiting cellular and viral protein translation. IFN-α/β-deficient mice as well as PKR−/− mice are extremely sensitive to

influenza infection (Balachandran et al., 2000). Several viruses, including RSV, have evolved mechanisms to escape the interferon system, which will be discussed below.

3.2. Adaptive Immune Response

Respiratory epithelial cells are the principal host cells for viral pathogens in lower respiratory tract disease. The degree of replication and the mechanism of spread along the epithelial layer depend on the virus family characteristics. Through the fusion (F) protein, RSV is capable of syncytium formation, which allows it to replicate and spread relatively undetected by the immune system for a relatively long period. The virus itself is directly responsible for cytopathology and viral envelope proteins are expressed on the surface of infected epithelial cells. Dendritic cells, lining the basal membrane of the respiratory epithelium encounter RSV, pick up viral antigens and migrate to mediastinal lymph nodes where viral antigen is presented to naïve CD4+ T-cells. Antigen presentation and co-stimulatory molecule expression lead to maturation to the T-helper phenotype. This then induces B-cell proliferation with the production of specific antibodies as well as proliferation of virus-specific cytotoxic CD8-cells.

Cellular responses are responsible for controlling and terminating acute infection with RSV. In primary infections, the adaptive cellular immune response develops within 10 days. These CD8+ cells can recognize and eliminate virus-infected epithelial cells resulting in perforin-mediated cytotoxity. Epithelial cells are nonprofessional antigen presenting cells (APC) expressing MHC Class l on the surface (Garofalo et al., 1996). When infected, epithelial cells present viral antigen in association with MHC Class l molecules. MHC Class l restricted antigen presentation to CD8+ cells, among other factors, may determine the strength of the cytotoxic response. In CD8-deficient mice, there is delayed viral clearance; however, these mice also exhibit decreased disease severity (Graham et al., 1991). Therefore, it is conceivable that CD8+ T-cells are crucial in viral clearance while a surplus of cytotoxicity may result in pulmonary injury.

In humans, a cytotoxic T-cell response is elicited against all viral proteins, except the G-(attachment)-protein, which is required for cell entry (Bangham et al., 1986; Hacking and Hull, 2002). It is suggested that a defective response against G-protein is directly associated with enhanced disease. However, G-protein can induce a CD4+ response in mice, which is associated with Th2-cytokine production and eosinophilia both during primary and secondary infection (Openshaw, 1995). The immune response to the F-protein is dominated by IFN-γ production and subsequent polarization toward a Th1-type cellular response, and therefore it has been postulated that responses to the other viral proteins can modulate the strong Th2-response to G-protein (Graham et al., 2000).

A stronger Th1-response seems to induce a more rapid viral clearance and milder disease (Bont et al., 1999; Legg et al., 2003). Besides activated T-cells, NK-cells also produce considerable amounts of IFN-γ (Hussell and Openshaw, 1998). IFN-γ has important antiviral effects and provides a link between adaptive and innate immune system. It can induce expression of TNF-related apoptosis inducing ligand (TRAIL) on immune cells, which has the potential to trigger apoptosis of

virus-infected cells (Sedger et al., 1999). *In vitro* findings suggest that RSV-infected cells *in vivo* are susceptible to killing by immune cells through the TRAIL pathway (Kotelkin et al., 2003). NK-cells are also thought to play a role in activating CD8+ cells, further modulating the degree of cytotoxicity (Hussell and Openshaw, 1998). In summary, in RSV lower respiratory tract infections, cytotoxic CD8+ T-cells are involved in viral clearance while the humoral response is required for the protection against reinfection. However, as has been discussed before, memory is incomplete and repeated infections with RSV are common. Both IgM and IgG as well as secretory IgA against RSV are formed in infants, and a more vigorous antibody response seems to be protective against RSV infections (Meurman et al., 1984; Welliver et al., 1989).

3.3. Immature Immune Response

RSV infections are most severe in the youngest age group, which is the least mature in terms of immunity to infections. Relative deficiencies in both innate and antigen-specific immunity in infancy have been characterized. These include delayed trafficking of immune cells, less-efficient antigen presentation by dendritic cells, and impaired production of IFN-γ by T-cells in response to antigen presentation (Bont and Kimpen, 2002).

The fetus derives maternal IgG-antibodies via the placenta fairly late in gestation. This partly explains why prematurity is an important risk factor for severe disease caused by RSV, as well as the physiological characteristics of the small airways. Antibody titers produced by infants are relatively low compared to older children. Trials with humanized monoclonal antibodies against RSV-F-protein have shown a 50% reduction in RSV lower respiratory tract-related hospitalizations in this high-risk group for severe disease (Impact Study Group, 1998).

The cytokine milieu at the time of infection is another factor possibly contributing to the occurrence of severe RSV bronchiolitis especially in the youngest age group. At birth, there is skewing toward a Th2-phenotype and RSV bronchiolitis was long thought to be a Th2-type disease. This role of Th2-skewing is an attractive concept, because it provides some explanation for the association between RSV bronchiolitis and the development of asthma. Asthma and allergy have long been acknowledged to beTh2-mediated conditions. However, convincing evidence that primary RSV infections are mediated by Th2 cytokines is lacking. Dendritic cells are thought to have an important function in skewing the Th1/Th2-ratio. Viruses may be important in maturation of dendritic cells, which can then drive differentiation of naive T-cells into either a Th2- or a Th1-phenotype. The role of regulatory T-cells that suppress both Th1 and Th2 differentiation has not been studied in RSV bronchiolitis so far.

3.4. Genetic Background

Gene polymorphism studies have been undertaken to identify a genetic background to explain the individual susceptibility to RSV lower respiratory tract infection. Several polymorphisms situated in genes relevant for the adaptive and innate immunity have been found to correlate with occurrence of RSV infection.

Polymorphisms of interleukin-4, IL-4R, and its receptor, have been associated with RSV bronchiolitis, which is consistent with the Th2-hypothesis (Choi et al., 2002; Hoebee et al., 2003). Very recently, a polymorphism of the gene coding for interleukin-10 (IL-10) was found as well (Hoebee et al., 2004). This is particularly interesting since IL-10 is a cytokine produced by T-regulatory cells and monocytes, thought to be primarily involved in development of allergy. Gene polymorphisms involved in innate immunity include surfactant proteins SPA and D (Lahti et al., 2002), the chemokine IL-8 (Hull et al., 2001), TLR-4 (Tal et al., 2004), and the chemokine receptor for RANTES and MIP-1α, CCR5 (Hull et al., 2003).

4. Virus Infection in the Immunocompromised Host

4.1. Immunocompromised Patients

Immunocompromised patients have a higher risk of developing severe disease from viral respiratory tract infections. In particular, the presence of defects in cellular immunity result in an increased duration of viral shedding and enhanced risk of developing severe disease.

Most cellular immunodeficiencies are iatrogenic in nature. An important cause is intensive immunosuppressive treatment. The number of pediatric patients undergoing organ or stem-cell transplantation is increasing and high doses of chemotherapeutic and immunosuppressive agents are often used in the pre- and posttransplant regimens. Immunosuppressive drugs are used in cancer treatment regimens and for a number of inflammatory conditions. Community acquired respiratory viruses such as RSV, rhinovirus, adenovirus, influenza A, influenza B, and the parainfluenza group are frequent causes of respiratory disease in these patients (Soldatou and Davies, 2003). Adenovirus infections have a particularly high risk of adverse outcome, mortality rates are high, and no effective treatment exists. The presence of lower respiratory tract infection and infection in the pre-engraftment phase of HSCT is believed to have a particularly poor prognosis (Khushalani et al., 2001). The risk of severe disease is higher during allogenic HSCT than autologous HSCT. Besides causing increased morbidity and mortality, respiratory tract infections are associated with a greater risk of delayed engraftment (Abdallah et al., 2003). In solid organ transplant patients, respiratory virus infections are also associated with a higher incidence of rejection (Wendt, 1997).

Prolonged shedding of respiratory viruses for weeks or months has been documented in HIV-infected adults and children. This has important implications for infection control in medical facilities. In addition, respiratory viral infection may result in increased HIV replication and, theoretically, HIV disease progression (King, 1997). In HIV-infected children, RSV infections are less limited by season (Madhi et al., 2000). However, generally, the course of RSV infections in HIV patients is not more severe, unless there is profound lymphopenia or pre-existing lung disease (Soldatou and Davies, 2003).

Other viruses may also cause respiratory complications in the immunocompromised patients. In particular herpesviruses, such as cytomegalovirus (CMV) and

varicella zoster virus, can cause severe pneumonia. With a CMV-negative donor and a CMV-positive recipient, there is an especially high risk of reactivation which may lead to severe disease. This reactivation also occurs with Epstein Barr virus (EBV), human herpes virus (HHV)-6, -7, and -8, although these are much less frequent causative agents of pneumonia.

4.2. Impaired Innate Immune System

The innate immune defense to viral respiratory tract infections consists of the mucosal layer, type 1 interferons, activated phagocytes, and NK-cells. The impact of primary defects in the innate immune defense has not been well documented. Phagocyte defects are primarily related to a higher incidence of bacterial infections. One indication that impaired phagocyte function also leads to increased severity of respiratory viral infection can be derived from a report of severe abnormalities on lung-CT-scans of RSV patients with phagocyte defects (Uzel et al., 2000). Interferon-gamma receptor deficiency, which may have implications for both the adaptive and innate immune system, has also been associated with increased susceptibility to viral respiratory pathogens (Dorman et al., 1999).

Chronic lung disease also increases susceptibility to respiratory viruses (Meert et al., 1989; Griffin et al., 2002). Premature patients with bronchopulmonary dysplasia are candidates for RSV-immunoprophylaxis because of their increased risk of developing severe lower respiratory tract infections.

In children with cystic fibrosis (CF), 39% are already hospitalized with respiratory virus infection in their first year of life. Furthermore, there is a correlation between viral infections in infancy and disease progression. Infants with CF suffering from a respiratory virus infection are at significant risk for lower respiratory tract disease, hospitalization, and deterioration in lung function that persists months after the acute illness (Hiatt et al., 1999). CF infants were found to be four times more likely to develop an LRTI compared with controls. It has been shown that CF-derived airway epithelial cells allow a higher degree of PIV replication and have an increased production of pro-inflammatory cytokines (Zheng et al., 2003). CF-derived epithelial cells are also unable to express NO-synthase 2, which results in a decrease production of nitric oxide (NO), which has antiviral capacity, reducing effects on replication. Furthermore, in CF-cells there is no viral induction of 2′5′-oligoadenylate synthetase (OAS), an enzyme that is normally induced by dsRNA and IFN-γ. OAS is involved in inhibition of cellular protein synthesis, thereby inhibiting viral replication.

5. Respiratory Viruses and Asthma

5.1. Virus Infections and Asthma Exacerbations

Respiratory viruses can be isolated from the secretions of approximately 75% of children and of more than half of adults during asthma exacerbations (Johnston et al., 1995; Lemanske, 2003). Recently, COPD exacerbations have also been attributed to viral infection by rhinovirus, RSV, and PIV (Seemungal and Wedzicha,

2003). The underlying mechanisms for this are, however, still a matter of debate. From experimental rhinovirus (RV) infections in humans, it has been shown that RV infection causes increased bronchoconstriction in atopic non-asthmatic and asthmatic individuals, while symptoms in normal individuals are relatively mild. This implies that induction of a wheezing episode requires both RV infection and a pre-existing tendency to develop allergic or asthmatic disease (Message and Johnston, 2001). RV-specific T-cell responses can be activated by either serotype-specific or shared viral epitopes. Cross-reactivity between RV-subtypes could result in vigorous T-cell responses and may amplify allergic inflammation.

Other proposed mechanisms linking viral infections to asthma exacerbation are epithelial dysregulation, airway remodeling, the immune response to virus, and alterations of neural responses (Message and Johnston, 2001; Gern, 2002).

Upregulation of ICAM-1-expression, which is the entry receptor for major group rhinoviruses, has been found in susceptible individuals. This may be one mechanism predisposing atopic individuals to RV-induced exacerbations. Rhinovirus can induce a number of inflammatory mediators (kinins, arachidonic acid) and cytokines (e.g., IL-1, IL-6, IFN-α/β, GM-CSF, TNF-α) that can further enhance inflammation. Th1 cytokines seem to have a general antiviral effect while a predominant Th2-cytokine response leads to enhanced disease, failure to clear the virus, and amplification of allergic inflammation (Message and Johnston, 2001). Eosinophil numbers were found to be increased in bronchial biopsies from both healthy and asthmatic human volunteers after experimental rhinovirus infection. This cell type is associated with allergic inflammation in the lung. In allergic rhinitis patients, the increased level of eosinophils in BAL even persisted for 6 weeks. These data suggest a potential role for eosinophils in virus-induced asthma, which can be either pathogenic or protective.

Virus-induced exacerbations of asthma tend to be resistant to treatment with corticosteroids and may require a different therapeutic approach. *In vitro*, blocking ICAM-1 has been tried with positive results, which may be of particular relevance to rhinovirus infections. The possibility of other immunomodulating drugs is being investigated and may be of significant benefit to future asthma treatment.

5.2. Viral Respiratory Tract Infections and the Inception of Asthma

A causal relationship between viral respiratory tract infections and asthma exacerbations is generally acknowledged. However, the suggestion that respiratory virus infection is a causal determinant in the development of asthma is highly controversial. According to the hygiene hypothesis, viral infection would be expected to have an inhibitory effect on the development of asthma (an allergy), and this is supported by a study from Matricardi et al. (1997), showing an inverse relation between hepatitis A seropositivity and atopy among soldiers. The hygiene hypothesis is based on the theory that the immune system is directed toward a more Th1-skewed immune response with each viral infection. However, this hypothesis is not supported by the observation that RSV infections, severe enough to cause bronchiolitis, are significantly associated with a higher incidence of asthma up to the age of 7–11

years (Stein et al., 1999; Sigurs et al., 2000). These data convincingly show a link between RSV bronchiolitis and recurrent wheezing in childhood. In a recent study, wheezing following RSV lower respiratory tract infection was found to develop independent of atopy (Bont et al., 2004). It may, however, be true that the transmission route, the organs involved, and exposure to microbial products may be important in determining the final effect of a virus infection on the development of asthma and allergy (Gern and Busse, 2002).

The link between RSV infection and atopy is even less clear than the one with recurrent wheezing and asthma, at least in humans. Animal studies have yielded conflicting results. One group found that RSV infection in mice enhances subsequent allergic inflammation (Schwarze et al., 1997), while others reported a decrease in allergic sensitization and BHR after RSV infection (Peebles et al., 2001). No proof exists that severe RSV infections are associated with atopy that persists into adulthood (Peebles, 2004).

A key question is whether the association with the development of asthma is merely an expression of increased susceptibility to both asthma and RSV-induced lower respiratory tract infections or whether true causality is involved.

The prevailing theory on this subject involves maturation effects of the Th1/Th2-balance. The system shifts from a Th2-polarization in fetal life, which is an optimal environment for the placenta, to a more balanced Th1/Th2-phenotype in adulthood. Most viruses are known to induce a Th1 cytokine response (IFN-γ). This theory states that when infections occur early in infancy, there is a reduced ability to react with an appropriate antiviral Th1-response. Low IFN-γ production may result in spread of the virus to the lower respiratory tract. This is in agreement with findings that in children with severe RSV lower respiratory tract infections, lower amounts of IFN-γ are produced (Bont, 2002). The dynamics of this shift toward a more balanced Th1/Th2 immune response may differ between individuals. Both environmental factors and genetic make-up may contribute to a slower maturation of Th1 competence in some individuals. Respiratory virus infections in infancy and an atopic sensitization to aero-allergens, both of which are related to Th2-skewed responses and intermittent wheeze, may than synergistically result in persistent wheeze (Holt and Sly, 2002). It is likely that more links between atopic sensitization and respiratory infections exist, while preventive RSV-IVIG treatment of children results in a decreased sensitization to aeroallergens as well. RSV-prophylaxis may therefore have a long-term benefit in the development of persistent wheeze (Piedimonte and Simoes, 2002; Wenzel et al., 2002).

Another theory linking viral infections in childhood to the development of asthma involves the pathologic effects of viral lower respiratory tract infections on airway physiology. Wall thickening with consequent increased resistance may predispose the airway to more infections and thus influence bronchial hyperreactivity (Bardin et al., 1992). However, it may also be true that small airways predispose to both asthma and airway symptoms from viral infections. Remodeling of the submucosal neural networks by RSV, as observed by Piedimonte et al., is also proposed to result in increased responsiveness to airway irritants (Piedimonte, 2002).

Walter et al. (2002) have proposed that paramyxoviral infection has the ability not only to induce acute hyperresponsiveness, but also to result in long-lasting

changes in airway behavior. From mouse-studies with a PIV (SeV), it has been concluded that viruses cause long-term effects in epithelial cells, associated with airway reactivity and goblet cell hyperplasia. Long-term effects are induced by the virus in the acute phase, and later on, the presence of virus is no longer required for the persistence of symptoms. It is speculated that primary paramyxoviral infection within the proper genetic background may result in chronic dysfunction of epithelial cell behavior. Their results have indicated that different mechanisms are responsible for the induction of the acute and the chronic response (Walter et al., 2002).

Several theories on the induction of asthma have thus been proposed. The current view is that virus infection modulates the development of an asthmatic phenotype in a susceptible host. The relationship is, therefore, not purely causal but certainly requires an intrinsic vulnerability.

6. Viral Evasion of the Immune System

The effectiveness of respiratory virus infections in the host depends partly on the ability to evade the immune system (Figure 4.2). While several viral evasion mechanisms have evolved, not all have been intensively studied in respiratory viruses.

6.1. Viral Entry

Viral entry into host cells is one of the first obstacles viruses have to overcome. Since the cell membrane is in principle impermeable to macromolecules, viruses

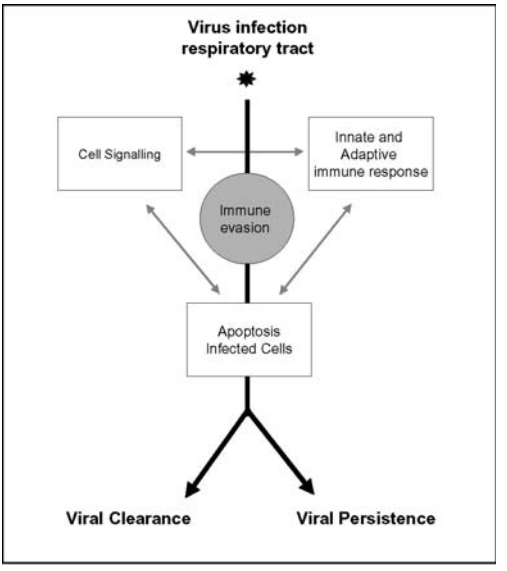

Figure 4.2. Evasion of the host response. The effectiveness of the virus in evading the host response mechanisms by interfering with cell signaling will determine whether the host is able to clear the virus or whether a state of viral persistence is established.

must first have an effective method to attach to the cell membrane. Some viruses bind putative cell surface receptors that do not simply play a role in viral attachment, but also allow viral entry by inducing endocytosis. For some viral pathogens, such as rhinovirus, these receptors have been identified (ICAM-1 and LDL-R), while for others, such as RSV, the receptor that is used for cellular entry has not been unequivocally defined. The CX3CR1-chemokine receptor, also known as fractalkine-receptor, may be involved and TLRs have also been proposed to play a role. Many enveloped viruses have glycosylated proteins, which not only bind to cellular receptors, but also have additional functions as membrane fusion factors, or receptor-destroying enzymatic activity. Membrane fusion factors such as the RSV F-protein also allow cell-to-cell transmission of virus, which keeps it relatively hidden from the cellular immune system (Smith and Helenius, 2004).

6.2. The Interferon Antiviral Response

Escaping the "interferon signaling system" is one of the common mechanisms most viruses have acquired. As mentioned earlier in this chapter, both IFN-α/β and IFN-γ have potent antiviral properties. Both types of interferon regulate transcription of a variety of target genes through activation of interferon inducible transcription factors. IFN-stimulated genes encode a variety of cellular enzymes, including PKR and 2′5 ′-oligoadenylate synthetase, both involved in inhibition of viral protein synthesis. Furthermore, interferons induce cellular apoptosis and upregulate MHC1 expression, targeting cells for CD8+ T-cell-mediated cytotoxicity. Additionally, IFN-γ activates the adaptive cellular immune system.

RSV infection leads to an increase in IFN-α/β, but does not induce IFN-γ from mononuclear and NK-cells as efficiently. Despite the fact that interferons are known for their antiviral properties, intranasal administration of either lFN-α/β or IFN-γ in the airway does not lead to reduction of symptoms of viral respiratory infections (Ramaswamy et al., 2004). This is suggestive of a viral mechanism to evade the host's interferon response.

The Paramyxoviridae family (Figure 4.3), responsible for a large part of children's respiratory infections, consists of two subfamilies based on the structural differences in the gene encoding for the polymerase complex (P-protein). The Paramyxovirinae subfamily members are able to block interferon-mediated promoter activity. These paramyxovirinae, to which parainfluenza-, measles-, and mumps virus belong to, have a P-gene that encodes for additional proteins besides the P-protein, the V-proteins. It is these V-proteins that have been found to be responsible for evading the interferon signaling pathways in this group of viruses. IFN-mediated transcription is predominantly mediated by signal transducers and activators of transcription (STAT). V-proteins have the ability to block interferon-mediated signaling by targeting STATs for proteosomal degradation (Horvath, 2004).

In contrast, RSV, belonging to the Pneumovirinae subfamily, fails to inhibit IFN-induced promoter activity. The pneumovirinae consist of only one genus, the pneumovirus, which also includes hMPV. In this subfamily, P-genes only encode for the P-protein and therefore RSV cannot block interferon-mediated signaling (Young et al., 2000). However, recently it was demonstrated that, although RSV does not

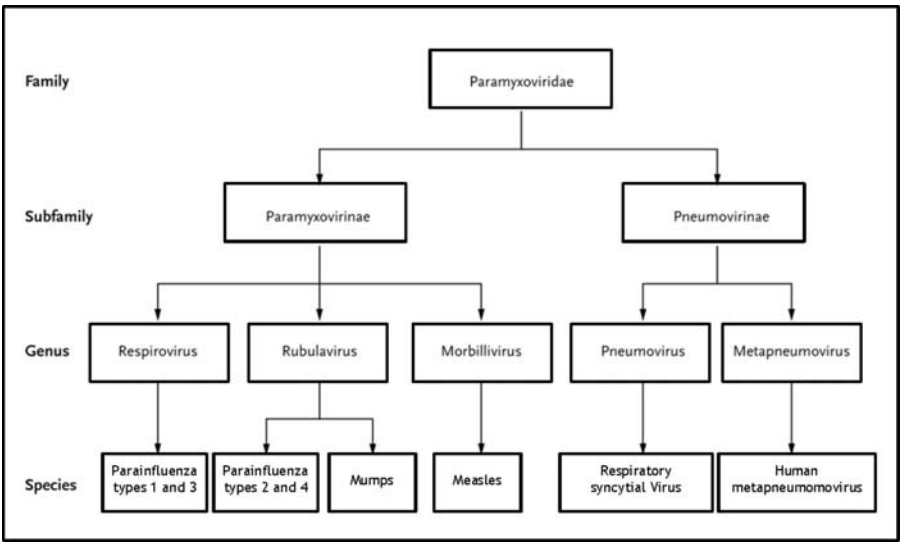

Figure 4.3. Classification of viral pathogens of the Paramyxoviridae family that infect humans. From McIntosh and McAdam (2004).

inhibit interferon-induced promoter activity, RSV replication is still resistant to IFN treatment of infected cells. Apparently, an alternative mechanism to circumvent the interferon antiviral response exists. This has been attributed to additional proteins, characteristic of these pneumoviruses (Spann et al., 2004). These are nonstructural proteins (NS1 and NS2) that have no homologs in the paramyxovirinae. However, the underlying molecular pathway has not yet been elucidated.

6.3. Evasion of Apoptosis

RSV infection of epithelial has been shown to lead to an upregulation of TRAIL-receptor expression on these cells (Kotelkin et al., 2003). Apoptosis of infected cells is an effective way to eliminate intracellular pathogens without damage to the surrounding tissue (Figure 4.4). However, several respiratory viruses have developed mechanisms to inhibit apoptosis. It has been demonstrated that RSV is able to effectively inhibit apoptosis of epithelial cells *in vitro*, in accordance with the limited pathology induced by RSV in epithelial cells during the first few days of the infection (Thomas et al., 2002). Eventually, necrosis is observed when mature viral particles are released from the cells, after 2–3 days. Furthermore, experiments with both adult and cord blood monocytes have shown a prolonged longevity of cells, when cultured in the presence of RSV (Krilov et al., 2000).

6.4. Immune Evasion Techniques by Adenoviruses

Of all respiratory viruses, viral evasion techniques of adenoviruses have been studied most intensively. It appears that approximately a third of the adenovirus

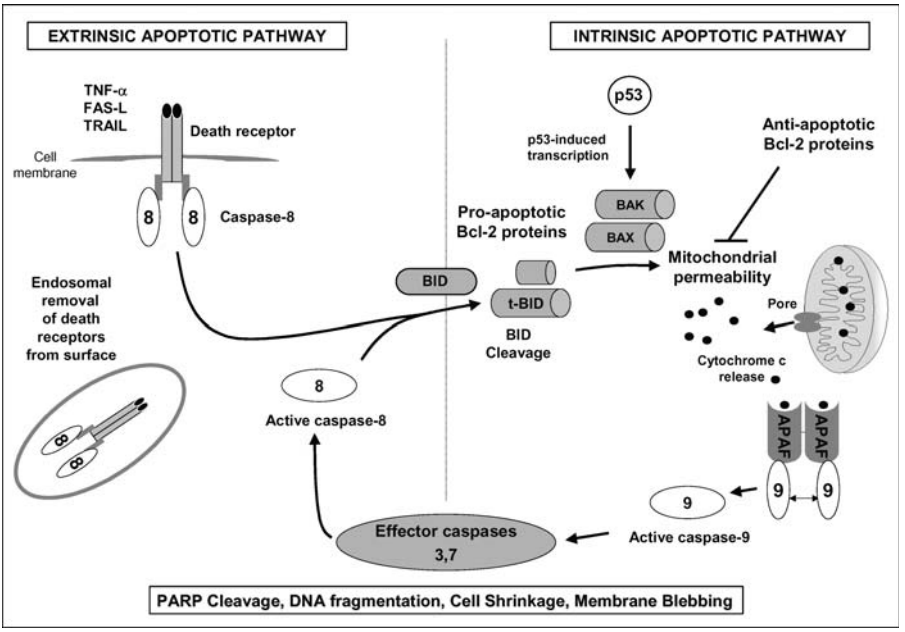

Figure 4.4. Extrinsic and intrinsic signaling pathways of apoptosis. Some viruses such as adenovirus interfere with cellular apoptosis. Cellular apoptosis can normally occur via activation of the extrinsic (death receptor) pathway or the intrinsic pathway. Both lead to activation of effector caspases 3 and 7, which will inevitably result in apoptosis. Via production of external factors, such as TNF-α, Fas-Ligand, and Trail, death-receptors are cross-linked, which leads to apoptosis. Adenovirus E3 proteins can remove these death receptors from the surface, thereby inhibiting extrinsic apoptosis. The intrinsic pathway is regulated by pro- and anti-apoptotic proteins of the Bcl2-family. The transcription factor p53 has anti-carcinogenic activity, induces the intrinsic pathway and promotes the transcription of pro-apoptotic proteins such as Bax and Bak. These proteins induce mitochondrial leakage of cytochrome *c*, which activates caspase 9, finally leading to effector caspase activation and apoptosis. Adenovirus proteins are involved in both inhibition of p53 and the functioning of Bax and Bak.

genome is devoted to counteracting innate and adaptive immune defenses (Burgert et al., 2002). Adenoviruses encode the protein E1A that blocks interferon-induced gene transcription. Through the VA-RNA protein that blocks activation of PKR, they also interfere with the antiviral enzymes that are synthesized under interferon control. Additionally, adenoviruses have developed several mechanisms to inhibit both constitutive and death receptor induced apoptosis (Figure 4.4). The E1B/55K protein inhibits p53-mediated apoptosis, E3/19K interacts with pro-apoptotic proteins Bax and Bak, whereas several E3 proteins are involved in removing Fas and Trail (death) receptors from the cell surface by promoting their degradation in lysosomes (Wold et al., 1999). Finally, adenoviruses interfere with recognition of infected cells by cytotoxic lymphocytes. The adenovirus E3/19K protein inhibits transport of MHC1 molecules to the cell surface, resulting in decreased viral antigen presentation to CD8+ cells.

Future studies may unravel further mechanisms of immune evasion that may also be important in viruses involved in lower respiratory tract disease. This knowledge

is likely to be crucial to the improvement of immunotherapies for prevention and treatment of viral respiratory tract infections.

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5

Chronic Granulomatous Disease: From Genetic Defect to Clinical Presentation

Johan Bylund, David Goldblatt, and David P. Speert

1. Introduction

Chronic granulomatous disease (CGD) is a rare, primary immune deficiency rendering the affected individuals hypersusceptible to bacterial and fungal infections. The underlying defect is an inherited inability of the patients' phagocytes to produce the reactive oxygen species (ROS) that are necessary for the full antimicrobial action of these cells. The ROS are normally produced by a membrane-bound enzyme complex, the NADPH-oxidase. Certain mutations in genes encoding this enzyme result in a non-functional oxidase and thereby abrogated ROS production and diminished bactericidal capacity. In addition to increased susceptibility to infection, CGD patients also regularly suffer from a number of different inflammatory complications, such as granuloma formation, inflammatory bowel disease, and lupus-like syndromes. These latter symptoms likely stem from what seems to be a propensity for exaggerated inflammatory reactions.

CGD was first described in 1950s (Berendes et al., 1957; Landing and Shirkey, 1957; Bridges et al., 1959), but the mechanisms to explain the increased susceptibility to infection remained unknown until evidence of defective neutrophil killing (Quie et al., 1967) and the absence of a respiratory burst in these cells (Holmes et al., 1966) was presented in mid-1960s. CGD is a rare disease and the overall incidence appears to vary geographically. Estimates range from 1 in 450,000 in Sweden (Ahlin et al., 1995) and 1 in 220,000 in Japan (Ishibashi et al., 2000) to 1 in 200,000 in the USA (Winkelstein et al., 2000), although it is likely that the actual figures are slightly higher since the disease may be under diagnosed or under reported.

In the early days after clinical characterization of the syndrome, the mortality of CGD patients was very high and a survey including the first 92 reported patients found 45 fatalities with the majority of deaths occurring before the age of 7 (Johnston and Baehner, 1971). Indeed in early reports, it was designated "fatal

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chronic granulomatous disease of childhood" (Bridges, 1959). Although this rather dreary outlook has improved considerably since then, with lower mortality rates and a longer average life expectancy (Winkelstein et al., 2000), CGD remains a very serious condition with profound morbidity and excess mortality.

The reason for the enhanced susceptibility to infection is clearly the lack of a functional NADPH-oxidase, resulting in defective phagocytic microbial killing. The exact mechanism by which the generation of ROS actually contributes to the killing is, however, a matter of debate. Furthermore, it is also unclear how the dysfunctional generation of ROS leads to enhanced inflammatory responses, although numerous hypotheses trying to reconcile the two have been presented. This chapter will deal with generally accepted facts concerning the leap from genetic abnormality to clinical presentations and review current controversies in these matters. Furthermore, it will provide an overview of existing and future therapeutic options and describe the gaps in our current understanding of CGD.

2. Biochemistry and Genetics of the NADPH-oxidase and its Deficiency

The leukocyte NADPH-oxidase, the defective oxidase in CGD, is found mainly in professional phagocytes of which the polymorphonuclear granulocyte, or neutrophil, is the most prominent. Other cell types expressing the NADPH-oxidase include monocytes, macrophages, other granulocytes, dendritic cells, and B lymphocytes (Babior, 1999). Neutrophils are the cells most commonly used for studying the NADPH-oxidase, mainly because of their crucial role in host defense and inflammation, but also due to their abundance in the peripheral blood and the relative ease with which these cells can be purified *in vitro*. The neutrophils are the most abundant cells among the human blood leukocytes and play an important role in combating the early stages of infection as well as in disposing of cell debris upon tissue damage. The recruitment of neutrophils to sites of infection or inflammation is a rapid process dependent on directed cellular migration: chemotaxis. This migration occurs along a gradient of chemical mediators and chemoattractants, of both exogenous and endogenous origin. After reaching the site of infection, the neutrophils phagocytose microbes and attack them with an impressive arsenal of antimicrobial substances and degrading enzymes in order to kill the invaders and prevent further spread. A proper and tightly controlled regulation of this process is of utmost importance, and failure to blunt the inflammation appropriately may cause serious tissue damage and result in a variety of inflammatory disease states.

The ROS formed as a result of NADPH-oxidase activation comprise one branch of the antimicrobial arsenal of neutrophils, often termed the oxygen-dependent branch. The neutrophils harbor two pools of NADPH-oxidase, one localized in the plasma membrane that releases ROS extracellularly upon activation, and one localized in the granule membranes that generate intracellular ROS (Dahlgren and Karlsson, 1999). After phagocytosis, the phagosome membrane contains NADPH-oxidase, partly derived from the plasma membrane, but also derived from the membranes of intracellular granules that fuse with the phagosome. In fact, subcellular fractionation studies have shown that only a minor fraction (approximately 5%) of a

resting cell's NADPH-oxidase resides in the plasma membrane and the remainder is found in the membranes of intracellular granules (Borregaard et al., 1983; Sengelov et al., 1992). Intracellular ROS can apparently also be generated without the formation of a phagosome (Karlsson and Dahlgren, 2002), but the significance of such intracellular ROS has not been clearly established, although they have been implicated as signaling molecules as will be discussed below. While ROS constitute the oxygen-dependent branch of the antimicrobial defense, the oxygen-independent branch includes antibacterial peptides/proteins and catalytic enzymes. Whether these systems work independently or are in reality two sides of the same story is currently a focus of considerable debate (discussed below). However, it is generally agreed that neutrophils from CGD patients are defective in killing various microbes and that this defect is due to the lack of a functional NADPH-oxidase.

The NADPH-oxidase is a complicated enzyme system with a core enzyme comprising at least five different components that are, in part, separated from each other in the resting cell (Figure 5.1). The membrane-bound subunits gp91^{phox} (the suffix PHOX stands for PHagocyte OXidase) and p22^{phox} together form a heterodimeric flavohemoprotein called cytochrome b_{558}. The cytosolic components p40^{phox}, p47^{phox}, and p67^{phox} exist as a complex that migrates to the membrane upon activation and associates with cytochrome b_{558} to form the active NADPH-oxidase. The proper assembly of these subunits into an active enzyme is a highly regulated process that also seems to involve one or more low-molecular weight guanine nucleotide binding proteins (G-protein) (reviewed in Babior, 1999; Clark, 1999).

The predominant form of CGD is a result of one of several known mutations in the gene encoding the gp91^{phox} subunit (Table 5.1). This gene is located on the X chromosome, and gp91^{phox} mutations are therefore inherited in an X-linked recessive (XLR) manner with males predominately affected. There are rare cases of female CGD patients with mutations in gp91^{phox}, expressing the CGD phenotype as a result

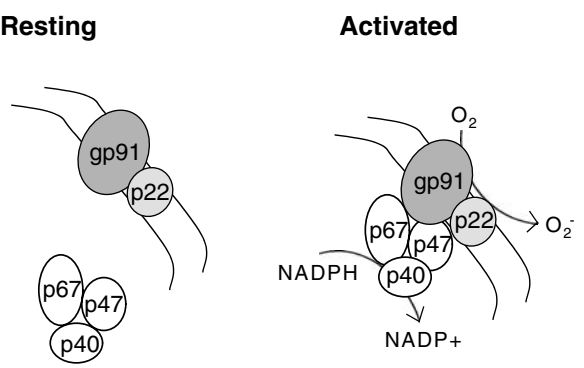

Figure 5.1. The NADPH-oxidase is a complex enzyme system comprising five different components that are separated in the resting cell (left). The cytochrome b_{558} consists of two subunits, gp91^{phox} and p22^{phox}, which reside in the membrane as a heterodimer. The cytosolic components p40^{phox}, p47^{phox}, and p67^{phox} exist as a complex that upon activation migrates to the membrane and associates with cytochrome b_{558} to form the active NADPH-oxidase (right), which transfers electrons from cytosolic NADPH to molecular oxygen on the other side of the membrane (extracellularly or intraphagosomally/intragranular).

Table 5.1. Genetic Deficiencies of 368 CGD Registry Patients

Genetic deficiency	Inheritance	% of patients
$gp91^{phox}$	XLR	70.4
$p22^{phox}$	AR	1.9
$p47^{phox}$	AR	12.2
$p67^{phox}$	AR	2.7
Unknown		12.8

XLR, X-linked recessive; AR, autosomal recessive. Data adapted from Winkelstein et al. (2000).

of skewed X chromosome inactivation (Anderson-Cohen et al., 2003). Mutations in the second cytochrome b_{558} subunit, $p22^{phox}$ are the genetic cause of only a small fraction of CGD cases, which are inherited in an autosomal recessive (AR) manner, affecting both sexes equally. Patients with molecular lesions in either $gp91^{phox}$ or $p22^{phox}$ typically lack expression of both cytochrome components in the membrane, indicating that expression of each subunit is a requirement for stable expression of the other (Parkos et al., 1989). Mutations in the cytosolic subunits $p47^{phox}$ or $p67^{phox}$ both display AR inheritance and cause the remaining CGD cases. To date, no individual with a mutation in $p40^{phox}$ has been described. Recently, a patient was described with a mutation in Rac2, one of the G-proteins involved in the regulation of the NADPH-oxidase (Kurkchubasche et al., 2001). This patient's neutrophils displayed a number of functional defects, including non-functional actin assembly, aberrant directed and random migration, impaired degranulation, and dysfunctional phagocytic ingestion and killing. However, his neutrophils were able to produce normal levels of ROS in response to phorbol myristate acetate (a protein kinase C agonist), even though the ROS production in response to other types of stimulation was impaired. Hence, this patient's leukocytes were clearly capable of producing ROS and it is thus questionable whether this Rac2 mutation should be regarded as CGD. The mutations resulting in CGD can be of a variety of different types, including deletions, frame shifts, and missense, nonsense, splice region, or regulatory region mutations (Ahlin et al., 1995). An exception is $p47^{phox}$ AR CGD that, in almost all cases, can be ascribed to the same GT deletion (Roesler et al., 2000). As a group, patients with AR CGD have a less severe disease phenotype than individuals with XLR disease. The latter display higher prevalence of infections, earlier age of diagnosis, and a significantly higher mortality compared to the AR group (Winkelstein et al., 2000). The basis for this difference is not totally clear, although evidence indicating that $p67^{phox}$ can make up for a lack of $p47^{phox}$ (Cross and Curnutte, 1995) and the fact that phagocytes from $p47^{phox}$—deficient patients are capable of producing minute, but detectable, amounts of ROS (Winkelstein et al., 2000) seem to imply that a small, residual ROS production could be of clinical significance.

3. Animal Models of CGD

As always when developing animal models for human disease conditions, the fact that fundamental differences may exist between humans and the model species

has to be taken into account. Although murine models are used extensively to study various aspects of neutrophil function, mice and men display several important differences regarding these cells. For instance, neutrophils are by far the most abundant white blood cells in humans, making up a total of 60–70% of the peripheral blood leukocytes, but are considerably less abundant in murine blood, contributing only approximately 10–15% (Chervenick et al., 1968; Fecho et al., 1998). Furthermore, murine neutrophils seem to lack the capacity to generate intracellular ROS, at least in the absence of phagosome formation (Bylund et al., 2003). The reason for these disparities are speculative but may implicate a different, and perhaps less critical role of neutrophils in murine immunity. Nonetheless, murine models of CGD have been developed by targeted disruptions of the genes encoding either $gp91^{phox}$ or $p47^{phox}$ in embryonic stem cells, resulting in XLR and AR CGD, respectively (Jackson et al., 1995; Pollock et al., 1995). The affected mice lack phagocyte production of ROS and display both an increased susceptibility to infections and exaggerated inflammatory responses, indicating that the models mimic the human disease fairly well. CGD mice exhibit a marked increase in susceptibility to microorganisms often isolated from CGD patients, including *Burkholderia cepacia* complex bacteria, *Aspergillus* spp., *Staphylococcus aureus* and *Salmonella typhimurium*, but also to other species like *Listeria monocytogenes* that are not a typical CGD pathogens (Dinauer et al., 2000).

CGD mice display an increase in neutrophil exudation in response to intraperitoneal thioglycolate challenge as compared to wild-type mice (Jackson et al., 1995; Pollock et al., 1995). In addition, after administration of sterilized *Aspergillus* hyphae to the lungs of $gp91^{phox}$ knockout mice, an exaggerated inflammatory response was seen that developed over time into chronic granulomatous lesions (Morgenstern et al., 1997).

The generation of reactive nitrogen species via inducible NO synthase (NOS2) is another oxygen-dependent antimicrobial system deployed by murine, and possibly also by human, phagocytes (Nathan, 1997). Mice with deficiencies in both NOS2 and the NADPH-oxidase ($gp91^{phox}$) exhibit high rates of spontaneous infections with a variety of commensal enteric bacteria, infections rarely encountered in mice with either one of these defense systems intact (Shiloh et al., 1999). Thus it appears that, at least in mice, these two oxidant-dependent antimicrobial systems are able to compensate for one another to some extent in controlling infections. Whether or not NO species play as important a role in human as in murine immune defense is a matter of debate; but as mentioned below, increased NO production has been proposed as one mechanism behind the decreased infection rates seen in CGD patients on IFN-γ prophylaxis (Ahlin et al., 1999). Moreover, although murine models provide invaluable tools for investigating various pathophysiological responses in CGD, the controversy over the significance of NO in humans reemphasizes the importance of not directly translating results from an animal model into the human setting.

4. Roles of ROS in Health and Disease

4.1. Microbial Killing

While it is quite clear that the inability of CGD neutrophils to produce ROS results in incompetence of these cells to kill certain microbes, the actual microbicidal

mechanism of ROS is still a subject of debate. The molecule generated by the activated NADPH-oxidase is superoxide (O_2^-), an oxygen radical that has low bactericidal potency. The O_2^- generated inside the neutrophil phagosome is converted to more toxic ROS, through conversion to H_2O_2, which is further processed into more bactericidal metabolites (Hampton et al., 1998). An example is the reaction between H_2O_2 and Cl^- to generate HOCl (also known as bleach), a very toxic compound to almost all microbes, but also quite short-lived. This reaction is catalyzed by the enzyme myeloperoxidase (MPO) that is a major constituent of the neutrophil azurophil granules (Borregaard and Cowland, 1997). The azurophil granules fuse with the phagosome during phagocytosis and thus deliver MPO to a site where it can catalyze the formation of HOCl in the proximity of the ingested microbe (Figure 5.2). Given the central role of MPO in the formation of bactericidal ROS, it is surprising that subjects with total or partial MPO deficiency—a condition far more common than CGD—generally do not display increased frequency of infections and are quite often asymptomatic (Lanza, 1998). This fact, together with other observations, has given rise to speculation that the ROS *per se* do not exert any direct bactericidal effects, but rather have indirect killing effects executed through activation of

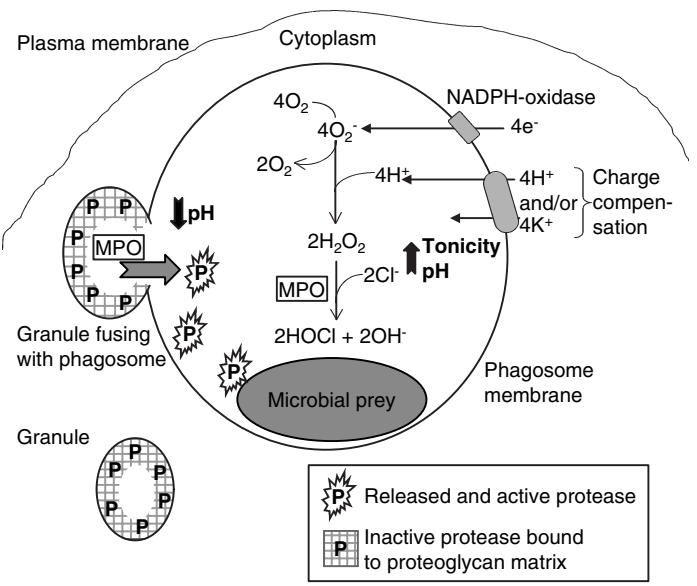

Figure 5.2. After phagocytosis of a microbial prey (dark gray) the NADPH-oxidase in the phagosomal membrane is activated transporting electrons over the membrane. This results in the generation of superoxide inside the phagosome that is converted into a variety of microbicidal ROS, such as HOCl catalyzed by the enzyme myeloperoxidase (MPO) that is delivered to the phagosome from azurophil granules. The electron transport across the phagosomal membrane represents a charge transfer that is compensated for by an influx of positively charged ions. This renders the phagosome hypertonic, which promotes the release and activation of granule proteases otherwise inactive and bound to an anionic proteoglycan matrix.

phagosomal proteases. Segal and co-workers recently published data showing that mice deficient in the granule proteases elastase and cathepsin G were ineffective at eradicating infections even though their ROS production was normal (Reeves et al., 2002). The microbicidal activity of human neutrophils in the presence of protease inhibitors was also inhibited to the same levels as CGD cells or cells treated with the NADPH-oxidase inhibitor DPI. These findings were not particularly unexpected and merely seem to suggest that both ROS and protease activities are required for optimal microbicidal activity. In normal neutrophils, the pH in the phagosome rises from 6 to 8 after formation, concomitant with the production of ROS. This pH increase occurs despite the fact that the phagosome fuses with azurophilic granules in which the pH is acidic (Segal et al., 1981). In contrast, phagosomes in CGD neutrophils do not display this rise in pH, suggesting that the NADPH-oxidase not only ferries electrons across the membrane but also transports protons in the opposite direction to compensate for the charge separation (Henderson and Meech, 2002). Segal and co-workers went on to show that the influx of protons is not enough to fully compensate for the electron influx and part of the charge compensation is due to a massive influx of K^+ rendering the phagosome hypertonic (Reeves et al., 2002). Neutrophil proteases such as elastase and cathepsin G are normally adsorbed to a highly charged acidic proteoglycan matrix and are in this state inactive and thus inhibited from destructive activation in the resting cell (Kolset and Gallagher, 1990). The hypertonicity generated by the K^+ influx is instrumental in releasing the proteases from the proteoglycan matrix, only once they are unleashed and active, can they attack the ingested microbe (Figure 5.2). Thus, according to the hypothesis of Reeves et al., the requirement for NADPH-oxidase activity in the killing of ingested microbes would not be direct, but instead indirect by ensuring that K^+ is transported into the phagosome to achieve the hypertonicity needed to activate the proteolytic enzymes. This view is highly controversial and there is currently a major debate as to whether ROS contribute directly to the phagosomal killing or not (Roos and Winterbourn, 2002). The theory mentioned above also needs to be elaborated further to explain how neutrophils kill bacteria under anaerobic conditions (Hampton and Winterbourn, 1995) and how CGD cells are capable of killing certain bacteria at all (Speert et al., 1994). Furthermore, a model in which ROS only provide means for the generation of hypertonicity in the phagosome does not leave a functional role for MPO. Segal and co-workers argue that MPO primarily acts as a detoxifying agent for H_2O_2 in order to protect the granule proteases from oxidative degradation (Reeves et al., 2002), although convincing evidence for this is lacking and several groups have presented data implicating a more important role for MPO in the killing of various microorganisms (Lehrer et al., 1969; Humphreys et al., 1989; Hampton et al., 1996; Aratani et al., 2002a, b).

It is likely that ROS do have direct antimicrobial effects, albeit perhaps less pronounced than hitherto thought, and efficient killing is most probably dependent on synergistic effects and co-operation among the several microbicidal effectors with which phagocytes are equipped. Whether or not ROS are directly bactericidal, it is clear that the lack of a functional NADPH-oxidase results in defective killing, which in turn explains the increased susceptibility to infection displayed by CGD patients.

4.2. Cell Death Processes

An explanation for the inflammatory complications often seen in CGD patients remains obscure. A common feature of these conditions is an abnormally exuberant inflammatory response perhaps due to dysfunctional negative feedback, resulting in improper termination of the inflammatory process.

One explanation proposed for heightened inflammation is based on the observation that ROS appear to be important mediators of cell death processes in neutrophils. Apoptosis, or programmed cell death, is the process by which "cellular corpses" are disposed of by the body in a physiological manner. Neutrophils are quite short-lived cells with a high turnover rate and they continuously undergo spontaneous apoptosis in a matter of hours to days in the tissues (Akgul et al., 2001). Since these cells are steadily produced in high numbers in the bone marrow, the apoptotic process is critical to the maintenance of cellular homeostasis, and changes in the rate of apoptosis can rapidly alter total cell numbers. Thus, in many bacterial and autoimmune inflammatory diseases, one important mechanism for neutrophil accumulation is delayed apoptosis (Simon, 2003). A hallmark of neutrophil apoptosis is the exposure of phosphatidyl serine on the cell surface. This molecule (among others) is recognized by other phagocytes, mainly macrophages, which subsequently remove the dying cells from the tissues. Neutrophils are filled with potentially tissue destructive and/or pro-inflammatory substances, and the phagocytic removal of intact, apoptotic neutrophils prevents the dying cells from releasing their cytotoxic content into the extracellular milieu, a release that would occur if the cells died by necrosis (Savill et al., 1989).

Many different factors have been described as modulators of the apoptotic rate. Anti-apoptotic stimuli ("survival signals") include inflammatory cytokines (e.g., IFNγ and microbe-derived molecules (e.g., lipopolysaccharide). Pro-apoptotic ("death signals") includes oxidative stress, radiation, and ligation of surface-located death-receptors (Akgul et al., 2001). Of special relevance in CGD are the findings that intracellular ROS production accelerates neutrophil apoptosis (Lundqvist-Gustafsson and Bengtsson, 1999) and that CGD neutrophils display delayed spontaneous apoptosis *in vitro* (Kasahara et al., 1997; Yamamoto et al., 2002). The vast amounts of ROS generated during phagocytosis have been implicated as important mediators of neutrophil apoptosis, and the uptake of a number of different microbes induces apoptosis in a radical-dependent manner (Watson et al., 1996; Rotstein et al., 2000; Perskvist et al., 2002; Kobayashi et al., 2003). The majority of these studies have employed neutrophils from normal donors in which ROS production was inhibited with an NADPH-oxidase inhibitor or various antioxidants to demonstrate that inhibition of ROS production inhibits apoptosis. In two studies, CGD neutrophils were used to confirm the importance of ROS in neutrophil apoptosis following phagocytosis (Coxon et al., 1996; Yamamoto et al., 2002); although ROS appear to be important in mediating apoptosis, there are alternative signaling systems present. Thus, CGD cells still become apoptotic, but the process is delayed as compared to control cells.

In a very recent paper by Kobayashi et al. (2004), microarray technology was used to assess differences in global gene expression between normal and CGD neutrophils. The results may provide some insight into the mechanisms behind

ROS-mediated apoptosis. After phagocytosis of opsonized latex beads, there were significant changes in 11 genes involved in apoptosis in normal neutrophils not observed in cells from patients with XLR CGD. Among the genes that failed to be up-regulated in CGD cells was BAX, a gene encoding a protein that plays a key role in the execution of apoptosis in eukaryotic cells (Oltvai et al., 1993) and contributes to neutrophil-mediated inflammation (Dibbert et al., 1999). Normal and CGD neutrophils expressed similar constitutive levels of BAX, but only normal cells increased the expression in response to phagocytosis (Kobayashi et al., 2004). Nonetheless, the molecular mechanisms behind the regulation of BAX and other important genes, in the regulation of which ROS appears to play a role, still remains to be elucidated.

Delayed apoptosis in CGD neutrophils is often regarded as a pro-inflammatory event, but there are also other closely related processes that could play important roles in the same setting. The clearance of apoptotic neutrophils by macrophages is a necessary step in termination of an inflammatory reaction. Dying phagocytes that are not disposed of will eventually become necrotic and leak both pro-inflammatory and/or tissue destructing substances. Thus, both apoptosis and clearance are probably anti-inflammatory in character and abnormalities in either of these processes could potentially result in the exuberant inflammatory responses characteristic of CGD. Recent work suggests that not only do apoptotic neutrophils have to expose phosphatidylserine on their surface in order to be cleared by macrophages, but also that this phospholipid has to be oxidized for efficient recognition to occur (Kagan et al., 2002). However, a lack of phosphatidylserine oxidation in CGD cells has not been shown, despite evidence for the proposed importance of NADPH-oxidase in this process (Arroyo et al., 2002). In fact, there is no published evidence of defective macrophage clearance of apoptotic neutrophils in CGD patients, although data suggesting such a defect have been obtained in the murine $gp91^{phox}$ knockout model (Hampton et al., 2002).

Another finding that also implies an abnormality in the clearing of apoptotic neutrophils in CGD was recently presented. The uptake of apoptotic neutrophils by macrophages is associated with the production of anti-inflammatory cytokines important for terminating the inflammatory process (Fadok et al., 1998). However, CGD macrophages were markedly inhibited in their production of anti-inflammatory cytokines during phagocytosis of apoptotic neutrophils from normal donors (Brown et al., 2003). Thus, ROS appear to be implicated in several stages of cell death and clearance, processes which are both crucial for the resolution of inflammation. This may partially explain the exaggerated inflammatory responses seen in CGD.

4.3. Regulation of Inflammatory Genes

One additional mechanism that has been proposed to explain the increased pro-inflammatory propensity of CGD patients is a dysregulation in the balance between production of pro- and anti-inflammatory cytokines. The recent microarray study by Kobayashi et al. mentioned above, not only described a defect in expression of apoptosis-related genes, but also an increased expression of pro-inflammatory

molecules (including chemokines/chemokine receptors, pattern recognition receptors, etc.) and a decreased expression of anti-inflammatory mediators (most notably genes involved in TGFβ signaling) in unstimulated CGD neutrophils as compared to controls (Kobayashi et al., 2004). These findings imply a pivotal role for ROS in the regulation of gene transcription with the capacity to induce certain genes and simultaneously repress others.

A somewhat contradictory view has been put forward regarding ROS and activation of NF-κB, a transcription factor capable of activating a number of genes involved in inflammation and other immunological processes (Tak and Firestein, 2001). Activation of NF-κB is generally regarded as a pro-inflammatory event and recent evidence has accumulated that ROS are involved in activation of this transcription factor (Flohe et al., 1997; Fan et al., 2002; Asehnoune et al., 2004). In one particularly interesting paper, a $p47^{phox-/-}$CGD mouse strain was crossbred with a strain harboring a NF-κB-driven luciferase reporter construct and these mice were unable to activate NF-κB to the same extent as mice with a functional NADPH-oxidase upon intratracheal infection with *Pseudomonas aeruginosa* (Sadikot et al., 2004). Furthermore, the CGD mice had markedly lower levels of the pro-inflammatory cytokine TNFα in their lungs after infection and were also impaired in bacterial clearance. These reports suggest that a lack of ROS production would lead to decreased NF-κB activation and subsequently decreased inflammatory responses, a fundamentally different scenario from that seen in CGD patients. To date, the involvement of ROS in NF-κB-mediated inflammatory responses in humans has not been directly studied using CGD cells, although numerous studies have presented data describing an increased production of pro-inflammatory cytokines upon stimulation compared to cells from healthy controls (Gallin and Buescher, 1983; Segal et al., 2000; Warris et al., 2003; Hatanaka et al., 2004). It may be that inherent differences between murine and human systems are behind these apparently contradictory results.

4.4. Other Effects of ROS with Possible Implications for the Development and Resolution of Inflammation

In addition to the effects of ROS discussed above, a number of other roles played by activation of the NADPH-oxidase and by the resulting free radicals have been described, providing exciting hypotheses for the enhanced inflammatory responses in CGD. For example, CGD neutrophils are unable to inactivate a variety of pro-inflammatory chemoattractants such as *N*-formyl peptides, leukotrienes, and the complement factor fragment C5a (Clark and Szot, 1982). These substances not only attract cells to an inflammatory focus, but are also involved in degranulation and activation of the NADPH-oxidase. Chemoattractants are thus involved at several stages in the inflammatory process of neutrophils and gradually alter them from circulating resting cells into actively cytotoxic cells in inflamed tissue. The inactivation of chemoattractants requires oxidation and is an inflammatory control mechanism that may be dysfunctional in CGD (Segal et al., 2000).

Another intriguing function of the NADPH-oxidase with possible implications for CGD pathology is the regulation of intracellular Ca^{2+} concentrations (Hallett, 2003). Ca^{2+} has long been known to be a pivotal regulator of a variety of cellular

processes and in resting neutrophils, cytosolic levels of this ion are much lower than extracellular (or intraphagosomal) levels (around a 10,000-fold difference). Upon neutrophil activation, membrane-bound Ca^{2+} channels open and a rapid influx of the ion to the cytosol occurs. This Ca^{2+} flux serves as an important mediator of cellular signaling pathways, leading to stimulation of various effector functions (e.g., degranulation and ROS generation) (Geiszt et al., 2001). In normal neutrophils, depolarization of the membrane usually follows activation (Demaurex et al., 1993); this depolarization dampens the influx of positively charged ions and thus keeps Ca^{2+} flux under control, thereby preventing over exuberant activation of cellular effector functions. It has long been known that the activation-induced depolarization of neutrophil membranes is absent in CGD cells (Seligmann and Gallin, 1980), and CGD neutrophils display exaggerated levels of Ca^{2+} influx. Quite recently, the defective depolarization and enhanced influx of Ca^{2+} in CGD cells were directly linked to the absence of a functional NADPH-oxidase (Rada et al., 2003). Furthermore, CGD neutrophils displayed enhanced degranulation in response to stimulation, resulting in enhanced release of elastase and increased activation of phospholipase A2 (Tintinger et al., 2001). These events are pro-inflammatory in nature and it was thus concluded that CGD neutrophils, due to their lack of functional NADPH-oxidase, become hyperactivated upon stimulation, which could explain the exaggerated inflammatory responses typical of CGD. These findings again highlight the electrogenic nature of the NADPH-oxidase and imply that the absence of ROS as microbicidal agents is far from the whole story behind CGD pathology.

5. Clinical Characterization of CGD and Current Therapies

5.1. Infections

The most typical clinical characteristic of CGD is recurrent, indolent infections, and although most patients are diagnosed at an early age, there are cases where the patient remains undiagnosed until adulthood. A recent survey of 368 CGD patients in the United States (Winkelstein et al., 2000) found the most common complication to be pneumonia (79%), followed by various abscesses (68%), lymphadenitis (53%), osteomyelitis (25%), and sepsis (18%). The pathogens responsible for the majority of infections in CGD patients are summarized in Table 5.2 and represent a distinctive set of bacteria and fungi.

The fungal species most commonly isolated from CGD patients, and also the leading cause of death, are members of the *Aspergillus* genus. *Aspergillus nidulans* appears to be particularly virulent in CGD patients but is rarely isolated from other immunocompromised patients at risk for aspergillosis. Moreover, an analysis of *Aspergillus* infections in CGD patients showed that although *Aspergillus fumigatus* was a more common pathogen than *A. nidulans*, the latter was more virulent and significantly more likely to cause fatal infection (Segal et al., 1998). These findings indicate a crucial role for ROS and an inadequacy of oxygen-independent killing mechanisms in the host defense against this fungal species, although the underlying reasons for this are yet to be determined.

Table 5.2. Infecting Organisms Recovered from 368 Patients in the CGD Registry

Type of infection	Organism	% of patients[a]
Pneumonia	*Aspergillus* spp.	32.6
	Staphylococcus spp.	9.2
	BCC	6.5
	Nocardia spp.	5.7
	Serratia spp.	3.8
	Other	23.6
Abscess	*Staphylococcus* spp.	28.0
	Aspergillus spp.	8.7
	Serratia spp.	7.6
	Other	23.4
Suppurative adenitis	*Staphylococcus* spp.	13.6
	Serratia spp.	4.8
	Candida spp.	3.8
	Other	9.2
Osteomyelitis	*Serratia* spp.	7.1
	Aspergillus spp.	5.4
	Other	7.6
Sepsis	*Salmonella* spp.	3.3
	BCC	2.2
	Candida spp.	1.9
	Other	9.2

BCC, *Burkholderia cepacia* complex.
[a] % of patients who had indicated organism isolated at least once from the infection shown. Data adapted from Winkelstein et al. (2000).

In addition to various *Aspergillus* species, infections with other fungi, such as *Candida albicans*, *Chyrosporium zonatum* (Roilides et al., 1999), and *Scedosporium apiospernum* (Jabado et al., 1998) have also been reported.

The bacterial species isolated from CGD patients are most often catalase-positive organisms, the most common being *S. aureus* (Table 5.1). Since catalase-negative organisms, for example, Streptococci and *Hemophilus influenzae,* are no more problematic in CGD patients than in the general population, it has been argued that endogenous generation of H_2O_2 from microbes in the phagosome could compensate (at least partly) for the lack of phagocyte-produced ROS. In the absence of bacterial catalase, the H_2O_2 could then react with, for instance, MPO and halides in the phagosome and form more toxic ROS, killing the catalase-negative pathogens. However, only a subset of catalase-positive organisms is generally encountered in CGD and recent studies have shown that *S. aureus* (Messina et al., 2002) and *A. fumigatus* (Chang et al., 1998) were equally virulent in a mouse model of CGD, irrespective of catalase expression. These studies suggest that the paradigm of catalase-dependent virulence in CGD is incomplete and that microbial virulence factors other than catalase mediate pathogenicity in these patients.

B. cepacia complex encompasses the bacterial species most frequently causing fatalities in CGD. This complex includes no less than nine discrete species of

which at least three (*B. multivorans*, *B. cenocepacia,* and *B. vietnamiensis*) have been recovered from CGD patients. These are also problematic pathogens in patients with cystic fibrosis (CF) (Speert, 2002). The *B. cepacia* complex infections in CGD patients are remarkably aggressive and the majority of the fatalities are caused by bacteraemic spread, a condition relatively uncommon with other bacterial organisms. This suggests that *B. cepacia* complex possesses virulence factors making it unique among bacterial CGD pathogens. *B. cepacia* complex bacteria have been shown to possess intrinsic resistance to many antibiotics and can, at least in the case of CF, be transmitted from one patient to other, making these pathogens especially difficult to handle clinically. In addition to antibiotic resistance, *B. cepacia* complex species are also highly resistant to cationic antibacterial peptides that are crucial components of the oxygen-independent killing machinery of phagocytes. Since CGD phagocytes are devoid of oxidative means of killing ingested microbes and thus rely completely on non-oxidative defenses, the resistance of *B. cepacia* complex to cationic peptides is one probable cause for their virulence in CGD patients. Neutrophils from CGD patients are incapable of killing *B. cepacia* complex *in vitro* while displaying normal killing of the phenotypically related *P. aeruginosa,* a species that is only rarely, if ever, isolated from CGD patients (Speert et al., 1994). Since both these organisms are catalase-positive, this finding again argues for the importance of virulence factors other than catalase.

5.2. Inflammatory Complications

Although the failure to eradicate bacterial and fungal infections is by far the most serious complication in CGD patients, there are also a number of other conditions, not obviously caused by infections, which are indicative of enhanced inflammatory responses. Most notable among these are persistent and exuberant tissue granuloma formations. Granulomas are usually defined as inflammatory lesions containing a variety of immune cells surrounding a foreign body such as a microbe. These are typically formed when invading microbes cannot be totally eliminated, and immune cells continue to release a variety of pro-inflammatory cytokines in response to the persistent nidus of infection. The failure to terminate an inflammatory response and shift the cytokine profile from pro- to anti-inflammatory, a shift that normally takes place upon elimination of an infection, could also contribute to granuloma formation. In line with this, granulomas in CGD patients are sometimes formed as a consequence of infections that are not properly cleared. However, the inflammatory sites (granulomas) are frequently sterile, and there are reports describing aberrant hyperactive cutaneous inflammatory reactions in the absence of infection (Gallin and Buescher, 1983; Segal et al., 2000). Granuloma formation in hollow viscera may lead to secondary complications, for example, obstructions of the gastric outlet, oesophagus, or urinary tract. These types of complications are seen in a substantial number of CGD patients and are more common in individuals with the XLR form of the disease than those with AR (Winkelstein et al., 2000).

Other non-infectious complications in CGD include enteritis/colitis and autoimmune diseases resembling systemic lupus erythematosus and Crohn's disease

(Segal et al., 2000). These and other less common complications could be explained by dysfunctionally exuberant inflammatory responses, and although no conclusive evidence explaining the exaggerated inflammation in CGD have been presented, many hypotheses and partial explanations have been proposed (as discussed above).

5.3. Current Therapy

In the above-mentioned review of *Aspergillus* infections, more than 30% of the infected CGD patients were asymptomatic at the time of diagnosis (Segal et al., 1998). Quite often, malaise and/or a vague feeling of infirmity can be the only symptoms of severe pulmonary infections. Thus, the normal care of CGD patients includes an increased awareness of the possible presence of infection, even if symptoms are absent, and a need to treat with antimicrobial agents on first suspicion of infection.

In general, treatment of CGD patients can be divided into prophylactic therapies, treatment of acute infections, and therapy for inflammatory conditions. The most common antibiotic used for prophylactic purposes is trimethoprim sulfamethoxazole. This agent has a broad activity against a variety of bacteria that are problematic in CGD. Its lipophilic properties allow it to be concentrated inside host cells, an effect that has been proposed to help phagocytes eliminate ingested bacteria without affecting the commensal anaerobic gut flora (Goldblatt, 2002). Retrospective analyses of CGD patients on trimethoprim sulfamethoxazole prophylaxis have shown that this intervention significantly reduces the rate of bacterial infection (Mouy et al., 1989; Margolis et al., 1990). For antifungal prophylaxis, itraconazole is the usual drug of choice. This lipophilic molecule is also concentrated within cells and displays high activity against *Aspergillus* species. In a group of patients given prophylaxis with itraconazole, only 10% developed *Aspergillus* infections, compared to over 34% in the untreated control group, suggesting that itraconazole prophylaxis is indeed quite effective against this fungal species (Mouy et al., 1994). Similar beneficial results of itraconazole, together with good tolerability of the drug, have been documented also in a more recent study (Gallin et al., 2003).

Interferon-γ (IFN-γ) is a particularly interesting prophylactic agent; it is an immunomodulatory cytokine with multiple effects on a variety of immune cells. A landmark study demonstrated that subcutaneous administration of IFN-γ in CGD patients partially restored phagocyte ROS production (Ezekowitz et al., 1988). Although later studies have failed to confirm these findings (Muhlebach et al., 1992; Woodman et al., 1992), the initial report was the basis for a large multi-center randomized double-blind, placebo-controlled study on the role of IFN-γ in preventing infections in CGD patients (The International Chronic Granulomatous Disease Cooperative Study Group, 1991). The study demonstrated that IFN-γ prophylaxis significantly reduced the frequency of serious infections, but no restoration of phagocyte ROS production could be detected. Interestingly, the positive effects of IFN-γ were restricted to certain geographical areas, and European patients appeared to be unaffected, displaying similar infection rates regardless of treatment. The reason behind this has never been fully explained, but as a consequence of the findings

there are currently geographical variations in the use of IFN-γ prophylaxis. One proposed mechanism for the salutary effects of IFN-γ is an increased production of nitric oxide (NO) (Ahlin et al., 1999). Since NO possesses antimicrobial properties and can be produced by a variety of immune cells, it is hypothesized to partially make up for the lack of ROS in CGD patients. There is, however, no explanation for why this mechanism would be absent in European CGD patients.

Therapy of established infections in CGD is particularly challenging, especially when the infectious agent cannot be readily identified. Therapy should be tailored to treat the suspected pathogen until cultures of tissue or good, normally sterile, specimens reveal the pathogen. For serious infections, such as liver abscess, osteomyelitis, and lobar pneumonia, a tissue biopsy may be the only way to establish an etiological diagnosis. Prior to obtaining culture results, or if an etiological agent cannot be established, therapy should be initiated on speculation of the most likely infectious agent. For instance, therapy of liver abscesses should include an anti-staphylococcal agent such as cloxacillin. Serious pneumonia should cover the entire range of infectious agents if the cultures remain negative; a regimen such as ceftazidime or meropenem (for Gram-negative bacteria including *B. cepacia* complex) plus cloxacillin plus amphotericin B can be used. Duration of therapy should be dictated by the response, but resolution of infection is slower than in immunocompetent patients and longer-term therapy is the rule.

Acute fungal infections are normally treated with high doses of amphotericin B. For particularly recalcitrant infections, itraconazole may be used in combination with amphotericin B, although there is no clear evidence that this regimen is superior to amphoterin B monotherapy, and there is *in vitro* evidence that the two drugs could be antagonistic (Kontoyiannis et al., 2000). If invasive fungal infection is established, therapy with itraconazole should be continued (at prophylactic dose) indefinitely. Newer antifungals, such as voriconazole, have been used to treat invasive aspergillosis in compromised patients and good responses were found in 47% of the subjects (Denning et al., 2002).

In order to treat the characteristic inflammatory complications of CGD patients, including hollow organ obstruction and colitis, immunomodulatory therapy is used. Steroids, both topical and systemic, usually have desirable and very rapid effect (Chin et al., 1987; Collman and Dickerman, 1990). One drawback in this treatment is steroid dependency in some patients with a requirement for long-term very low-dose administration. A variety of other immunomodulatory agents have also been used, including thalidomide (Nielsen and Valerius, 1986), cyclosporin (Rosh et al., 1995), sulphasalazine, and granulocyte colony-stimulating factor (Myrup et al., 1998).

5.4. Newer Therapies

Bone marrow transplantation has been shown to be potentially curative in CGD, although it has been associated with unacceptably high rates of morbidity, mortality, and graft failure except in patients with an HLA-identical sibling donor (Leung et al., 1999; Seger et al., 2002). Attempts to reduce conditioning related toxicity are continuing, using T cell depleted stem cell grafts following "mini" conditioning of the

patients with cyclophosphamide, fludarabine, and anti-thymocyte globulin, and are producing encouraging results (Horwitz et al., 2001). For patients without an HLA identical donor, gene therapy remains an attractive, potentially curative treatment modality. The recent description of successful gene therapy for X-linked SCID has provided increased impetus in this field (Cavazzana-Calvo et al., 2000). A number of pre-clinical studies have established the potential for gene therapy for CGD. The *ex vivo* transduction of human CD34 positive peripheral blood stem cells (PBSC) from patients with each of the four forms of CGD has been achieved while the genetic correction of knockout mice with both $gp91^{phox}$- and $p47^{phox}$-deficiency has been demonstrated (Bjorgvinsdottir et al., 1997; Mardiney et al., 1997). Gene therapy for patients with $p47^{phox}$-deficiency has been described using *ex vivo* retroviral transduction of CD34 positive PBSCs (Malech et al., 1997). This procedure proved to be safe although expression of NADPH-oxidase positive neutrophils in the circulation of patients was only transient. Autologous stem cells were reinfused without any conditioning thus limiting the survival advantage of the reinfused stem cells. It is likely that some form of conditioning of the patients, such as that attempted in the "mini" transplants described above (Seger et al., 2002) will be required to enable transfected stem cells to survive. Such studies are currently being planned and will hopefully provide a realistic chance of a cure for CGD.

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6

Novel Primary Immunodeficiencies

Capucine Picard and Jean-Laurent Casanova

1. Introduction

In the last 10 years, new primary immunodeficiencies have been identified that provide a molecular explanation for severe pediatric infections previously thought to be idiopathic. These new hereditary immunodeficiencies are associated with severe and/or recurrent infections caused by a single family of microorganisms, in contrast to what is observed in "classic" primary immunodeficiency (Notarangelo L et al., 2004). Standard immunologic explorations such as white blood counts, lymphocyte counts, vaccine serology, immunoglobulin levels, and complement were normal. However, the affected children were susceptible to infection by a single type of microorganism, and such infections were in some cases fatal. The aim of this chapter is to describe few new syndromes involving genetic predisposition to infectious diseases: IL-12-IFNγ axis deficiency (Mendelian susceptibility to mycobacterial disease), STAT-1 deficiency (predisposition to mycobacterial and viral diseases) and NEMO, IκBα and IRAK-4 deficiencies (predisposition to infections caused by pyogenic bacteria) (Table 6.1).

2. Mendelian Susceptibility to Mycobacterial Disease

Inherited disorders of the IL-12/23-IFNγ axis involve selective susceptibility to weakly pathogenic mycobacteria and *Salmonella* (Doffinger et al., 1999; Dorman and Holland, 2000; Dupuis et al., 2000; Remus et al., 2001; Ottenhoff et al., 2002). These primary immunodeficiency diseases (PID) are Mendelian disorders caused by mutations in five genes involved in IFNγ-mediated immunity: *IFNGR1* and *IFNGR2*, encoding the two chains of the receptor for IFNγ, a pleiotropic cytokine secreted by NK and T cells; *STAT1*, encoding a molecule essential to the IFNγR signaling pathway; *IL12B*, encoding the p40 subunit of IL-12 and IL-23, potent IFNγ-inducing cytokines secreted by macrophages and dendritic cells; and *IL12RB1*, encoding the β1 chain of the receptor for IL-12 and IL-23, which is expressed on NK and T cells (Casanova and Abel, 2002; Ottenhoff et al., 2002).

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Table 6.1. Novel Primary Immunodeficiencies

			Infections					
Gene	Form	Inheritance	Mycobacteria	*Salmonella*	Virus	Pyogenic bacteria	Fungus	EDA
IFNRG1	Complete	AR	++	+	+	−	−	No
	Partial	AR	++	+	−	−	−	No
		AD	++	+	−	−	±[a]	No
IFNGR2	Complete	AR	++	−	+	−	−	No
	Partial	AR	++	+	−	−	−	No
IL12RB1	Complete	AR	++	++	−	−	±[b]	No
IL12B	Complete	AR	++	++	−	−	−	No
STAT1	Partial	AD	++	−	−	−	−	No
	Complete	AR	++	−	++	−	−	No
NEMO	Hypomorphic	XR	+	+	+	++	+	Yes/No
IKBA	Hypermorphic	AD	−	+	+	++	+	Yes
IRAK4	Amorphic	AR	−	−	−	++	−	No

[a] One Partial Dominant (PD) *IFNGR1* deficient patient presented one infection caused by *Histoplasma capsulatum*.
[b] One Complete *IL12RB1* deficient patient presented a single infection caused by *Paracoccidioides brasiliensi*.

2.1. Complete Interferon-γ Receptor 1 Deficiency

Recessive complete interferon-γ receptor 1 (IFNγR1) deficiencies (MIM 107470) result in a complete loss of cellular responsiveness to IFNγ, due to mutations precluding the expression of INFγR1 on the cell surface (null mutations) (Newport and Levin, 1994; Jouanguy et al., 1996; Pierre-Audigier et al., 1997; Altare et al., 1998b; Cunningham et al., 2000; Dorman and Holland, 2000; Rosenzweig et al., 2002; Koscielniak et al., 2003) or recognition of the ligand IFNγ (expression of a non-functional receptor) (Jouanguy et al., 2000; Allende et al., 2001). Affected patients therefore fail to respond to IFNγ by producing IL-12 production and have high serum IFNγ concentrations after infection (Fieschi et al., 2001). Twenty-three patients have been reported to date. These patients suffer from disseminated infections caused by environmental mycobacteria (EM) and bacille de Calmette et Guérin (BCG) (Dorman et al., 2004), and have impaired granuloma formation (Emile et al., 1997). A few of these patients presented non-typhoid salmonellosis disease (Dorman et al., 2004) and one patient presented repeated invasive infection caused by *Listeria monocytogenes* (Roesler et al., 1999). Three patients had respiratory viral infections and one patient had a fatal Kaposi's sarcoma (Dorman et al., 1999; Camcioglu et al., 2004). Hematopoietic stem cell transplantation (HSCT), once the mycobacterial disease is under control (Horwitz et al., 2003), is the only curative treatment for these patients (Reuter et al., 2002), (Roesler J, et al., 2004). Null recessive *IFNGR1* mutations are responsible for the susceptibility of these patients to severe and often fatal mycobacterial infections, frequently occurring early in childhood.

2.2. Complete Interferon-γ Receptor 2 Deficiency

Two children with complete IFNγR2 signaling chain deficiency (MIM 147569) have also been reported (Dorman and Holland, 1998, 2000). Mutations in

the gene encoding IFNγR2 lead to a complete loss of cellular responsiveness to IFNγ. Both children with complete IFNγR2 deficiency had severe, early-onset infections due to *Mycobacterium fortuitum* and *M. avium* for one, and BCG and *M. abscessus* for the other, both requiring continuous multi-drug therapy (Dorman and Holland, 1998; Dorman and Holland, 2000). No mature granulomas were observed. An episode of curable herpes simplex virus (HSV) esophagitis was also reported (Dorman et al., 1999). The immunological features of both patients are indistinguishable from those due to complete recessive IFNγR1 deficiency. Thus, null recessive *IFNGR2* mutations, like null recessive *IFNGR1* mutations, cause susceptibility to severe, early-onset mycobacterial infections with impaired granuloma formation.

2.3. Partial Recessive IFNγR1 and IFNγR2 Deficiencies

Two siblings with recessive partial IFNγR1 deficiency (MIM 107470) have also been reported (Jouanguy et al., 1997). A mutation causing an amino-acid substitution in the extracellular domain was identified and the encoded receptor was detected with specific antibodies at the cell surface. The cells of the patients responded to only high concentrations of IFNγ. One of these children had disseminated BCG and *Salmonella enteritidis* infections with a favorable outcome. This child's sibling, who had not been vaccinated with BCG, had curable symptomatic primary tuberculosis. These children had well-circumscribed and well-differentiated tuberculoid granulomas. These children are currently 19 and 22 years old and have no ongoing treatment. One patient with partial IFNγR2 deficiency (MIM 147569) has also been reported (Doffinger et al., 2000). A homozygous nucleotide substitution was found in *IFNGR2*, resulting in a single amino-acid substitution in the extracellular domain. The encoded receptor was weakly but significantly detected on B cells. The young adult patient concerned had presented BCG and *M. abscessus* infections is now well at 24 years of age. Thus, a diagnosis of recessive partial IFNγR1 or IFNγR2 deficiency should be considered in children with mycobacterial and *Salmonella* infections with a mild clinical and histological phenotype.

2.4. Partial Dominant IFNγR1 Deficiency

Patients from 26 unrelated kindreds were found to have a dominant form of partial IFNγR1 deficiency (MIM 107470) (Jouanguy et al., 1999; Arend et al., 2001; Villella et al., 2001; Sasaki et al., 2002; Dorman et al., 2004). These patients have a heterozygous small frameshift deletion in *IFNGR1*, downstream from the segment encoding the transmembrane domain. This deletion decreases, but does not abolish, cellular responsiveness to IFNγ. The clinical phenotype is characterized by EM and BCG infections, often affecting the bones (Sasaki et al., 2002; Dorman et al., 2004). A total of 45 children with this condition have been identified. Seventeen of the 19 children inoculated with live BCG vaccine developed clinical infection, 31 patients had infection caused by EM, *M. avium* in most cases, and *M. bovis* in one case. Only two patients presented non-typhoid salmonellosis disease and one patient presented one episode of infection caused by *Histoplasma capsulatum* (Dorman et al., 2004). Eight of these patients are asymptomatic, but two died of disseminated mycobacterial infection at the ages of 17 and 27 years. The survivors are currently well and aged

between 2 and 62 years. Most have no prophylactic treatment. A diagnosis of partial dominant IFNγR1 deficiency should be considered in patients with mycobacterial osteomyelitis. The clinical outcome of patients with partial dominant IFNγR1 deficiencies, such as that of patients with partial recessive IFNγR1 and IFNγR2 deficiencies, appears to be better than that of children with complete IFNγR deficiency, probably because there is some residual IFNγ signaling.

2.5. IL-12Rβ1 Deficiency

Fifty-four patients with recessive complete IL-12Rβ1 deficiency (MIN 601604), with no cellular expression (Altare et al., 1998a; de Jong et al., 1998; Aksu et al., 2001; Altare et al., 2001; Sakai et al., 2001; Elloumi-Zghal et al., 2002; Caragol et al., 2003; Fieschi and Casanova, 2003; Fieschi et al., 2003; Lichtenauer-Kaligis et al., 2003; Staretz-Haham et al., 2003) and one patient with a complete recessive form in which the affected receptor was expressed have been reported (Fieschi et al., 2004). The patients were found to be homozygous or compound heterozygous for nonsense, splice, and missense mutations; small insertions, large deletions, and deletions/insertions in *IL12RB1* (Fieschi et al., 2003). The cellular phenotype of IL-12Rβ1-deficient patients is a lack of response to IL-12 and IL-23, with low levels of IFNγ production. The clinical phenotype is characterized by EM/BCG infections and half the patients had non-typhoid salmonellosis (Fieschi and Casanova, 2003). Twenty-six of the 35 children inoculated with live BCG vaccine developed clinical infection, 12 children had infections caused by EM, and three patients had infections caused by *M. tuberculosis* (Fieschi and Casanova, 2003). Twenty-five patients presented non-typhoid salmonellosis and one patient presented a single episode of infection caused by *Paracoccidioides brasiliensi*. Five of these patients are asymptomatic, but eight died of mycobacterial or *Salmonella* infection before the age of 8 years. The survivors are currently well and aged between 1 and 33 years. Most have no prophylactic treatment.

2.6. IL-12 p40 Deficiency

Nineteen patients with complete recessive IL-12p40 deficiency (MIM 161561) have been reported (Altare et al., 1998c; Elloumi-Zghal et al., 2002; Picard et al., 2002; Fieschi and Casanova, 2003). The mutations in *IL12B* concerned are small insertions or large deletions. Two founder mutational events have been identified in four kindreds from Saudi Arabia and in two kindreds from the Indian subcontinent (Picard et al., 2002). Children with this deficiency produce abnormally low levels of IFNγ due to a lack of stimulation through IL-12. This defect can be partially corrected in a dose-dependent manner with exogenous recombinant IL-12 (Picard et al., 2002). All 16 children inoculated with live BCG vaccine developed clinical infection, two children had infection caused by environmental mycobacteria and one patient had an infection caused by *M. tuberculosis*. Five patients presented non-typhoid salmonellosis and one patient presented a single episode of infection caused by *Nocardia asteroides*, an acid-fast agent closely related to *Mycobacterium* (Picard et al., 2002). One of these children is asymptomatic, but six died of infection between the ages of 2 and 11 years. The survivors are currently well and aged

between 5 and 14 years. Most have no prophylactic treatment. Granuloma formation is preserved but often multibacillary in these patients (Picard et al., 2002). The prognosis is good for patients with IL12-Rβ1 and IL-12p40 deficiencies due to the low clinical penetrance of primary mycobacterial infection. Children with primary mycobacterial disease can mount a fully protective immune response against a secondary mycobacterial disease. However, the high incidence of salmonellosis in these patients suggests that IL-12 is required for immunity against this disease.

2.7. Partial STAT1 Deficiency

Two kindreds with the same heterozygous mutation in *STAT1* causing partial dominant STAT-1 deficiency (MIM 600555) have been described (Dupuis et al., 2001). STAT-1 is a critical transducer of IFN-mediated signals, either as STAT-1 homodimers—designated gamma-activating factor (GAF)—or as a STAT-1/STAT-2/p48 trimers—known as interferon-stimulated gamma factor 3 (ISGF3). This heterozygous *STAT1* mutation decreased the cellular response to IFNγ more strongly than the cellular response to IFNα. Clinically, one patient suffered from disseminated BCG infection with tuberculoid granulomas, whereas the other had disseminated *M. avium* infection. They are now 38 and 12 years old and well. The first patient had two asymptomatic children heterozygous for the *STAT1* mutation. This observation suggests that STAT1 and GAF are necessary for human IFNγ-mediated mycobacterial immunity. In conclusion, the partial STAT1-deficient patients had clinical and cellular phenotypes (i.e., susceptibility to mycobacterial disease and impaired GAF activation) similar to those of patients with partial IFNγR deficiency.

2.8. Complete STAT1 Deficiency

Two children from two unrelated kindreds with complete STAT1 deficiency have also been reported (Dupuis et al., 2003). These patients carried homozygous mutations, which abolished completely the cellular responses to both IFNγ and IFNα. Both patients had disseminated curable BCG disease. One patient died of recurrent encephalitis caused by HSV1 and the second died of a non-documented illness thought to be viral in origin. Viral illnesses in children with complete STAT1 deficiency suggest that the STAT1-dependent response to human IFNα/β is necessary for viral immunity. The patients with complete STAT1-deficient patients had severe, early-onset viral infections.

3. Inherited Disorders of the NF-κB Signaling Pathway

This group of inherited disorders leads to impaired NF-κB signaling and greater susceptibility to pyogenic bacteria (Picard et al., 2003b; Orange et al., 2004; Puel et al., 2004). Patients with anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) syndrome carry either X-linked recessive hypomorphic mutations in *NEMO* or autosomal dominant (AD) hypermorphic mutations in *IKBA*. Other patients with autosomal recessive amorphic mutations in *IRAK4* present a more

restricted, purely immunological defect, with specific impairment of the Toll and interleukin-1 receptor (TIR)–IRAK signaling pathway (Puel et al., 2004).

3.1. NEMO Deficiency

X-linked anhidrotic ectodermal dysplasia with immunodeficiency (XL-EDA-ID) is a rare primary immunodeficiency (MIM 300291). To date, 35 patients with XL-EDA-ID (Abinun, 1995; Abinun et al., 1996; Zonana et al., 2000; Aradhya et al., 2001; Doffinger et al., 2001; Jain et al., 2001; Kosaki et al., 2001; Mansour et al., 2001; Dupuis-Girod et al., 2002; Orange et al., 2002, 2004; Carrol et al., 2003; Nishikomori et al., 2004) have been reported. Patients with XL-EDA-ID carry hypomorphic mutations in the gene encoding NF-κB essential modulator (NEMO), a protein essential for the activation of the ubiquitous transcription factor NF-κB. In the immunologic work-up, the only known consistent abnormality is a lack of serum antibodies to carbohydrates, which has been found in most patients reported (Doffinger et al., 2001; Picard et al., 2003b; Puel et al., 2004). Some patients have hyperIgM syndrome (Doffinger et al., 2001; Jain et al., 2001) and a few patients have NK cell abnormalities (Orange et al., 2002). EDA-ID is characterized by hypohidrosis, widely spaced cone- or peg-shaped teeth, and hypotrichosis. However, the range of clinical manifestations appears to be broad, from both the developmental and infectious standpoints. Two patients carrying the X420W mutation had osteopetrosis and lymphedema (defining XL-OL-EDA-ID) (Mansour et al., 2001; Dupuis-Girod et al., 2002). In contrast, some children had an exclusively infectious phenotype, with no sign of EDA (Orange et al., 2004). The infectious phenotype is characterized mostly by infections due to encapsulated pyogenic bacteria, such as *Haemophilus influenzae* and *Streptococcus pneumoniae*, or *Staphylococcus aureus* (Abinun, 1995; Abinun et al., 1996; Zonana et al., 2000; Aradhya et al., 2001; Doffinger et al., 2001; Jain et al., 2001; Kosaki et al., 2001; Mansour et al., 2001; Dupuis-Girod et al., 2002; Orange et al., 2002; Carrol et al., 2003; Nishikomori et al., 2004; Orange et al., 2004). Infections caused by weakly pathogenic mycobacteria, such as *M. avium* and *M. kansasii*, were also diagnosed (Doffinger et al., 2001; Dupuis-Girod et al., 2002). Other infectious diseases found include salmonellosis, pneumocystosis, and viral illnesses, caused by HSV and CMV (Doffinger et al., 2001). Infectious episodes were marked by a poor clinical and biological inflammatory response (Dupuis-Girod et al., 2002). Half the patients with EDA-ID died from invasive infection, demonstrating the severity of this disorder. Thus, NEMO deficiency is a disorder in which a PID may or may not be associated with developmental abnormalities.

3.2. IκBα Deficiency

One patient with an AD form of EDA-ID was initially reported (Courtois et al., 2003), followed by a child and his father a year later (Janssen et al., 2004). The three patients with AD-EDA-ID carried the same hypermorphic mutation in *IKBA*, encoding an inhibitor of NF-κB. All were heterozygous for this mutation, the mutant allele exerting a dominant-negative effect over the wild-type allele. Interestingly, the father of the second patient presented a milder clinical and immunological phenotype,

probably due to complex mosaicism (Janssen et al., 2004). Both children had a lack of memory T cells and an impaired T-cell response to both antigenic and CD3 mitogenic stimulation *in vitro*. They also displayed hyperIgM syndrome and had the EDA phenotype. From the age of 2 months onwards, they suffered from recurrent opportunistic infections and chronic diarrhea caused by pyogenic bacteria, *Pneumocystis carinii*, and virus. They presented also failure to thrive. The two children were treated by HSCT at the age of 1 year and 2.5 years. The father of the second patient suffered from recurrent *Salmonella typhimurium* infection. Thus, a diagnosis of IκBα deficiency should be considered in children with EDA and severe immunodeficiency with impaired T-cell immunity.

3.3. Interleukin-1 Receptor-Associated Kinase-4 Deficiency

Inherited interleukin-1 receptor-associated kinase-4 (IRAK-4) deficiency (MIM 607676) is an autosomal recessive disorder first described in three unrelated patients (Picard et al., 2003a). A fourth patient was subsequently reported (Medvedev et al., 2003). Nine other patients have since been identified (C. Rodriguez-Gallego, H. Chapel, A. Enders, L. Marodi, and J.L. Casanova, unpublished data) (Currie et al., 2004) (D. Speert, personal communication). All the causal mutations concern the kinase domain of the protein (missense mutations and small insertions). The blood cells of the patients fail to produce pro-inflammatory cytokines upon stimulation by all known Toll-like receptor (TLR) agonists, IL-1β and IL-18. One patient was reported to display antibody responses to protein and polysaccharide antigens (Day et al., 2004). Clinically, IRAK-4 deficient patients had recurrent infections caused by pyogenic bacteria, mostly Gram-positive, with little or no fever and inflammatory responses. The leading pathogen causing infections in these patients is by far *S. pneumoniae*, which was found in the 10 patients with proven IRAK-4 deficiency and caused blood-borne invasive disease (septicemia, meningitis, or arthritis). The second most frequently detected infectious organism is *S. aureus,* often responsible for skin infections, but occasionally also for infection of the liver or septicemia. Invasive disease caused by Gram-negative bacteria was diagnosed in these patients on only two occasions (*Neisseria meningitidis* and *Shigella sonnei*). Although sudden infections may strike at any age, the global trend is an improvement with age, as attested by two adult patients doing well off all treatment at 22 and 30 years of age (Medvedev et al., 2003; Chapel, submitted). However, IRAK-4 deficiency is a life-threatening disease, resulting in the deaths of six of the 13 patients. A diagnosis of IRAK-4 deficiency should be considered in children presenting recurrent pyogenic infection with poor inflammatory responses.

4. Conclusion

Knowledge of the molecular basis of these new immunodeficiencies causing Mendelian susceptibility to mycobacteria, viruses, and pyogenic bacteria is required for a thorough understanding of their physiopathology, and for the treatment of affected children and genetic counseling. Novel PIDs should be sought in patients

with unexplained infectious disease, even if all standard immunological explorations are normal as common infectious diseases may be favored by as yet unknown Mendelian immune disorders. The description of novel primary immunodeficiencies associated with susceptibility to pathogens is an exciting perspective, which will not only increase our understanding of immunity to pathogens, but will also be of benefit to the patients.

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7

Periodic Fever

Sarah S. Long

1. A Case

A 4-year-old adopted boy of mixed African American and Caucasian race was referred for consultation because of recurring fever. Before the age of 1 year, he had begun to have monthly febrile illnesses without diagnoses. By 2 years of age, febrile episodes were periodic, occurring approximately once in a month and had a characteristic pattern of sudden rise in temperature to 38.6–39°C, vomiting on the first day, seeming stiffness, poor appetite, and swollen neck glands. Episodes lasted 3 days. By 3 years of age and continuing to the time of referral, episodes had the same character and periodicity. However, an additional feature was sudden mood change during episodes with crankiness and temper tantrums. He had been evaluated during several episodes by his pediatrician who found no organ-specific findings. Fever was modestly responsive to acetaminophen and ibuprofen.

Review of systems revealed no history of skin rash, respiratory tract symptoms, headache, arthritis, mouth ulcers, or diarrhea during episodes. He is completely well between episodes, swollen neck glands recede, appetite and mood are excellent, and growth and development are normal. He is very active and has difficulty in concentrating unless attention is given one-on-one. Past history reveals no medical problems as a neonate, infant, or toddler. He has never had a serious infection. He has received few courses of antibiotics. He has received all immunizations appropriate for age, which have been associated with self-resolving high fevers (39–41°C). He received measles, mumps, rubella, and varicella vaccines without incident. Natural family history is unknown. Of note, his adoptive parents and siblings are not ill when the patient is ill. They live rurally.

Physical examination revealed a healthy appearing, bright, well-grown boy who had difficulty in sitting quietly. Height and weight were 75th percentile for age. Examination was normal. He had no mouth ulcers or gingivitis, enlarged or abnormal tonsils, and skin or joint abnormalities. Cervical lymph nodes were present, normal in consistency and less than 1.5 cm bilaterally. Abdominal examination revealed no tenderness, guarding, or hepatosplenomegaly. There were no abnormalities on neurologic

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examination. He had normal strength, reflexes, dexterity, and recall of recent and distant events.

Review of relevant laboratory tests performed over the recent years by his pediatrician included range of Hb 10.2–11.8 g/dl, white blood cell (WBC) count of approximately 10,000/mm^3 during healthy periods and elevated WBCs up to 21,000–30,000/mm^3 with normal or relative neutrophilia and mild left shift ($<$8% band forms) during febrile episodes. Platelet counts never exceeded 350,000. Sedimentation rate ranged from 20 to 30 mm/hr during healthy periods and was elevated to 70–90 mm/hr during episodes. Screening serum chemistries and hepatic enzymes were normal on multiple occasions. Serum immunoglobulins were normal for age; IgD level, 2 mg/dl. Serum antibody tests for multiple viruses including HIV were unrevealing. Hemoglobin electrophoresis was normal. Bacterial cultures of throat, blood, and urine on occasions of fever were negative. Multiple diagnoses were pursued, following the approach and considering conditions delineated in Section 2. Denouement is presented in Section 4.

2. Approach to the Child with Recurring Fevers

The history is paramount. Diagnoses are considered and excluded based on the history. It focuses on the specifics of illness and intervening periods, the environment, and the genetic background.

2.1. History of Episodes and Intervening Periods

Children normally can have up to 10 self-limited viral illnesses per year for the first 2–3 years of life. Those in out-of-home childcare have more. Many are associated with fever, and if the child is otitis-prone, fever can predominate as the clinical "problem" to the family. The child with multiple, self-limited illnesses, with different target organs identified (especially the respiratory tract), with no serious or unusual infection identified, and good growth and development should not be pursued for a defect in host immune response. When other family members or contacts share similar, temporally related illnesses, the likelihood of a host defect is remote even when the patient's symptoms are "worse" as long as they are self-limited.

However, the diseases and syndromes of noninfectious periodic fever should be considered when (1) the cardinal feature of the illnesses is fever; (2) episodes recur after symptom-free intervals; and (3) episodes have a predictable course and lack respiratory tract symptoms. Additional questions target the features of the episodes, the rapidity of return to health at conclusion of episodes, time intervals between episodes, and family history. The family history seeks genetic background of the child as well as whether older family members or siblings have had similar problems, autoimmune or autoinflammatory illness or amyloidosis. History of adverse events or triggering of a typical episode by immunizations is sought.

2.2. Physical Examination

The growth chart supplies critical information, the major question being whether there is recovery of weight between episodes and steady incremental velocity of height

and weight over time. Inflammatory bowel disease can masquerade as recurring fevers (not with clockwork periodicity and usually of low grade and associated with mild gastrointestinal complaints or iron deficiency anemia or both). Examination must be thorough, primarily to exclude target organ systems of other diseases that may appear in early stages as a recurring fever problem. These include malignancies, autoimmune, endocrine, and metabolic disorders. Considering periodic fever syndromes, special attention is paid to mouth ulcers and gingivitis, rashes, abnormalities of joints and nodes that sometimes have been overlooked when attention has been focused on high fever and apathy during episodes.

2.3. Laboratory Evaluations and Imaging Studies

Laboratory tests useful in evaluation of children with periodic fever begin simply, with the broad goal of either supporting the continued consideration of periodic fever syndromes or suggesting some other diagnosis. It is rare that simple tests *confirm* a specific periodic fever syndrome diagnosis and it is rare that tests for unusual causes of infectious relapsing fever will be revealed in the absence of a specific exposure history.

Simple tests, such as complete blood counts and ESR, screening serum chemistries including hepatic enzymes and albumin, and quantitative immunoglobulins including IgA, D, and E, should be performed. Usually, prior to referral, multiple tests have been performed. The consultant should ascertain whether cultures have been performed during at least two episodes and prior to antibiotic therapy on urine as well as on throat specimen for *Streptococcus pyogenes* if pharyngitis symptoms or signs have been present.

Unlike the usefulness of imaging tests, especially computed tomography, in evaluation of children with prolonged fever of unknown origin, imaging has little conceivable role in children with clinically established periodic fever.

3. Differential Diagnosis of Periodic Fever

Periodic fever is defined for this discussion as recurrent episodes of unwellness in which fever is the cardinal feature, which have a predictable and similar set of symptoms, last days to weeks, and are separated by symptom-free intervals. For some disorders, the *episodes* or *recurrences* have consistent or "*clockwork*" *periodicity* while in others they do not. Patients with autoinflammatory disorders in which periodic fever is one finding but is overshadowed by others such as urticaria, arthritis, and multiorgan dysfunction are not likely to be referred to infectious diseases consultants and are not considered here. These include Muckle–Wells syndrome, familial cold autoinflammatory syndrome (FCAS), neonatal onset multisystem disease (NOMID), and chronic infantile neurologic cutaneous and articular syndrome (CINCA). Disorders considered here have the cardinal feature of periodic fever, and are considered in order of frequency. PFAPA is most common, has no etiology defined or confirming laboratory tests. Only cyclic neutropenia has both easily confirming laboratory test (neutropenia) and a known genetic mutation. The others are rare, have no confirming laboratory tests but have specific diagnostic gene mutations. Several excellent reviews of the hereditary periodic fever syndromes are published (Centola et al., 1998; Drenth and van der Meer, 2001; McDermott, 2002; Grateau, 2004.

3.1. Periodic Fever, Aphthous Stomatitis, Pharyngitis, and Cervical Adenopathy

3.1.1. Epidemiology and History

PFAPA (periodic fever, aphthous stomatitis, pharyngitis, and cervical adenopathy) is a non-hereditary, autoinflammatory disorder first described in 12 children from Tennessee and Alabama (Marshall et al., 1987). Now >200 cases have been described or anecdotally reported (real incidence is likely exponentially greater) representing all racial backgrounds and continents except South America and Africa. Western and East Europe, the United Kingdom, and the Middle East account for most cases outside the United States. There is no universally agreed case definition, registry, or diagnostic test. Population based incidence figures do not exist. There may be underrepresentation of African American and Hispanic children in the United States; referral bias cannot be excluded as a reason. In the United States, most pediatric infectious diseases subspecialists have made this diagnosis repeatedly; where investigators have numbers of children with PFAPA, it outnumbers exponentially diagnoses of cyclic neutropenia and other genetic periodic fever syndromes. Feder et al. (1992) reported a beneficial effect of cimetidine, serendipitously found, on course of episode and frequency of recurrences. Abramson et al. (1989) reported unexpected resolution of PFAPA after tonsillectomy in three cases. Except for unique cases of PFAPA in which Epstein-Barr virus infection with aberrant antibody response was found (Lekstrom-Himes et al., 1996) and a case of disseminated *Mycobacterium chelonae* in a normal child (Ryan et al., 1996), no infectious causative agent or autoimmune or autoinflammatory disorder has been established. Marshall et al. (1987) noted dramatic abortion of episodes with a single dose of corticosteroid, a finding reiterated in every subsequent report (Long, 1999; Padeh et al., 1999; Thomas et al., 1999).

3.1.2. Clinical Features

Case series are the sum evidence of features of disease; however, a constellation emerges that distinguishes PFAPA as a syndrome separate from others (Table 7.1). Slight male predominance and onset before 3 years (and almost always before 5 years of age) are typical. The child has a brief prodrome of clinginess and "glassy eyes" and then sudden temperature rises to 39–40.5°C followed by poor appetite and energy, chills but no rigors. Fever is the cardinal feature, is modestly if at all responsive to acetaminophen or ibuprofen, and lasts 3–4 days. During the episode, a few shallow, not-so-painful ulcers may appear in the mouth. All symptoms and signs cease after 4–5 days. Episodes occur at intervals of 21–36 days, typically 28 days, with a predictable clockwork periodicity unique to each patient. The only laboratory abnormalities are mildly elevated WBC during episodes (typically 13,000/mm^3) with neutrophilia and sometimes mildly excessive band forms. Platelets are normal or modestly elevated (<400,000/mm^3); ESR is elevated, usually <60 mm/hr. Hemoglobin is characteristically unaffected, urinalysis is normal as are serum hepatic enzymes. Between episodes, children have no lingering symptoms, seem uncommonly energetic, and have good appetite. They do not have recurrent, unusual, or severe infections. Parents report fewer "regular colds" than siblings or contemporaries. Any laboratory abnormality reverts to normal between episodes.

Table 7.1. Differentiating Features of Periodic Fever Syndromes

	PFAPA	Cyclic neutropenia	Familial mediterranean fever (FMF)	Hyper-IgD syndrome (HIDS)	TNF receptor-associated periodic syndrome (TRAPS)
Onset <5 years	Usual	Usual; often <1 year of age	Common; peak onset middle of first decade	Common	Uncommon but variable
Length fever episode	4 days	5–7 days	2 days	4 days	Weeks; sometimes days
Periodicity	3–6 weeks (28 days)	21 days in >90%	Irregular intervals: weekly, every 3–4 months or less often	4–8 weeks or irregular	Irregular intervals; varies weeks to years
Associated symptoms/ signs	Pharyngitis 65–70%; aphthous stomatitis 65–70%; cervical adenopathy 75–85%	Ulcers, gingivitis, periodontitis; otitis media and sinusitis; rare peritonitis; rare Gram-negative bacillary or clostridial septicemia	Polyserositis; erysipelas-like rash; scrotal pain and swelling	Abdominal pain, diarrhea in young; arthralgia; rashes; splenomegaly; immunizations trigger	Migratory muscle pain; pseudocellulitis; conjunctivitis, periorbital edema; other
Ethnic/ geographic/ genetic features	None	No ethnic autosomal dominant	Jewish, Armenian, Arab, Turkish autosomal recessive	Dutch, French, others autosomal recessive	Irish and Scottish but variable including Mediterranean descent autosomal dominant
Laboratory findings	Mild ↑ WBC, neutrophils, ESR	Absolute neutrophil count <200 cells/mm^3	Elevated acute phase reactants for 3–5 days	Elevated acute phase reactants; variable↑ IgA and IgD	Elevated acute phase reactants
Etiology/ diagnosis	Unknown/ none	Chromosome 19 *ELA2* mutation(s) leading to mutant neutrophil elastase; apoptosis myeloid cells	Chromosome 16 *MEFV* missense mutations	Chromosome 12 *MVK* mutations leading to ↓ mevalonate kinase and isoprenoids	Chromosome 12 *TNFRSF1A* mutations leading to ↓ soluble TNF receptor superfamily type 1A
Treatment	None established (see text)	Recombinant G-CSF; aggressive periodontal care; aggressive treatment suspected septicemia	Colchicine	Simvastatin (preliminary) etanercept (preliminary)	Corticosteroid etanercept (preliminary)

3.1.3. Treatment and Outcome

A follow-up study of 94 children cared for in Tennessee and Connecticut over a 10-year period was reported (Thomas et al., 1999). Mean age at follow-up was 8.9 years. Episodes retained characteristics as on diagnosis. Mean duration of PFAPA was 4.5 years; only 41% of children had resolution over the mean follow-up of 3.3 years. Cimetidine was tried in less than one-third for treatment or prophylaxis; it was somewhat-to-very effective in 90%. Prednisone (most given 1 or 2 doses of 1–2 mg/kg/day) was somewhat-to-very effective in 90%. Inexplicably, in experience of most experts, use of prednisone although beneficial for an episode, decreases the time interval of wellness before the next episode. Tonsillectomy and adenoidectomy was performed in 47 of 94 children and was somewhat-to-very effective (usually the latter) in 86% of children.

There is no etiology known or confirming the diagnostic test. Sometimes, a single dose of prednisone at the onset of an episode may be useful as a "test"; rapid resolution is expected in children with PFAPA. Unfortunately, it is not known how often febrile episodes due to other periodic fever syndromes might respond thusly and corticosteroids can affect febrile responses in a variety of infectious diseases.

3.2. Cyclic Neutropenia

Cyclic neutropenia is a rare hematologic disorder characterized by regular cycling of the absolute neutrophil count in peripheral blood to nadir of <200 cells/mm^3, and a symptom complex manifesting during the neutropenic nadirs. Because blood monocytes, reticulocytes, platelets, and lymphocytes can have similar but less profound periodic oscillations as neutrophils, the disorder sometimes is called cyclic hematopoiesis.

3.2.1. Epidemiology and History

The first case of cyclic neutropenia was reported by Leale in 1910. Reiman described the autosomal dominant pattern of inheritance in 1949, subsequently describing the natural history and clinical associations of mucosal ulcerations and skin infections. Occasional cases are sporadic, and adults with acquired cyclic neutropenia also have been described. In 1989, Hammond et al described the treatment response to granulocyte colony-stimulating factor (G-CSF), more than 10 years before the defect and mechanism of disease were established. In 1999, mutations of the gene for neutrophil elastase (*ELA2*) were discovered as the molecular mechanism of the biologic clock in cyclic hematopoiesis (Horwitz et al., 1999), and accelerated cellular apoptosis was hypothesized as the cellular mechanism for cyclic neutropenia by Aprikyan et al. in 2001. We now know that all cases of cyclic neutropenia as well as most cases of severe congenital neutropenia are due to mutations of *ELA2* (Dale et al., 2002).

3.2.2. Clinical Features

Features of cyclic neutropenia are shown in Table 7.1. Diagnosis usually is made in infants or toddlers because of recurrent fever with clockwork periodicity,

pharyngitis, mouth ulcers, and lymphadenopathy. Some cases are diagnosed because of recurrent cellulitis or furunculosis. In comparison with the mild oral signs and symptoms in PFAPA syndrome, children with cyclic neutropenia have predominant complaints of deep and painful mouth ulcers that often last more than 1 week. Gingivitis and periodontitis is common (not a finding in PFAPA). Recurrent bacterial otitis media, sinusitis, and pharyngitis are frequent complications, but recurrent cellulitis and furunculosis from insect bites, minor cuts, or abrasions typically on the hands and in the perianal areas distinguish cyclic neutropenia. Some patients have few and relatively minor complicating bacterial infections but invasive, fatal infections can occur. Acute bacterial peritonitis and septic shock as well as overwhelming Gram-negative bacillary or clostridial septicemia resulting from colonic ulcers during the period of neutropenia have been described. Bacterial complications occur only during the periods of neutropenia. Although ulcers, gingivitis, and periodontal disease linger, the child usually is well before the next episode.

3.2.3. Etiology and Diagnostic Tests

During the neutropenic period, peripheral neutrophil levels fall to <200 cells/mm^3 for 3–5 days. The count then usually rises to about 2,000 cells/mm^3 (the lower limit of normal) where it remains until the next neutropenic period. Monocytes oscillate with neutrophils and some patients have detectable, non-critical fluctuations in reticulocytes and platelets as well. If bone marrow is examined at the onset of neutropenia, recognizable early myeloid precursors are seen but postmitotic neutrophils are absent. Recovery is rapid, with cells from promyelocytic to band neutrophilic forms seen. Neutropenia precedes the heralding clinical features of fever, stomatitis, and tender lymphadenopathy, and may be in recovery phase at the time of presentation. Children with compatible history (as well as most children in whom the diagnosis of PFAPA is considered) should have cyclic neutropenia evaluated by twice weekly complete blood counts performed beginning during the interval of wellness and continuing through the next febrile episode.

Cyclic neutropenia is inherited as an autosomal dominant disorder with full penetrance but varying severity of clinical manifestations. In general, an affected parent of a child with cyclic neutropenia has not been recognized because of milder clinical and laboratory manifestations. Genetic abnormality is localized to chromosome 19p13.3, resulting in mutation(s) of *ELA2* and its neutrophil elastase protein product. Diagnosis is confirmed by genetic testing.

3.2.4. Treatment and Outcome

The morbidities of cyclic neutropenia include: (1) pain, discomfort, and time lost due to febrile episodes; (2) periodontal disease frequently resulting in shedding or necessary extraction of decidual teeth in childhood; (3) common non-serious or moderately serious bacterial infections and rare life-threatening invasive bacterial and possibly fungal infections; (4) increased rate of spontaneous abortion in affected women; and (5) transmission of the disorder to approximately 50% of offspring. No tendency to malignancy has been noted in children with cyclic neutropenia.

Availability of recombinant G-CSF has greatly changed the management of congenital severe neutropenia and cyclic neutropenia (Hammond et al., 1989; Dale et al., 1993, 2002). The majority of children with cyclic neutropenia are treated with G-CSF daily or on alternate days at doses of 5 μg/kg/day or less. More than 90% of children with typical cycles of approximately 3 weeks have responded and have greatly reduced occurrence of the immediate complications of neutropenia.

3.3. Familial Mediterranean Fever

Familial Mediterranean fever (FMF) is one of the inherited autoinflammatory syndromes in which seemingly unprovoked or minor stress or trauma causes inflammation without autoantibodies or autoreactive T lymphocytes.

3.3.1. Epidemiology and History

FMF is the most frequent periodic fever syndrome, typified by periodic (*not* clockwork or predictable) attacks of fever and serositis/synovitis. Although occurring probably since biblical times, FMF was described as a distinct entity only in 1945 (reviewed by Eliakim et al., 1981). Goldfinger (1972) reported effectiveness of colchicine therapy. In 1997, the International FMF Consortium and the French FMF Consortium independently cloned the mutant *MEFV* gene, the former group naming the protein product pyrin (indicating relationship to fever) and the latter group calling it marenostrin (meaning "our sea," indicating prevalent relationship to the Mediterranean Sea).

FMF is an autosomal recessive disease and the most prevalent inherited periodic fever syndrome, affecting more than 10,000 individuals worldwide. FMF is almost completely restricted to non-Ashkenazi Jews, Armenians, Arabs, and Turks (Ben-Chetrit and Levy, 1998). More than 90% of Jewish FMF patients are of Sephardic or Middle Eastern origin. Heterozygous carrier frequency is greater than 1 in 10 North African Jews and Armenians. Data suggest that *MEFV* mutations probably appeared in the Middle East from whence they spread to Europe, to North Africa, and to Armenia more than 2,500 years ago. It is also speculated that such frequent and persistent mutational gene frequency must have conferred survival advantage probably against an infectious pathogen (Aksentijevich et al., 1999). Availability of genetic diagnosis will help categorize occasional patients diagnosed with FMF from other continents and seemingly nonendemic backgrounds.

3.3.2. Clinical Features

MEFV mutations are more varied and complex than originally described. Single genotype–phenotype correlations are not solid and multiple genotypes exist. Description of manifestations as if FMF were a single disease are valuable only as generalities. Symptoms of FMF can begin before the age of 2 years (in up to 20% of patients) and two-thirds of affected individuals have manifestations before 10 years of age. Less than 10% have onset after 30 years of age. A typical attack is heralded by abdominal pain and then rise in temperature to 40°C followed by chills; fever lasts 12 hr to 3 days and is rarely the only manifestation. Abdominal pain is present

in >90% of patients, can be accompanied by diarrhea, begins suddenly a few hours before fever, and persists 1–2 days after defervescence. This autoinflammatory acute peritonitis can simulate all other causes of "acute abdomen" and attacks can follow surgical trauma to peritoneal serosa as from a biopsy or appendectomy. A report of 20% homozygosity for FMF gene among Sephardic Jewish and Arab children with diagnosis of functional abdominal pain suggests a possible spectrum of symptoms (Brik et al., 2001). Chest pain of pleuritis occurs in 25–80% in multi-series reports; it is the presenting manifestation in <10% of patients. Pericarditis is less common (<1% of cases) and may occur with pleuritis. Small fluid collections can be detected by various imaging techniques when serosal involvement occurs. Arthritis is common and has characteristics that vary with ethnicity. Acute pain and effusion in wrist, ankle, or knees, occurring asymmetrically and resolving completely over days is most typical. An erysipelas-like exanthem reported in 7–40% of patients almost invariably affects the extensor surface of the lower leg and dorsum of the foot and usually is unilateral. It fades spontaneously within 2–3 days. Other organs can be involved less frequently; acute scrotal edema and pain was described in 17% of patients in one pediatric Israeli series (Gedalia et al., 1992) and occurred as a singular feature in some cases years before the diagnosis of FMF. Splenomegaly can occur in 30–50% of patients.

3.3.3. Etiology and Diagnostic Tests

There is no specific laboratory marker for FMF. During febrile attacks, nonspecific elevations in inflammatory mediators, fibrinogen, C-reactive protein, neutrophils, and sedimentation rate occur. If sampled, serosal or synovial fluid shows neutrophilia. Proteinuria (>0.5 g protein/24 hr) in patients with FMF is highly suggestive of amyloidosis. At least 28 mutations in *MEFV*, most clustered in one exon, have been described on the short arm of chromosome 16. FMF is the result when missense mutation(s) occurs in both copies of the gene encoding pyrin/marenostrin protein; the mechanism of clinical effect is as yet unclear. Pyrin is mainly expressed in neutrophils and monocytes. The "pyrin domain" (PVD) of this protein is a member of the death-domain-fold superfamily, and is involved in the apoptotic pathway through caspase recruitment, and also in the regulation of interleukin 1 (IL-1) cytokine and the production of nuclear factor kappaB (NFκB) transcription factor. Although reduced activity of C5a inhibitor in serosal or synovial fluid of patients with FMF is described, this test is not readily available and genetic testing is the confirmatory method of choice, with some limitations. Genetic laboratories usually screen for the five most frequent mutations (accounting for 85% of FMF). Single mutant-allele disease and double mutant-allele nondisease also are described.

3.3.4. Treatment and Outcome

Colchicine is the treatment of choice and is highly effective in preventing attacks. Mechanism of action is unclear but it is concentrated in neutrophils where it acts on microtubules, possibly up-regulating *MEFV* gene expression. Therapy prevents attacks in 60% of individuals with FMF and significantly reduces the number of attacks in an additional 20–30%. Adherence to therapy is important as attacks

can follow within days of discontinuance. Regardless of efficacy in prevention of attacks, colchicine therapy arrests or prevents amyloidosis, the life-threatening complication of FMF.

3.4. Hyper-Immunoglobulinemia D and Periodic Fever Syndrome

3.4.1. Epidemiology and History

Hyper-immunoglobulinemia D and periodic fever syndrome (HIDS) was first described in several Dutch patients by van der Meer et al. in 1984. It was also known as Dutch-type periodic fever. Most patients are White and are from western European countries. Sixty percent are Dutch or French. HIDS has been thought to be rare. The HIDS registry in the Netherlands currently has data on over 200 patients worldwide. Surprisingly, the defect is an enzymatic one, mapped to mutations in the *MVK* gene on the long arm of chromosome 12 (Drenth et al., 1999; Houten et al., 1999). Mevalonate kinase is an intermediary enzyme in the cholesterol metabolic pathway and isoprenoid biosynthesis pathway. In patients with HIDS, activity of mevalonate kinase is reduced to 5–15% of normal; serum cholesterol is slightly reduced; and during attacks, urinary level of mevalonic acid is slightly elevated. Autosomal Mendelian recessive inheritance is described. Frequency of the susceptibility gene in the Netherlands is 1:350 individuals. Most affected patients are compound heterozygotes for missense mutations in the *MVK* gene. Less than 1% of the patients have complete deficiency of mevalonate kinase, which is associated with infantile mevalonic aciduria, the rare inherited disorder characterized by dysmorphic features, failure to thrive, mental retardation, ataxia, recurrent fever attacks, and death in early childhood. Both the fatal mevalonic aciduria neurologic syndrome and HIDS with recurrent fever attacks without neurologic symptoms are caused by a functional deficiency of mevalonate kinase. Recently, Simon et al. (2004) identified five adults with neurologic signs and symptoms and mevalonate kinase deficiency (a phenotypic overlap between syndromes), suggesting that there may be a continuous spectrum of disease. It is not understood how mevalonate kinase deficiency is linked to the autoinflammatory periodic fever syndrome. Elevated serum levels of IgD ($>$100 IU/ml or 14 mg/dl) are characteristic but not universal especially in children under 3 years; elevated levels of IgA (e.g., $>$5 times the upper limit of normal) are characteristic ($>$80%) but not universal (Drenth et al., 1994; Saulsbury, 2003). These probably are markers of dysregulation rather than causes of disease. Although abnormalities of tumor necrosis factor (TNF)-alpha are not the primary cause of HIDS, plasma TNF-alpha levels are elevated in HIDS patients during attacks. Takada et al. (2003) described a favorable preliminary experience with treatment of HIDS patients with the TNF-alpha inhibitor, etanercept.

3.4.2. Clinical Features

HIDS patients' recurrent attacks of fever usually begin in the first year of life, after which clinical features become stereotypical (Drenth et al., 1994). An attack is heralded by chills followed by rapid rise of temperature to $\geq$39°C that lasts for

4–6 days with gradual defervescence. Headache, abdominal pain with vomiting, diarrhea, or both accompany most attacks. Cervical lymphadenopathy is common and frequently prominent. Arthralgias or arthritis of medium joints (knees, ankles, wrists) and erythematous macular, papular, or petechial rash predominantly on extremities are less common. Painful aphthous ulcers in the mouth or vagina occur in occasional patients. Orchitis recently has been described. Patients are well between attacks; growth is unimpaired. Periodicity typically involves recurrences of episodes every 4–6 weeks, but more sporadic recurrences as well as clockwork episodes with shorter intervals are possible. A characteristic feature is triggering of attacks by immunization, injury, or stress.

3.4.3. Etiology and Diagnostic Tests

HIDS is suspected clinically when periodic fever has begun in infancy and includes additional associated clinical symptoms. Serum IgD and IgA levels if both elevated are diagnostic. Screening for *MVK* mutations is confirmatory. More than 20 mutations have been identified, but one mutation, V3771, is present in >80% of patients. Screening for this mutation is a first step; if not found and suspicion is high, sequencing of the gene to detect other mutations is possible. Measurement of mevalonate kinase activity in leukocytes or urine is not recommended as the test is difficult to perform accurately, levels vary over only a modest range and with activity of illness, and usefulness is supplanted by confirmatory gene analysis.

3.4.4. Treatment and Outcome

There is no established treatment for HIDS. Beneficial effect of simvastatin (which inhibits a hydroxy-methylglutaryl-coenzyme-A reductase in the isoprenoid pathway) and etanercept (which inhibits TNF-alpha) has been reported (Takada et al., 2003; Simon et al., 2004). There does not appear to be increased mortality or amyloidosis associated with HIDS. With increasing age, frequency and severity of febrile episodes tends to decrease.

3.5. Tumor Necrosis Factor Receptor-Associated Periodic Syndrome

3.5.1. Epidemiology and History

The tumor necrosis factor receptor-associated periodic syndrome was first described by Williamson et al. in 1982 in a large Irish family and was called familial Hibernian fever. Although most of the reported families with TRAPS (tumor necrosis factor receptor associated periodic syndrome) are of Irish and Scottish descent, a wide variety of ethnic origins have been described recently, including French, Dutch, Belgian, Italian, Portuguese, Puerto Rican, African American, Mexican, Japanese and populations of Mediterranean origin where FMF is endemic such as Sardinian/Sicilian, Sephardic Jewish, Armenian, and Arab (Dodé et al., 2002). Although inheritance is autosomal dominant, there is variable penetrance and occasional sporadic cases appear. Through linkage analysis, Mulley et al. (1998) mapped the susceptibility gene

for this familial periodic fever syndrome to the short arm of chromosome 12. Missense mutations in the *TNFRSF1A* gene were linked with familial Hibernian fever by McDermott et al. (1999) and the name TRAPS suggested. Since then, at least 25 gene mutations have been identified, the vast majority in exons 2–4 in the extracellular domains. Aganna et al. (2003) showed heterogeneous gene defects in patients with familial TRAPS-like syndromes but found that *TNFRSF1A* mutations were not commonly associated with sporadic (nonfamilial) TRAPS-like cases. As in FMF and HIDS, TRAPS mutations are in the death-domain fold superfamily involved in protein–protein interactions that are required for apoptosis or anti-apoptosis signaling. Hypothesized pathogenesis of TRAPS is that missense mutations lead to structural abnormalities causing failure of shedding of TNF-alpha receptor from its intracellular site to the extracellular circulation where binding to TNF-alpha would prevent ongoing TNF-alpha induction of cytokine secretion, activation of leukocytes, fever, and cachexia. Uncontrolled inflammation is the result. Galon et al. (2000) described beneficial effect in TRAPS patients of etanercept, a dimeric recombinant fusion protein that binds both soluble and cell-bound TNF-alpha thus attenuating its biologic effect.

3.5.2. Clinical Features

The prototypical clinical syndrome described in familial Hibernian fever is only one manifestation of an ever growing group of genotypes—phenotypes of TRAPS. Some cases are FMF-like and some HIDS-like and, in fact, kindreds have been described with combined defects. Age of onset varies from a few weeks to >40 years of age. Most often, first symptoms occur in school-aged children. Attacks of fever are heralded by severe localized pain and tightness of one muscle group, which is migratory, in most patients. Skin rashes, tender, and raised erythematous plaques simulating cellulitis occur most frequently on extremities and migrate distally. Painful conjunctivitis and periorbital edema also distinguish TRAPS. Less specific but frequent symptoms are abdominal pain, arthralgia, testicular pain, and pleuritic chest pain. Duration of fever and other symptoms usually is greater than 1 week, but shorter and mild, nonspecific symptomatology or fever alone is described. Magnetic resonance imaging has suggested involvement of subcutaneous tissue, fascia, and muscles; biopsies have revealed mononuclear cell infiltration of fascia without myositis (Hull et al., 2002). Attacks come at irregular intervals weeks to years apart and can be triggered by minor infections, physical or emotional stress. Neutrophilia, elevated C-reactive protein and sedimentation rate are typical findings during attacks. Elevated serum immunoglobulins, including IgA and IgD, can be present.

3.5.3. Etiology and Diagnostic Tests

In patients with symptoms suggestive of TRAPS, identification of mutations in the *TNFRSF1A* gene is the definitive diagnostic test. More than 28 described mutations lead to qualitative or quantitative abnormalities in TNF receptor family type 1A proteins. Soluble *TNFSFR1A* is inappropriately low (<1 mg/ml). During attacks, serum levels may rise paradoxically to within normal limits. Unless gene analysis is accessible, measurement of serum soluble *TNFSFR1A* level between attacks might serve as a

screening test for patients with periodic fever and symptoms that overlap syndromes. A recent case report of genetic diagnosis of TRAPS in a man who had been diagnosed with PFAPA (with atypical features) at age 8 years highlights situations where gene analysis will be invaluable (Saulsbury and Wispelwey, 2005; Long, 2005).

3.5.4. Treatment and Outcome

Attacks of TRAPS respond dramatically to high dose of oral prednisone (>20 mg in adults) but response wanes over time. Colchicine has no effect. Etanercept is highly effective, reported to result in long-term remissions in some patients after a single course. Prognosis is mainly related to the presence or absence of amyloidosis, reported to occur in 10–25% of affected families. Certain mutations in *TNFRSF1A* and other modifier genes may be associated with amyloidosis, and the presence of amyloidosis in one family member cautions occurrence in another.

4. Case Denouement and Summary

The diagnosis of PFAPA was made in this 4-year-old boy of mixed racial background whose fever attacks occurred with clockwork periodicity every 4 weeks, lasted 3 days, and were followed by intervals of total well being. Inflammatory indicators during attacks and unremarkable quantitative serum immunoglobulins were compatible with this diagnosis. A 3-month trial of cimetidine was ineffective in preventing fever episodes. Tonsillectomy was performed, yet attacks continued with similar periodicity. At the age of 7 years, he was re-evaluated. History revealed that attacks had similar features except that mood changes and aggressive behavior were more marked and abdominal pain with vomiting was a new manifestation. Growth and development were normal. Physical examination was normal. Serum immunoglobulins were normal (including IgD 5 mg/dl and IgA 72 mg/dl). Genetic testing revealed mutations on chromosome 12 of *MVK* gene, compatible with HIDS. He will have a trial of etanercept therapy.

Identification of specific gene mutations rather than categorization by clinical syndromes or markers of inflammation undoubtedly will help clarify these early descriptions of periodic fever syndromes and advance management. (See one commercial laboratory resource for gene testing at http://www.genedx.com) Discovery of the genetic or infectious cause of PFAPA will greatly aid its accurate diagnosis. It is likely that there will continue to be genotype–phenotype disparities and unique abnormalities in patients with periodic fever syndromes. Such experiments of nature may provide a better understanding of other autoinflammatory syndromes and targeting of potential therapies. They also afford a glimpse at the complexly integrated mechanisms that balance innate responses in healthy individuals.

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8

BCG Vaccine

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1. History of BCG

All current strains of BCG (Bacille Calmette-Guerin) are derived from a strain developed by Leon Calmette and Camille Guerin at the Pasteur Institute, Lille, in the early 20th century (Sakula, 1983). Nocard had isolated a virulent *M. bovis* strain from a cow with tuberculous mastitis. This bovine strain, "Lait Nocard," was sent to Calmette (Grange et al., 1983; Oettinger et al., 1999). He and Guerin had been working on bovine tubercle bacilli, that is, *M. bovis*. Between 1908 and 1921, 230 passages were completed (Corbel et al., 2004). There was an associated decrease in virulence of this strain in different animal species over this period (Sakula, 1983) and administration to humans as a vaccine began in 1921 (Sakula, 1983; Bryder, 1999). Even after this, for more than 10 years controversy raged over whether, in fact, the BCG strain was attenuated. Unfortunately, the original strain was lost during the First World War, and the exact process(es) that led to the attenuation of this *M. bovis* strain is uncertain (Grange et al., 1983).

2. Phylogeny of BCG

2.1. How BCG Differs from *M. bovis*

BCG strains lack the deletion region, or region of difference, RD1, which is present in *M. bovis* and *M. tuberculosis* (Mahairas et al., 1996). There are also deletions in *M. bovis* relative to *M. tuberculosis* (Behr et al., 1999; Gordon et al., 1999, 2001). The absence of RD1 is common to all BCG strains, and presumably this mutation occurred early in the evolution of BCG. Certain BCG strains lack RD2, and others RD3 as well (Behr et al., 1999) (Figures 8.1 & 8.2). However, it is the loss of RD1 that is the major reason for the attenuation of BCG (Mahairas et al., 1996; Pym et al., 2002). Restoration of RD1 to BCG by gene knock-in led to both a change in the morphology of the BCG colony and more virulent behavior in both immunocompetent and immunodeficient mice. In contrast, restoration of RD3, RD4, RD5, RD7, and RD9

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did not result in different colonial morphology or increased virulence in immunocompetent mice, and restoration of RD3, RD5, and RD7 did not result in increased virulence in immunodeficient mice. However, others have shown that complementation with RD1 did not result in BCG Pasteur or BCG Russia regaining the virulence of *M. tuberculosis* or *M. bovis*. It has been proposed that mutations at other unlinked sites may be responsible for this further decease in virulence (Pym et al., 2002).

3. The Immunological Response to BCG

3.1. Host Response to *M. tuberculosis*

The immune response of the human host to *M. tuberculosis* is complex and incompletely understood. Cell-mediated immunity, particularly T cells, plays a central role in the host response to *M. tuberculosis* (Schluger and Rom, 1998; Boom et al., 2003).

CD4+ T cells, CD8+ T cells, and interferon-γ (IFN-γ) have all been shown to be important in the immune response to *M. tuberculosis*. Macrophages and other T cell subsets, including γδ T cells, also appear to be important in the response (Schluger and Rom, 1998; Schluger, 2001; Boom et al., 2003). CD4+ T cells can be phenotypically divided according to their cytokine profiles (Mosmann and Sad, 1996; Schluger and Rom, 1998). Th1 cells secrete IL-2 and IFN-γ and are involved in the inhibition of pathogenic bacteria and in protective immunity (Schluger and Rom, 1998). Th2 cells secrete Il-4, IL-5, and IL-10 and are involved in the recruitment of eosinophils and in production of IgE. Cytokines produced by Th2 cells shift the host immune response toward impaired immunity (Schluger and Rom, 1998). Both cell types secrete other and common cytokines (Mosmann and Sad, 1996; Schluger and Rom, 1998), but investigators have utilized IFN-γ and IL-4 as markers of a predominantly Th1 or Th2 host immune response to *M. tuberculosis* and BCG.

There are both animal and human data supporting the importance of the role of IFN-γ in host defense against *M. tuberculosis* (Schluger and Rom, 1998; Ellner et al., 2000; Boom et al., 2003), suggesting a predominantly Th1 response (Schluger and Rom, 1998; Schluger, 2001). The exact role of IFN-γ in the protective response is as yet unclear (Boom et al., 2003), and although it may be an imperfect marker of immunity to tuberculosis (TB), it is currently the most promising candidate. Further studies are needed to determine the exact role of IFN-γ in the host response (Ellner et al., 2000).

3.2. Host Response to BCG

Animal and human studies have shown that administration of BCG is associated with a Th1 response rather than a Th2 response, demonstrated by the combination of elevated IFN-γ levels and low IL-4 levels (Kemp et al., 1996; Marchant et al., 1999; Brandt et al., 2002; Hussey et al., 2002; Lee et al., 2004).

It is possible that immune responses are also dependent on the route of administration and/or dose of BCG. In mice, lower doses of BCG led to a mainly Th1

(IFN-γ) response while higher doses were associated with a mixed Th1/Th2 response, irrespective of how BCG was administered (Power et al., 1998). Kemp et al. (1996) compared intradermal and percutaneous BCG vaccine in healthy adults, and demonstrated a significantly greater lymphoproliferative and IFN-γ response to *M. tuberculosis* following intradermal administration. Interestingly, in both the groups the duration of vaccination site pain and the magnitude of the tuberculin skin test (TST) reaction correlated with the lymphoproliferative and IFN-γ responses.

Hussey et al. (2002) examined the effect of routes of administration and timing of vaccination of infants vaccinated at birth and 10 weeks of age. There was a trend to greater lymphoproliferative responses and a Th1-type (IFN-γ) cytokine response with intradermal rather than percutaneous vaccination and delaying vaccination until 10 weeks of age. Children in the Gambia who received BCG vaccine at birth or 2 months of age also developed a Th1 response as measured by higher levels of IFN-γ in response to PPD compared to unvaccinated controls. The lymphocytes of these vaccinated children also responded to both intracellular and extracellular antigens of *M. tuberculosis*. Moreover, the Th1 response was associated with the induction of immunological memory at the age of 1 year with high levels of IFN-γ but low levels of IL-5 and IL-13. Interestingly, significant levels of IL-4 developed in unvaccinated infants between the ages of 2 and 4 months. The development of the Th2 response in these children is not understood, but it has been suggested that it may be due to exposure to environmental mycobacteria (Marchant et al., 1999).

It has also been suggested that alterations in the immune response due to chronic infections, such as helminthic infections, may be a factor in varying BCG efficacy. Helminthic infections have been shown to induce a Th2-type immune response (Mosmann and Sad, 1996). Indeed, in Ethiopia treatment of helminthic infections with albendazole prior to BCG vaccination has been associated with significantly increased proliferative and IFN-γ responses to tuberculin PPD (Elias et al., 2001).

As BCG is associated with a Th1 response, there has been interest in determining whether the Th1 response to BCG decreases the future risk of atopic diseases such as asthma, which seem to be associated with a predominantly Th2 response (Robinson, 2004). However current data are conflicting (Aaby et al., 2000; Gruber et al., 2001; Pahari et al., 2002), and it has been suggested that genetic factors may also be important in these differences (von Hertzen and Haahtela, 2004). Moreover, in children in Guinea-Bissau the presence of a BCG scar and a positive tuberculin reaction has been associated with decreased mortality even when exposure to TB was controlled for (Garly et al., 2003). This contrasts findings from North America and the United Kingdom, where no decrease in non-TB-related deaths was observed, despite high levels of observed efficacy (Aronson and Aronson, 1952; Aronson et al., 1958; Medical Research Council, 1963). It has been hypothesized that the Th1 response could be favorable in conferring resistance to major infections and thus lead to a decrease in mortality (Garly et al., 2003).

Despite the vast numbers of BCG that have been administered, its mechanism of action is still unknown. There is currently no immunological investigation in humans or in animal models that predicts protective efficacy following vaccination with a TB vaccine. There is also no serological marker of immunogenicity or even a

surrogate marker of protection (McMurray, 2003), unlike other vaccines such as influenza or meningococcal vaccines. Certainly, the conversion of a tuberculin skin test (TST) following BCG vaccination does not necessarily translate to protection (Comstock, 1988; Al-Kassimi et al., 1995; Behr and Small, 1997). Better tests of immunogenicity to TB vaccines are needed.

4. Efficacy of BCG

4.1. Early Studies of BCG Efficacy

4.1.1. Student Nurses in Norway

Heimbeck (1948), in his 1924–1926 study of student nurses, found that only 2 of 151 students tuberculin-positive prior to commencing work with tuberculous patients developed clinical TB disease compared to 51 of 185 tuberculin-negative students. This led to a program of (voluntary) BCG vaccination of the students before commencing work, and therefore contact with tuberculous patients. Between 1924 and 1926, the rate of TB in tuberculin-negative BCG-vaccinated students was 24.1/1,000 observation years compared to 141.2/1,000 observation years in those who were tuberculin-negative and unvaccinated, that is, who developed primary infection during the study period. Similarly, mortality was reduced from 14.6 to 2.1/1,000 observation years. Heimbeck also noted that outside the hospital setting, BCG-vaccinated individuals in Oslo had 1/4 the rate of TB disease of those who were tuberculin-negative and unvaccinated.

4.1.2. Trial in American Indians

Aronson et al. (1958) conducted a placebo-controlled trial which began in the 1930s in over 3,000 American Indians ranging from infancy to over 60 years of age, who were followed initially for 15 years. This gave more than 21,000 person years of observation in the BCG-vaccinated group and more than 19,000 person years of observation in the placebo group. The BCG-vaccinated group had a markedly lower TB-related mortality after 15 years (Aronson and Aronson, 1952). A follow-up study of over 99% of the original subjects found there was still a marked difference in TB-related mortality (83 vs. 467/10,000 subjects) 20 years after vaccination with BCG.

4.2. BCG Trials

4.2.1. Medical Research Council Trial

This trial in England commenced in 1950 with over 50,000 school children aged 14 to 15½ years. Two vaccine strains were used: the Danish strain administered intradermally and *M. microti*, the vole bacillus, administered percutaneously. Children who had a positive TST to three tuberculin units or 100 tuberculin units were excluded from the vaccine groups and were analyzed separately. Active case-finding took the form of postal enquiries, home visits, chest X-rays (CXRs), and

tracking via the National Health Service. In addition, a blinded researcher assessed cases suggestive of TB (Medical Research Council, 1956). After 15 years, the annual incidence of TB in children given the BCG vaccine was 0.28/1,000 participants and 0.29/1,000 participants in those given the vole bacillus vaccine. In comparison, the annual incidence in the unvaccinated children admitted to the trial with those who received the BCG vaccine was 1.28/1,000 participants and the incidence in those children admitted with those who received the vole bacillus vaccine was 1.50/1,000. This gave a protective efficacy of 78.4% (99% Confidence intervals, CI: 69–86%) and 80.8% (99% CI: 68–91%) for the BCG and vole bacillus vaccines, respectively (Medical Research Council, 1972). Over 20 years post-vaccination, there were 10 cases of tuberculous meningitis and miliary pulmonary TB; all occurred in the control subjects. Despite the large study population there were insufficient cases of TB to draw a conclusion regarding efficacy between 15 and 20 years, although average efficacy over the 20-year period was 77% for both vaccines (Medical Research Council, 1972; Hart and Sutherland, 1977).

4.2.2. Tuberculosis Prevention Trial, Madras

This study was designed to examine the efficacy of BCG vaccination against culture-positive or smear-positive pulmonary TB. In the 1960s, only two areas of South India did not have a BCG vaccination program. The Chingleput area had a much higher prevalence of TB than the alternative area, which was part of the Bangalore district, and thus the former was chosen for this trial. Recruitment began in 1968 and ceased more than 2 years later. Skin testing was offered to those 1 year of age or older with either PPD-S (from the Statens Serum Institut) or PPD-B (from Battey Hospital, USA, and subsequently identified as *M. intracellulare*).

Two freeze-dried BCG strains were used: the Copenhagen strain 1331 and the Pasteur strain 1171 P2. Each vaccine was administered at a higher (0.1 mg) and lower (0.01 mg) dose. A placebo group made up the fifth arm of the study. During recruitment, over 366,000 individuals were registered. Of these, over 351,000 were eligible and 77.8% of these received either a BCG vaccine or the placebo.

The prevalence of TB infection as defined by a PPD-S reaction $\geq$12 mm was 50% in this population. This increased from childhood to a maximum at about 35 years of age in females (70% infected) and to about 25 years of age in males (80% infected), before leveling off in both genders. In contrast, reactions of 10 or more mm to PPD-B were already present in more than one-third of children aged 1–4 years and were almost 100% by the age of 15. There were 2,042 culture-positive cases from 206,609 subjects, that is the prevalence of TB disease was 1,068/100,000. The prevalence of radiological diagnoses of TB disease was 1,429/100,000, relatively more in the elderly ($\geq$65 years) and the young (10–14 years). It was suggested that the former represented healed lesions on CXRs being labeled as active disease and the latter hilar lymphadenopathy. Diagnosis of TB was made by sputum microscopy and culture, and by CXRs. Consequently, no diagnoses were made in any subjects under the age of 10 years initially and while the age at which CXRs were performed was lowered to 5 years in follow-up, no microbiological diagnoses at any stage were made in children younger than 10 years. The study design also excluded any diagnoses of extrapulmonary TB.

Re-recruitment was performed every 2½ years, continuously moving between villages. Follow-up was conducted initially every 7½ months and every 10 months subsequently to track those without a CXR or with an abnormal CXR and to instigate contact tracing. In addition, the trial coordinators facilitated passive case-finding including at local medical institutions.

After 7½ years of follow-up, there was no protective effect of BCG observed in the vaccinated groups, even when subgroups were analyzed according to age, one or two positive cultures, or vaccine strain. While most cases arose from those infected at recruitment, new cases which arose from those with a TST of 7 mm or less showed a slight but not statistically significant trend to a higher incidence in the vaccinated group. The investigators discarded the possibility of the effect of the strains or primary infection occurring soon after vaccination before there was a vaccine-related cell-mediated immune response. It was also felt that any effect of environmental mycobacteria would not be large enough to "abolish" any effect of BCG (Baily, 1979, 1980; Tuberculosis Research Centre, Chennai, 1999).

4.2.2.a. Consequences of the "Chingelput Trial"

The results of the trial came as a major shock to the global TB scientific community. Soon after the publication of the results, a WHO Scientific Group excluded the possibilities of errors in data processing or field operations, but raised the possibilities of (i) the effects of environmental mycobacteria, (ii) specific characteristics of the South Indian variant of *M. tuberculosis* which made it less virulent, resulting in both a longer infectious period and making itself a more effective vaccine than BCG, (iii) the high prevalence of adult TB disease resulting from a high level of exogenous reinfection or endogenous reactivation, and/or (iv) the specific host response to BCG vaccination (World Health Organization, 1980a). The possibilities of any effect of the freeze-dried form of the BCG vaccine or malnutrition in the study population were subsequently discarded (Springett and Sutherland, 1970; World Health Organization, 1980b).

However, it has also been proposed that the results of the Chingleput trial could not necessarily be applied to other countries (World Health Organization, 1980b). There is also a consensus that the results cannot be applied to infant BCG vaccination (Baily, 1979; World Health Organization, 1980b).

More recently, it has been proposed that that the very high level of exposure to *M. avium intracellulare* protected the study subjects to the same extent as BCG (Smith et al., 2000). It has also been hypothesized that despite this, there was a high prevalence of TB in this trial due to exogenous, airborne reinfection rather than endogenous reactivation (ten Dam and Pio, 1982; Smith et al., 2000).

4.3. Meta-analyses of BCG Efficacy

Rodrigues et al. (1993) performed a meta-analysis that set out to examine the efficacy of BCG against pulmonary tuberculosis, tuberculous meningitis, and miliary tuberculosis. The observed efficacies of BCG against pulmonary TB from 10 randomized controlled trials (RCTs) were heterogeneous to a highly significant degree and thus it was felt inappropriate to combine the results from the trials. The combined

protection against meningeal and miliary TB from four of these RCTs without statistically significant heterogeneity ($p > 0.20$) was 86% (95% CI: 65–95%). With the inclusion of a fifth trial with only two cases, the heterogeneity became statistically significant ($p = 0.013$) and combined efficacy was 81% (95%: 62–91%). However, the authors cautioned that the number of cases in each RCT was small. The combined protection from eight case–control studies (without significant heterogeneity, $p > 0.15$) was 75% (95% CI: 61–84%). It has been suggested that the reasons for the relative homogeneity of the observed efficacy of BCG against these forms of TB may be a consequence of cases being younger and thus be less likely to be exposed to protective atypical mycobacterial infections or to be subject to waning efficacy of the vaccine (Rodrigues et al., 1993). It has also been proposed that BCG may be immunologically more effective in halting dissemination of *M. tuberculosis* than in preventing pulmonary disease (Wiegeshaus and Smith, 1989).

Colditz et al. (1994) analyzed 14 prospective and 12 case–control studies. The combination of results from 11 trials showed a relative risk (RR) of TB of 0.49 (95% CI, 0.34–0.70), that is, a protective effect of 51% against mainly pulmonary TB. This was similar to the RR of 0.50 (95% CI, 0.39–0.64) or protective efficacy of 50% calculated from the combination of 10 case–control studies. Seven trials that reported tuberculous deaths gave a combined RR for death from TB for BCG vaccinees of 0.29 (95% CI, 0.16–0.53), that is, a 71% protective effect of BCG. Based on five case–control studies that examined tuberculous meningitis as an outcome, the RR was 0.36 (95% CI, 0.18–0.70), that is, a protective effect of BCG of 64%.

Subsequently, a separate meta-analysis was performed to examine the efficacy of BCG vaccination in newborns and infants. Five prospective trials of BCG

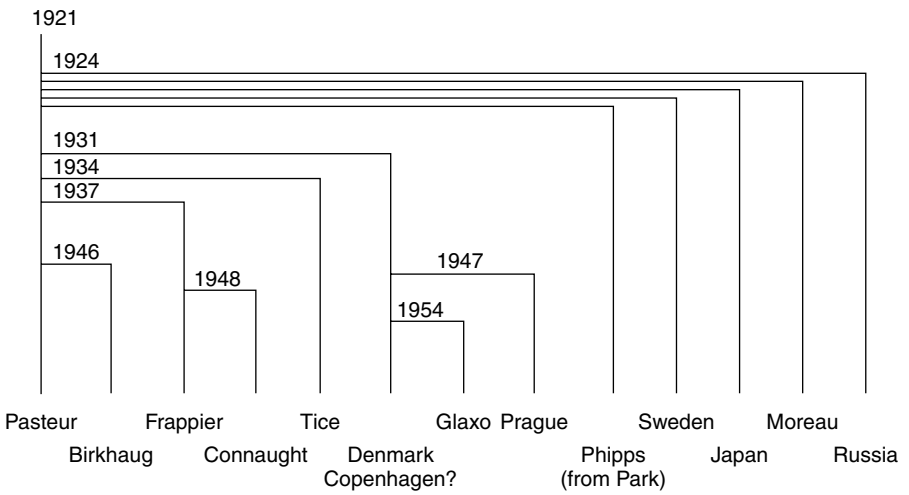

Figure 8.1. Genealogy of BCG strain dissemination. Vertical axis scales to time. Horizontal dimension does not scale to genetic difference. Reprinted from Behr, M.A. and Small, P.M. A historical and molecular phylogeny of BCG strains. *Vaccine* **17** (7–8), 915–922, Copyright 1999, with permission from Elsevier.

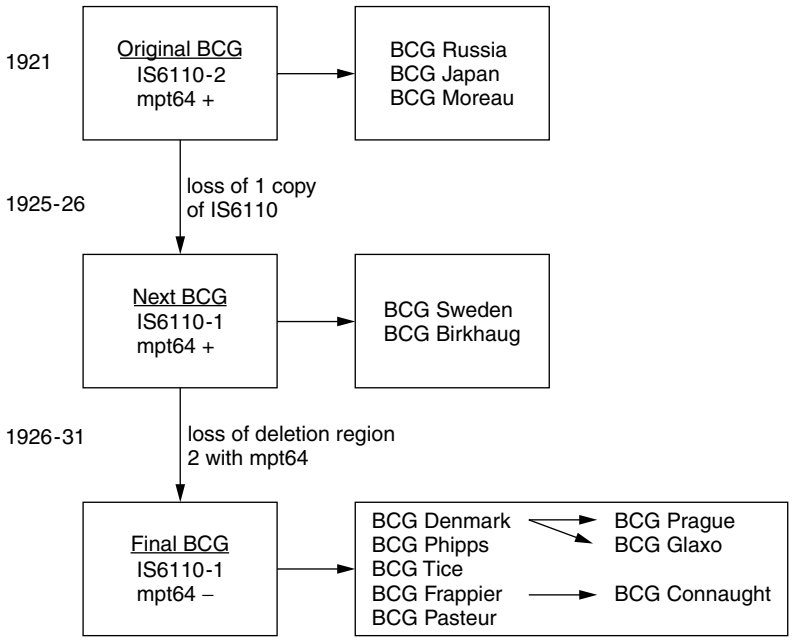

Figure 8.2. Evolutionary framework of BCG strains based on variability of IS6110 and mpt64 typing. Reprinted from Behr, M.A. and Small, P.M. A historical and molecular phylogeny of BCG strains. *Vaccine* **17** (7–8), 915–922, Copyright 1999, with permission from Elsevier.

vaccination in the 1st year of life and 11 case–control studies were included in this analysis. The combined RR from four RCTs was 0.26 (95% CI, 0.17–0.38) and the combined odds ratio (OR) from nine case controlled studies was 0.48 (95% CI, 0.37–0.62). With regards to TB deaths, the combined RR from five RCTs, which examined infants from households both with and without TB, was 0.35 (95% CI, 0.14–0.88). However, the most recent of these trials was reported more than 40 years ago, and three of the five trials were reported in the 1940s. Three case–control studies reported a total of 108 histologically or culture-confirmed cases, giving a combined OR of 0.17 (95% CI, 0.07–0.42). Five case–control studies reported a total of 181 cases of TB meningitis with a combined OR of 0.36 (95% CI, 0.18–0.70). For protection against disseminated TB, three case–control studies gave a combined OR of 0.22 (95% CI, 0.12–0.42) (Colditz et al., 1995).

Notably, of the 29 included trials in this latter analysis, 20 individually reported a statistically significant beneficial effect of BCG before the meta-analysis. A caveat is that for some of the case–control studies, determination of BCG vaccination status was based solely on the presence or absence of a BCG scar. Not all BCG vaccinees develop a BCG scar and this may partially account for the lower observed efficacy in these studies compared to the four RCTs in this meta-analysis. It is also interesting to note that the RCTs were all conducted further from the equator than the case–control studies, and that the former found higher observed efficacies of BCG. Within the case–control studies, the observed efficacy against severe

forms of TB or laboratory-confirmed TB was higher than for all TB cases, suggesting that diagnostic error may have been higher for the other TB cases (Colditz et al., 1995). This seems probable given that children are culture-positive much less frequently than adults (Shingadia and Novelli, 2003).

It has been suggested that the meta-analysis approach:

1. Could overestimate BCG efficacy by combining the results from trials with new, less-effective strains with results from earlier trials with ancestral strains (Behr and Small, 1997).
2. May be erroneous due to the association between latitude and observed efficacy, as the former may be an imperfect surrogate for other possible ecological factors (Fine, 1995).
3. Is not appropriate due to the heterogeneity between included studies (Fine, 1995).

4.4. Duration of Efficacy of BCG

A review of the duration of the efficacy of BCG examined 10 trials which reported BCG efficacy in TST-negative individuals, used randomized (or systematic, for early trials) allocation, and provided adequate results to calculate efficacy for differing periods after vaccination. Ten years after vaccination, the efficacies were no longer heterogeneous. The average efficacy of BCG after 10 years was 12% (95% CI, (−5–27%) by fixed effect meta-analysis and 14% (95% CI, (−9–32%) using random effects meta-analysis. It was concluded that there was no good evidence of a protective effect of BCG more than 10 years following vaccination (Sterne et al., 1998).

Aronson and Aronson, in their original study of American Indians, had suggested that the efficacy of BCG might decrease 10 years after vaccination (Aronson et al., 1958). A 60-year follow-up study of over 2,700 of the original study participants in the trial of BCG vaccination in American Indians found that the case rate between 1948 and 1998 was 66/100,000 person years in the BCG-vaccinated group and 138/100,000 years in the control group. This gave an estimated vaccine efficacy of 52% (95% CI, 27–69%) against TB disease. Over this period, there was a slight but not statistically significant decrease in the efficacy of BCG, less so in women than in men ($p = 0.02$). The efficacy of prevention of pulmonary TB was also 52% (95% CI, 14–74%) and efficacy was 44% (95% CI, (−22–75%) against death due to TB. There were few cases of miliary and meningeal TB in this population and an estimate of efficacy against these forms of disease was not reported (Aronson et al., 2004).

4.5. Explanations for Variability in Observed BCG Efficacy in Trials

4.5.1. Latitude and Environmental Mycobacteria

In a meta-analysis of BCG vaccine efficacy, Colditz et al. (1994) calculated that differences in latitude accounted for 41% of the variance between studies. Subsequently, Fine (1995) has provided strong evidence that studies with a higher observed BCG efficacy were conducted at higher latitudes. Studies such as the

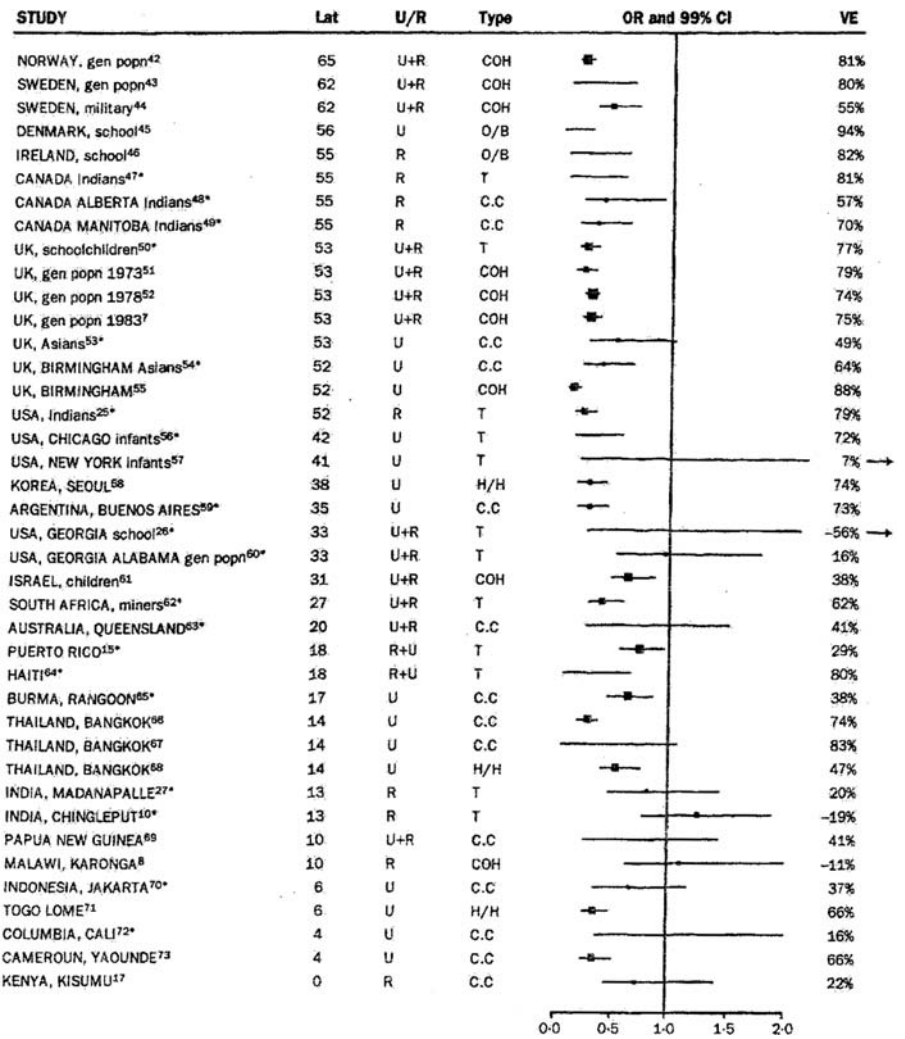

Figure 8.3. Summary of published estimates of efficacy of BCG against pulmonary tuberculosis in trials and observational studies, by latitude. This includes published trials involving random or alternate allocation of vaccine, and both retrospective and prospective observational studies. *Studies included in a review (Colditz, 1994) which concluded that latitude could explain 41% of the between-study variance. Total series of studies shown here also shows strong heterogeneity (χ^2[39df] = 394, $p < 0.00001$) and a significant trend with latitude (χ^2[1df] = 151, $p < 0.00001$). Lat = latitude in degrees (north or south) from equator; U/R = urban or rural; Type = study type (T = trial, COH = cohort, C–C = case control, O/B = outbreak, H/H= household contact study); OR = odds ratio; VE = vaccine efficacy (1 – OR). Reprinted and references from Fine, P.E.M. 1995. Variation in protection by BCG: Implications of and for heterologous immunity. *Lancet*, **346** (8986), 1339–1345, Copyright 1995, with permission from Elsevier.

Chingleput study which found a lower or even negative effect of BCG tended to be conducted at lower latitudes and this trend is statistically significant (Figure 8.3). Fine hypothesized that environmental mycobacteria provided protection against TB and this effect might account for a reduction in the observed efficacy of BCG. In the

1960s, Palmer and Long (1966) had demonstrated that guinea pigs inoculated with atypical mycobacteria survived longer after challenge with virulent *M. tuberculosis* than those who had not been inoculated. Exclusion of subjects with atypical mycobacterial infections (based on weakly reactive TSTs to lower doses of PPD or reactive TSTs to only higher doses of PPD, as in the MRC trial) (Medical Research Council, 1956) should lead to a higher observed efficacy (Medical Research Council, 1956; Palmer and Long, 1966; Comstock, 1994). However, others have suggested that the effect of atypical mycobacteria is confounded by uncertainty whether subjects with a weakly reactive TST were excluded by a higher dose of PPD (Comstock, 1994). In two RCTs of BCG vaccination in adolescents and young adults in Malawi and in adolescents in the United Kingdom, prevaccination IFN-γ responses to *M. tuberculosis* PPD and TST responses to tuberculin PPD were higher in the Malawian study population than in the UK school children. Following BCG vaccination, IFN-γ responses increased more in the UK study population than in the Malawian study population. It has been proposed that this disparity is due to a difference in sensitization by environmental mycobacteria (Black et al., 2002). In addition, following on from the work of Palmer and Long, Brandt et al. (2002) determined, in a murine model, that the ability of BCG to multiply and the number of BCG-specific IFN-γ producing cells in draining lymph nodes were decreased in mice which had been sensitized with environmental bacteria. Following challenge with *M. tuberculosis*, BCG also led to less protection in terms of bacterial numbers in mice which had been sensitized to atypical mycobacteria compared to those which had not been previously infected.

4.5.2. BCG Strain

Colditz et al. (1994) proposed that population variations, vaccinees of differing ages, and use of different vaccine strains have not been important factors in explaining the differences in the observed efficacy in vaccine trials. In contrast, a subsequent analysis suggested that four of five BCG strains lost efficacy with an increasing number of passages (Behr and Small, 1997). Strains also differ in the numbers of culturable particles per dose (Milstien and Gibson, 1990). Furthermore, Comstock (1994), noting variations including physical, genetic, and protective characteristics between strains, stated that it was difficult not to believe that there were differences in the efficacy of different strains, and that "clearly BCG is not BCG is not BCG; there are many BCG vaccines."

4.5.3. Different Routes of Administration of BCG Vaccine

As discussed earlier, the route of administration may result in differences in the human immune response. Different methods of administration have been used in different studies, including early studies which used the oral route (Comstock, 1994). Both the multiple puncture and intradermal route have been used subsequently in trials such as the MRC trial (Medical Research Council, 1956; Comstock, 1994). The effects of routes of administration were not considered in the meta-analyses performed by Colditz et al. (Colditz et al., 1994, 1995). However, it is unclear as to how important the route of administration is for efficacy and more studies are needed.

4.5.4. Genetic Differences Between Populations

There have been differences in the observed BCG efficacy when the same strain has been administered in two different populations (Fine, 1995). Perhaps, the best example of this is the Copenhagen strain 1331 that showed essentially zero efficacy in South India (1979), but 78% protection in the MRC trial (Medical Research Council, 1972). However as discussed above, this observation is subject to possible confounding effects, especially exposure to atypical mycobacteria. Genetic differences may be important with tuberculous disease. For example, in a small sample of adults pulmonary tuberculosis has been associated with a particular HLA haplotype (Goldfeld et al., 1998). In addition, the recent elucidation of genetic immune defects including partial and complete forms of deficiency of the IFN-γ receptor has led to the suggestion that milder forms may contribute to the differing susceptibilities of different populations to TB (Jouanguy et al., 1997). How these genetic differences affect the efficacy and immune response to BCG remain unclear.

4.5.5. Methodological and Statistical Rigor

Clements et al. (1983) performed a methodological and statistical appraisal of eight major BCG trials. Four sources of possible bias were identified: (i) susceptibility bias (confounding), (ii) surveillance bias (unequal follow-up between control and vaccinated groups), (iii) diagnostic-testing bias (investigations not applied equally to control and vaccinated groups), and (iv) diagnostic-interpretation bias (assessment of whether an outcome is due to TB is made with knowledge of the subject's vaccination status). The North American Indian, MRC, and Chicago trials that had the highest observed efficacy were also more highly rated in terms of methodological and statistical quality. They not only found a BCG efficacy of $\geq$75%, but also had the narrowest 95% confidence intervals. However, four of the remaining five trials had confidence intervals that exceeded 45%. It has been suggested that overall evidence indicates that BCG could be highly protective and that conflicting data might in part be due to bias or underpowered studies.

5. Complications of BCG

5.1. Classification of Complications

Complications of BCG vaccine include an abnormal BCG primary complex, disseminated BCG infection, and post-BCG syndromes or diseases clinically associated with vaccination (Lotte et al., 1984).

5.2. Frequency of Complications

The abnormal BCG primary complex accounts for the most common complication and includes development of local ulcers or abscesses, the Koch phenomenon, or regional suppurative lymphadenitis. The rate of these local complications has been estimated at 0.387 per 1,000 vaccinees less than one year of age and 0.025 per 1,000 vaccinees between 1 and 20 years of age. Certain side effects such as otitis media or retro-pharyngeal abscesses were seen with oral BCG only (Lotte et al., 1988).

The exact incidence of disseminated BCG disease is difficult to determine but has been estimated at two cases per one million vaccinees (Lotte et al., 1988). Reasons for the difficulty in obtaining a better estimate mainly stem from its rarity, incomplete reporting (Lotte et al., 1988), and the challenges in obtaining data in resource-poor countries where BCG is given in HIV-endemic areas but HIV-testing is not routine (Talbot et al., 1997).

5.3. Specific Complications

5.3.1. Local Lymphadenitis

This mainly occurs in draining lymph nodes following BCG administration, that is axillary or cervical for injections given into the deltoid, and inguinal for those given in the thigh. The lymph nodes may be either non-suppurative or suppurative. Conservative therapy is recommended for non-suppurative cases, although data are lacking for optimal management (Goraya and Virdi, 2002; Goraya and Virdi, 2001). The optimal therapy for suppurative lymphadenitis is also uncertain, and conservative management, antituberculous chemotherapy, incision and drainage, and surgical excision have all been employed (Lotte et al., 1988). In the immunocompetent individual, gradual resolution with or without spontaneous drainage generally occurs.

5.3.2. Disseminated BCG Disease

This is usually associated with an immunocompromised vaccinee (Talbot et al., 1997). Immune defects associated with disseminated BCG disease have included acquired immunodeficiency syndrome (AIDS), chronic granulomatous disease, severe combined immunodeficiency (Casanova et al., 1995; Talbot et al., 1997), IL-12 deficiency (Altare et al., 1998) or abnormalities of the IL-12 receptor (Remus et al., 2001; Elloumi-Zghal et al., 2002), and partial or complete IFN-γ receptor deficiency (Jouanguy et al., 1996, 1997). Interestingly, a study of 155 adults with advanced AIDS showed that none had positive blood cultures for BCG despite the presence of positive cultures for *M. tuberculosis* and *M. avium* (Marsh et al., 1997). Optimal antimycobacterial therapy for disseminated BCG disease is still unclear, and mortality is high despite treatment (Talbot et al., 1997).

6. BCG Vaccination Policies

6.1. World Health Organization Policy

6.1.1. WHO Indications for BCG Vaccination

1. Infants as soon as possible after birth in highly endemic countries.
2. Children at particular risk of exposure in otherwise low endemic areas.
3. Anyone exposed to multidrug-resistant TB (World Health Organization, 2004).

6.1.2. WHO Contraindications to BCG Vaccination

1. Impaired immunity, that is, symptomatic HIV infection (see Section 6.1.3), known or suspected congenital immunodeficiency, leukemia, lymphoma, or generalized malignant disease.
2. HIV-infected, asymptomatic infants in a low burden area (see Section 6.1.3).
3. Patients undergoing immunosuppressive treatment (corticosteroids, alkylating agents or antimetabolites, or radiotherapy).
4. Pregnancy.

6.1.3. WHO Recommendations Regarding BCG Vaccination and HIV Infection

1. HIV-infected infants may receive BCG vaccine only when asymptomatic and living in areas where TB is highly endemic.
2. In HIV-endemic areas, all healthy neonates should be given BCG.
3. HIV-infected, asymptomatic infants in a low-burden area should *not* be vaccinated.
4. Infants or children with symptomatic HIV-infected should *not* be vaccinated.
5. Long-term follow-up of BCG-vaccinated HIV-infected infants and BCG-vaccinated infants of known HIV-infected mothers is desirable (World Health Organization, 2004).

6.2. Policy in the United States

The Centers for Disease Control (1996) recommends BCG vaccination for children only if they are:

1. "Exposed continually" to an untreated or ineffectively treated case of infectious pulmonary TB and the child and infectious case cannot be separated or long term prophylaxis cannot be provided, or
2. "Exposed continually" to a case of multidrug resistant TB and the child and infectious case cannot be separated.

6.3. Variations Within Europe

Of 47 countries reporting on BCG immunization policies to WHO, 34 give the first BCG at birth and 8 after infancy (Fine et al., 1999). Some countries have a universal policy, while others have adopted a selective vaccination policy. Revaccination is recommended in some countries. Recommendations regarding the age of universal vaccination of older children and BCG vaccination of healthcare workers also differ between countries (Trnka et al., 1998). By contrast, the Netherlands has never recommended universal BCG (Fine et al., 1999).

6.4. Policy in the United Kingdom

6.4.1. Indications for BCG Vaccination

The Department of Health in the United Kingdom recommends BCG vaccination in various circumstances, in individuals who have not previously had BCG,

have negative skin tests (apart from babies aged less than 3 months), and no other contraindication, including

1. Health service staff at risk of occupational exposure
2. Children younger than 2 years of age who are contacts of a smear-positive case
   a. "Newly born babies" who are TST-negative 3–6 months after commencing prophylactic isoniazid chemotherapy
   b. Other children: Not previously BCG-vaccinated, following chemoprophylaxis, and "if appropriate"
3. New entrants to the United Kingdom from countries with a "high prevalence" of TB who are tuberculin-negative
4. UK-born children of immigrants from countries with a "high prevalence" of TB
5. Schoolchildren aged 10–14 years
6. At parents' or individual's request
7. Re-immunization: subjects who give a history of previous BCG immunization should be reimmunized only if the characteristic scar is absent and the tuberculin test is negative (Department of Health, 1996).

6.4.2. Contraindications to BCG Vaccination

1. Individuals with positive TSTs
2. Individuals who are immunosuppressed
   a. Those receiving immunosuppressive treatment including corticosteroids or general radiation (inhaled steroids are not a contraindication)
   b. Those in whom the normal immunological mechanisms may be impaired
3. Individuals suffering from malignancies such as leukemia, lymphoma, Hodgkin's disease, or other tumor of the reticuloendothelial system
4. Individuals known or suspected to be HIV-infected, including infants born to HIV-infected mothers until their status is clarified
5. Individuals who:
   a. Are febrile
   b. Have generalized septic skin conditions
   c. Are pregnant (Department of Health, 1996).

6.4.3. Selective Versus Universal BCG Vaccination

The United Kingdom has a policy of selective neonatal BCG vaccination based on presumed risk factors. However, these have proved challenging to implement due to administrative and logistic difficulties in identifying at risk infants, the tendency for high-risk populations to come from different ethnic backgrounds than the majority white population, and the time involved in assessment of risk status (Pharoah et al., 1996; Tseng et al., 1997). Moreover, the high rate of TB notifications in London (Tuberculosis Section, Communicable Disease Surveillance Centre London) leads to the question of whether there should be a program of universal BCG immunization in London. Indeed, several high-prevalence boroughs in London have already adopted universal neonatal BCG vaccination programs. However,

questions still remain as to when a universal program should be adopted (Pharoah et al., 1996).

7. The Future

The optimal approach for BCG vaccination programs in TB prevention is unclear. Different vaccination strategies have been adopted in different population. One option is a prophylactic (pre-infection or priming) vaccine that could be administered to vaccinees who have not been infected with *M. tuberculosis* or had a significant level of exposure to atypical mycobacteria (Olsen and Andersen, 2003; Doherty, 2004). Candidates for this strategy are recombinant BCG (knocking-in genes such as RD1) or *M. tuberculosis* (knocking-out genes) (Agger and Andersen, 2002; Doherty, 2004). Another option is a booster or sensitizing vaccine for those previously vaccinated or already sensitized by atypical mycobacteria (Olsen and Andersen, 2003; Doherty, 2004). Candidates for this approach are subunit vaccines administered as recombinant protein or DNA, or genes expressed in a vector such as a virus. Mucosal or oral vaccines have also been examined as possible options. A third option is a vaccine against latent infection to prevent future TB disease. This approach is very challenging due to the chronic nature of TB and the need to target the immense numbers of latently infected individuals (Doherty, 2004).

8. Conclusion

BCG has been used for over 80 years and is one of the most widely administered vaccines globally. However, there is uncertainty with both its efficacy in the field and the human host response to the vaccine. BCG vaccine does appear to be protective against severe forms of TB, for example, tuberculous meningitis and miliary TB. While BCG is safe in immunocompetent children and seems to be safe even in asymptomatic HIV-infected children, there is also a paucity of data on the efficacy and safety of BCG in HIV-endemic areas where BCG is particularly widely used.

Hopefully, future large-scale, prospective randomized studies of modified BCG vaccines or novel TB vaccines in areas of highly endemic TB will build on current and future animal models to provide us with this much sought-after evidence.

Lastly, it should always be remembered that BCG vaccination plays but one role alongside prompt diagnosis, directly observed therapy, appropriate chemoprophylaxis, and infection control and public health measures in the control of TB (World Health Organization, 2004).

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9

Helminth Infections of Children: Prospects for Control

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1. Introduction

The major soil-transmitted helminth (STH) infections, ascariasis, trichuriasis, and hookworm infection, together with schistosomiasis, occur in an estimated 2 billion people in the developing countries (de Silva et al., 2003; Hotez et al., 2006). It has been suggested that the STHs and schistosomes are among the most common human pathogens, and it is not unusual for a single individual, especially a child, to harbor several different species at the same time. According to the World Health Organization (WHO), these infections account for more than 40% of the disease burden due to tropical diseases, exclusive of malaria (WHO, 2002a).

A characteristic feature of STH and schistosome infections is that their morbidity is non-linearly related to the number of adult worms an individual harbors (sometimes referred to as the "worm burden") (Anderson and May, 1992; Hotez et al., 2005). Usually, only individuals with medium and heavy worm burdens suffer severe morbidity. The WHO estimates that of the approximately 300 million people who harbor heavy worm burdens, most are school-aged children (WHO, 2002a). Data collected over the past two decades indicate that heavy worm burdens in children result not only in adverse health effects, but also in cognitive and educational impairments (Drake et al., 2000). In contrast, other important helminth infections such as lymphatic filariasis and onchocerciasis exert their major effects on adult populations; the major approaches to their control have been reviewed elsewhere (Hoerauf, 2003).

Because of the emerging importance of pediatric helminthiases, the WHO and World Bank have focused international attention on identifying and implementing cost-effective methods for helminth control. To date, the major approach has been to reduce the worm burdens and their corresponding morbidity through the periodic

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and frequent administration of two anthelminthic drugs, namely a benzimidazoles anthelminthic (BZA), for example, albendazole or mebendazole to treat STH infections, and praziquantel (PZQ) to treat schistosome infections. It is likely that global pediatric helminth control will rely heavily on using school systems and school teachers for drug delivery (Partnership for Child Development, 1997; Savioli et al., 2002). The benefits and caveats of school-based anthelminthic chemotherapy for global control efforts are discussed, as are current efforts to develop anthelminthic vaccines for hookworm infection and schistosomiasis.

2. Global Disease Burden of Helminth Infections

The most recent estimates from the published literature and other computerized database searches indicate that 1,221 million, 795 million, and 740 million cases of ascariasis, trichuriasis, and hookworm infections occur worldwide, respectively (de Silva et al., 2003) (Table 9.1). Almost all of these infections are in the developing nations of the tropics and subtropics, with the highest prevalence occurring in the World Bank regions of sub-Saharan Africa, China, and East Asia, and the Pacific Islands. An even greater number of people, roughly 3–4 billion, are at risk for acquiring STH infections in these regions (de Silva et al., 2003). Of the 187 million cases of schistosomiasis worldwide, most are caused by *Schistosoma haematobium* (119 million cases) and *S. mansoni* (67 million cases). The majority of the world's schistosomiasis disease burdens occur in sub-Saharan Africa; this includes 112 million cases of *S. haematobium* infection and 54 million cases of *S. mansoni* infection (van der Werf et al., 2003). Only 1 million cases are caused by the Asian schistosomes *S. japonicum* and *S. mekongi* (Hotez et al., 2006).

Although the global prevalence estimates for STH and schistosome infections are impressive, specific knowledge regarding the prevalence of a helminth infection is often less informative than knowing the worm burden. Generally speaking, morbidity from helminths is proportional to the worm burden. In endemic communities, all of the STH and schistosome infections exhibit an aggregated distribution where most individuals harbor a few worms and a minority harbor disproportionately heavy

Table 9.1. Prevalence of STH and Schistosome Infections

Infection	People at risk (millions)	Estimated number of infections (millions)
Ascariasis	4,211	1,221
Trichuriasis	3,212	795
Hookworm	3,195	740
Schistosomiasis	Not determined	187
S. haematobium		119
S. mansoni		67
S. japonicum complex		1

Modified from de Silva et al. (2003) and van der werf et al. (2003).

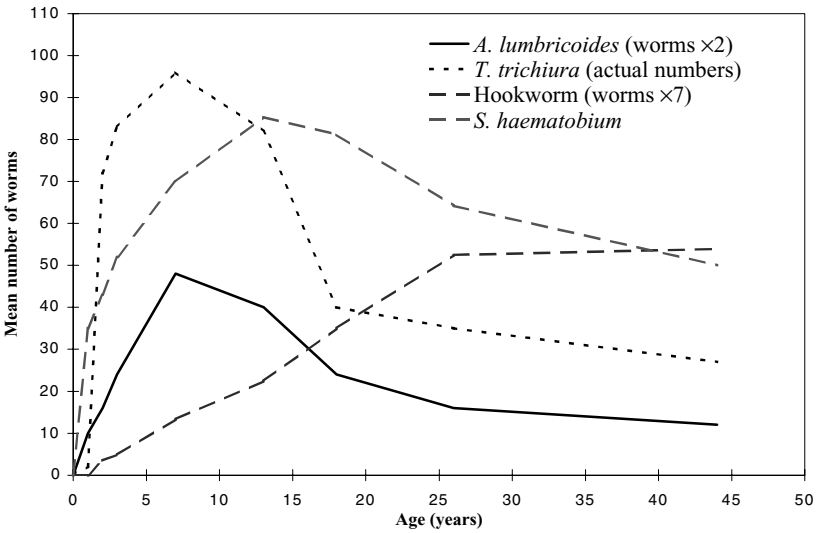

Figure 9.1. Age-associated infection intensity of helminth infections caused by *Ascaris lumbricoides*, *Trichuris trichiura*, hookworm, and *S. haematobium*, modified from Hotez et al. (2006).

worm burdens (Anderson and May, 1992). Generally, worm burdens are indirectly estimated using quantitative fecal egg counts, and WHO defines light, moderate, and heavy infections on this basis (WHO, 2002a).

Helminth infection intensities are markedly age-dependent (Anderson and May, 1992; Bundy, 1995). Since intensity is linked to morbidity, age-intensity profiles help to identify the populations that are vulnerable to the different STHs and schistosomes (Hotez et al., 2006). As shown in Figure 9.1, for ascaris and trichuris infections the age-intensity profiles are convex in shape, with intensity peaking in children 5–15 years of age (Bundy, 1995). For schistosomiasis, the convex pattern is pushed slightly to the right so that the peak worm burdens occur in adolescents and young adults, and declines thereafter, possibly due to the acquisition of acquired immunity. Practically speaking, this means that school-aged children, more than any other group, harbor the largest worm burdens (World Bank and World Health Organization, 2003). The only major exception is hookworm infection, for which the age-intensity profile exhibits greater variability. In some communities, hookworm intensity increases with age until adulthood, and then either plateaus or even continues to increase (Bethony et al., 2002; Brooker et al., 2004; Hotez et al., 2004). Women of reproductive age and pregnant women are particularly vulnerable to the clinical effects of heavy hookworm burdens that could result in anemia (Bundy et al., 1995). Therefore, hookworm is an important maternal–child health problem in developing countries (Hotez et al., 2004).

The observation that the highest intensity helminth infections occur in school-aged children has important clinical and public health implications (Hotez, 2000). The most common and universal clinical consequence of heavy STH and schistosome infections is their ability to cause physical growth stunting (Stephenson et al., 1989; Dickson

et al., 2000; Hotez, 2000). Because this phenomenon occurs so widely, it can be argued that helminth infections are the world's leading endocrinopathy (Hotez, 2000). The mechanisms by which helminths cause physical growth retardation are not known.

In addition, to impairing physical growth, each specific type of helminth infection produces a unique syndrome. For instance, heavy ascaris infections in children can result in acute intestinal obstruction or hepatobiliary ascariasis, the latter occurring when adult ascaris worms enter the enterohepatic portal system (de Silva et al., 1997; Crompton, 2001). Heavy pediatric trichuris infections cause trichuris colitis and trichuris dysentery syndrome, which can lead to rectal prolapse (Bundy and Cooper, 1989), while heavy hookworm infections result in moderate and severe iron-deficiency anemia and protein loss leading to hypoalbuminemia (Hotez et al, 2004; Stoltzfus et al, 1997a; 1997b; 2000; Brooker et al, 1999). Heavy schistosome infections can result in liver fibrosis and hepatosplenomegaly or severe bladder wall pathology leading to hydronephrosis (Richter, 2003).

There is also evidence that chronic moderate and heavy STH and schistosome infections associated with cognitive and intellectual deficits (Sakti et al., 1999; Drake et al., 2000). Further studies indicate that helminth infections are associated with reduced school attendance (Bleakley, 2003). Therefore, helminth infections have a major impact on the education of children in the developing countries (World Bank and World Health Organization, 2003).

The physical and intellectual cognitive impairments caused by helminth infections are usually insidious and not easy to measure objectively. Furthermore, helminth infections do not commonly result in death, although some estimates indicate that they cause more than 150,000 deaths annually (WHO, 2002a). Therefore, apart from determining their prevalence, the global burden of STH and schistosome infections has traditionally been under estimated by clinicians and public health workers. However, using the disability-adjusted life year as a metric of disease burden, the Global Burden of Disease study has confirmed the enormous health impact of STH and schistosome infections (WHO, 2002b). As shown in Table 9.2, the disease burden caused by STH infections and schistosomiasis worldwide is roughly

Table 9.2. Burden of Disease in DALYs from Helminth and Other Common Childhood Conditions

Disease	DALYs (000)
Measles	26,495
Pertussis	12,464
Meningitis	6,420
STH and schistosomiasis	6,466
STH infections	4,706
Leukemia	4,660
Hookworm infection	1,825
Schistosomiasis	1,760
Trichuriasis	1,649
Ascariasis	1,181
Dengue	653

Data from WHO (2002b).

equivalent to the global disease burden of bacterial meningitis, half the disease burden of pertussis, and one-quarter that of measles (WHO, 2002a).

3. Anthelminthic Drugs in the Control of Childhood Helminth Infections

Efforts to control STH and schistosome infections have traditionally depended on the sanitary disposal of feces, and health education about the proper use of latrines. In regions and periods where economic growth has been substantial, such as in the Southern United States during the first-half of the 20th century or in post-World War II Korea and Japan, such efforts have been effective at reducing helminth infection prevalence and intensity (Hotez, 2002). However, in the absence of economic development the impact of sanitation and health education on helminth control has been both slow and incremental (Asaolu and Ofoezie, 2003). The WHO and World Bank have determined that the most cost-effective way to control the harmful effects of STH and schistosome infections in the developing regions is through population-wide treatment with either a single dose of albendazole (400 mg) or mebendazole (500 mg) (Montresor et al., 2002; The Medical Letter Inc., 2002; WHO, 2002a, b). Increasingly, emphasis has been placed on the school-based treatment programs in order to focus efforts on children who are at the greatest risk for acquiring heavy STH infections, especially ascariasis and trichuriasis (Montresor et al., 2002; WHO, 2002a; World Bank and World Health Organization, 2003). Targeting school-aged children with heavy ascaris and trichuris infections has the added benefit of reducing community-wide transmission (Bundy et al., 1990). In regions where schistosomiasis is co-endemic, especially sub-Saharan Africa and Brazil, school-based treatment programs include the addition of a single dose of PZQ (40–60 mg/kg) to periodic de-worming programs (Fenwick et al., 2003). These drugs are inexpensive, have undergone extensive safety testing, and have now been used in hundreds of thousands of children with only a few, minor side effects (Utzinger and Keiser, 2004).

The control of STH and schistosome infections is cost-effective only when anthelminthics are administered to all school-aged children in a community regardless of their infection status. This concept is supported by the recently summarized observations that (1) the clinical appearance of these infections is often insidious and without specific symptoms; (2) individual diagnosis would require the continuous availability of laboratory supplies and trained microscopists, which add considerably to the costs of control; and (3) the drugs are safe and, because they are available as generics, inexpensive (Montresor et al., 2002; WHO 2002a; World Bank and World Health Organization, 2003; Hotez et al., 2006). In areas of intense transmission ($>$70% prevalence and more than 10% of the cases with moderate and heavy infection), the WHO recommends a treatment frequency of two or three times a year for helminth infections in order to maintain host worm burdens below the threshold that would otherwise result in morbidity. However, in areas with lower intensity of transmission, once-yearly interventions are usually sufficient to control morbidity (WHO, 2002a).

These observations and recommendations have provided a framework for the adoption of a resolution at the 54th World Health Assembly in 2001 (Resolution 54.19), which calls for the regular administration of a BZA and/or PZQ to at least 75% of all school-aged children at risk for morbidity by 2010 (www.who.int/worm-control). The primary focus on school-aged children reflects the observations, also recently summarized, that (1) overall, such children typically harbor the largest worm burdens of any age group; (2) chronic STH and schistosome infections in childhood lead to physical growth suppression, and repeated anthelminthic de-worming results in improved catch-up growth; (3) early intervention against schistosomiasis prevents the development of hepatosplenic disease and other sequelae (Richter, 2003); (4) de-worming can improve children's learning and school attendance; and (5) de-worming can reduce the transmission of ascaris and trichuris infections in the entire community and therefore has a community-wide major externality (Montresor et al., 2002; World Bank and World Health Organization, 2003; Hotez et al., 2006). Since the adoption of resolution 54.19, several non-governmental organizations, such as the Partnership for Child Development and the Partnership for Parasite Control, have promoted schools in the developing countries as promising venues for administering BZAs and PZQ (World Bank and World Health Organization, 2003). School-based de-worming programs have been shown to be cost-effective, especially when the anthelminthic drugs are administered by teachers in a supervised setting of training and support. If widely implemented, school-based de-worming could in time become one of the world's largest health program (Horton, 2003).

4. Downstream Alternatives to School-Based De-worming: Rationale and Promise for Developing Anthelminthic Vaccines

4.1. Anti-hookworm Vaccines

There are concerns that school-based de-worming programs may have less of an impact on the prevalence, intensity, or transmission of hookworm infection compared to other STH infections. The reasons for this include (1) unlike ascaris and trichuris infections, the highest intensity hookworm infections frequently occur in populations outside of the school-aged period—such populations would not benefit from exclusively school-based programs (Bundy, 1995); (2) for similar reasons, school-based programs will not reduce hookworm transmission in the community (Chan et al., 1997); (3) albendazole and mebendazole have variable efficacy against hookworms (Hotez et al., 2005); (4) both children and adults usually become re-infected with hookworm infection within a few months (Albonico et al., 1995), which necessitates repeated and frequent use of the drugs; and (5) the efficacy of mebendazole may decrease with frequent and repeated use (Albonico et al., 2003), and there are concerns that such heavy reliance on BZAs might promote drug resistance (Albonico, 2003).

As an alternative or complementary approach to school-based de-worming, the development of a safe and cost-effective vaccine would provide an important

new tool for the control of hookworm infection. The feasibility of developing an anti-hookworm vaccine is based on the previous development of a live irradiated third-stage infective larval (L3) canine vaccine (Miller, 1971), which was marketed in the United States during the early 1970s (Miller, 1978). Although it is not feasible to develop a live L3 vaccine for humans, the observation that many of the target antigens linked to L3 vaccination are secreted proteins has led to intensive study of these macromolecules (Hotez et al., 2003). The major L3 secreted proteins have now been identified, isolated, cloned, and expressed, and then preclinically tested as recombinant vaccines (Hotez et al., 2003). They include two cysteine-rich proteins belonging to the pathogenesis-related protein superfamily, ancylostoma secreted protein 1 (ASP-1) and ASP-2. ASP-2 was selected as the lead candidate because it is partially protective in laboratory hamsters and dogs challenged with animal hookworm L3 (Goud et al., 2004), and it was antigenic in individuals who have low-intensity hookworm infection (Jeff Bethony, unpublished observation). The ASP-2 ortholog from the human hookworm *Necator americanus* (*Na*-ASP-2) was cloned and expressed as a 21.3 kDa recombinant protein in the yeast *Pichia pastoris*. The purified protein was formulated with alhydrogel. Tentatively, a US Phase 1 dose-escalating trial to test the safety and immunogenicity of the *Na*-ASP-2 Hookworm Vaccine has been planned, as has a Phase 2 trial in an endemic region of Brazil.

4.2. Anti-schistosomiasis Vaccines

The Institut Pasteur in Lille is developing and testing a recombinant vaccine against schistosomiasis caused by *S. haematobium*. The vaccine (Bilhvax) is comprised of a recombinant glutathione-S-transferase, which is expected to reduce parasite egg output and therefore diminish urinary tract pathology that would otherwise result from parasite egg deposition in the bladder (Capron et al., 2002a, b). Bilhvax was shown to be safe and immunogenic in vaccine trials in France, Senegal, and Niger (www.who.int/tdr). It is hoped that vaccination would reduce the burden of *S. haematobium* disease, possibly including long-term sequelae such as hydronephrosis and squamous cell bladder cancer. A second vaccine for *S. mansoni* infection based on a novel fatty acid binding protein is also being developed in Brazil (Tendler et al., 1996).

5. Conclusions

The relative cost-effectiveness of administering an anthelminthic vaccine versus repeated and frequent anthelminthic de-worming has not yet been determined. Although the cost of generic BZAs and PZQ has been reduced to well below $US 0.50 per dose, there are additional costs associated with maintaining an infrastructure for their consistent delivery and monitoring (Guyatt, 2003). Similarly, while the current costs of recombinant vaccines are high, in the case of hookworm between $3 and $10 per dose, it is anticipated that these costs will be reduced substantially following larger scale fermentation and off-shore manufacture in the middle-income countries such as Brazil, China, or India.

Another important consideration for implementing control programs that include anthelminthic vaccines will be the identification of target populations suitable for vaccination. While school-aged children are an obvious vulnerable population, there are currently not many school-aged vaccine programs in the developing countries. Therefore, an anthelminthic vaccine program must be considered in the context of current periodic de-worming efforts. Over the next decade, several new vaccines for school-aged children, older adolescents, or young adults are expected to come on-line. These include papillomavirus vaccines to prevent cervical cancer, and vaccines for group B streptococcus. It will be of great interest to package such vaccines together for use in the developing countries.

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10

Rabies on the Doorstep

David A. Warrell

1. Introduction

Apparently, the United Kingdom had been free of indigenous rabies since 1902. However, in 1996, a rabies-infected Daubenton's bat (*Myotis daubentonii*) was found at Newhaven on the south coast of England. It seemed possible that this sick bat had been carried in by boat or blown across the Channel by prevailing winds. In 2002, an infected juvenile bat of the same species was found on the Lancashire Canal, which runs between Preston and Tewitfield in northwestern England. Both the bat's age and location favored the possibility that it had acquired rabies in England. In both these cases, the rabies-related virus involved was European bat lyssavirus (EBLV) type 2a (Whitby et al., 2000; Johnson et al., 2002). EBLV 2 had been implicated in three human deaths from rabies in mainland Europe (Lumio et al., 1986; World Health Organisation 1987; Roine et al., 1988).

On November 19, 2002, a 55-year-old male natural history artist was admitted to hospital in Aberdeen, Scotland, complaining of 5-days' increasing pain in the left arm, paraesthesiae of both arms, and dysphagia. He was found to be feverish (38.5°C), inappropriately disinhibited, dysarthric, with nystagmus, areflexic arms, ataxia, and dysaesthesia of the left arm. CT and MRI of the brain were essentially normal, as was the cerebrospinal fluid (CSF). On the fifth day of his hospital admission, he became acutely confused, aggressive, and agitated, and his Glasgow Coma Score declined to 6. The repeat CT scan was normal, but the CSF protein was elevated and there was a mild lymphocyte pleocytosis. The next day there was further deterioration, with pulmonary collapse, respiratory failure, a further decline in Glasgow Coma Score to 3, excessive salivation, and flaccid quadriplegia with extensor plantar responses. He died on the 14th day of hospital admission. A more detailed history revealed that he had been bitten frequently by bats in the course of his work as a bat conservationist in Angus, Scotland. Most recently, he had been bitten on the left hand 19 weeks earlier by a Daubenton's bat. Although the Department of Health had recommended pre-exposure vaccination against rabies for licensed bat handlers (UK Health Departments, 1996), the patient had apparently refused this protection. PCR revealed the presence of EBLV type 2a in saliva

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sampled on days 7 and 9 of his hospital admission. This diagnosis was confirmed at autopsy by immunofluorescence, RT-PCR, virus isolation, and other techniques (Fooks et al., 2003; Nathwani et al., 2003). In continental Europe since 1977, more than 700 cases of EBLV infection have been found in insectivorous bats, mainly in Denmark, the Netherlands, Poland, and Germany (Muller, 2000).

2. Implications of Having Enzootic EBLV in Britain

The discovery of EBLV in the United Kingdom raises a number of questions demanding urgent answers.

2.1. How prevalent is EBLV among British bats?

Over the past 18 years, three infected Daubenton's bats have been found by passive surveillance in the United Kingdom involving 4,000 bats of which about 75 were *M. daubentonii*. The third of these EBLV-2a-positive Daubenton's bats was found sick near the River Thames in central Staines, Surrey, in September 2004 and later died (Fooks et al., 2004). Active surveillance of target bat species (*M. daubentonii* and *Eptesicus serotinus*) in selected geographical areas was started in 2003. So far, the prevalence of seropositivity to EBL-2 in the sample Daubenton's bats has ranged between 2% and 8% (Fooks, 2004). A recent sero-survey in northwestern England found that, among 100 bats tested, 5–14% of Daubenton's were seropositive for EBLV type 2. In other parts of Europe in 2001, 158 EBLV seropositive bats were found, 4.4% of all the positive results in wild mammals tested.

2.2. Can EBLV be transmitted to other mammals?

Four cases of transmission to humans, four to sheep, and one to a stone marten (*Martes foina*) in Germany (Müller et al., 2004) have been detected. A serological survey of cats in Denmark revealed one animal with evidence of EBLV-1 infection (Tjørnehøj, et al., 2004). However, in Europe, rabid terrestrial mammals are usually assumed to have been infected by foxes and so the virus is rarely typed. As a result, EBLV infections may have been missed.

2.3. How effective are standard rabies vaccines and rabies immune globulin against EBLV?

Serological and challenge studies in mice have proved inconclusive (Warrell and Warrell, 2004). Protection varied both with the genotype of EBLV used in the challenge and with the rabies vaccine strain employed for immunization. However, there is some reassurance in the fact that, among the hundreds of people bitten by EBLV-positive bats in the Netherlands and one in the United Kingdom, all were given post-exposure prophylaxis with conventional rabies vaccines and none has developed rabies. None of the four known human victims of EBLV in Europe had received any rabies vaccine.

2.4. To whom should pre- and post-exposure prophylaxis be given in the United Kingdom?

Pre-exposure vaccination would seem to be entirely reasonable for registered bat handlers and for all others who work with or handle bats, together with veterinarians who have unusual habitual exposure to bats. Post-exposure vaccination should be given to people bitten by bats, especially by Daubenton's bats.

3. Other Rabies-Related Viruses

The discovery of previously unsuspected enzootic EBLV in British bats and the tragic outcome of a bite inflicted on a bat handler were reminiscent of the emergence of another rabies-related virus, Australian bat lyssavirus (ABLV) (serotype 7). In 1996 and 1998, two women, one of whom acted as a bat carer, died of rabies-like illnesses in Queensland (Samaratunga et al., 1998; Hanna et al., 2000). They had been infected with a new lyssavirus transmitted by flying foxes (genus Pteropus) and insectivorous bats. In November 2004, near Townsville, Queensland, a flying fox which wrapped itself round the head of a 4-year-old boy and bit him on the face was found to be infected with ABLV.

Apart from classic rabies virus (genotype 1), which is globally distributed, six other rabies-related lyssaviruses are now known to have caused fatal encephalitis in humans. Mokola (genotype 3) and Duvenhage (genotype 4) have been found in dogs, cats, rodents, and insectivores in Africa; EBLV (genotypes 5 and 6), Australian bat lyssavirus, and the recently described Aravan, Khujand, West Caucasian, and Irkut viruses are enzootic in a number of different insectivorous bat species in parts of the former Soviet Union (Table 10.1). After the retreat of the European fox rabies epizootic in the second half of the 20th century, western Europe was left virtually free of classic rabies, but EBLV has been found in Spain, the Netherlands, Germany, Poland, Hungary, France, Switzerland, Denmark, Russia, Ukraine and, most recently, the United Kingdom. In Europe, the numbers of several species of bats have been in decline and these animals have been the subject of intensive conservation efforts. The increasing number of people engaged in looking after sick bats ("bat carers") has undoubtedly increased exposure of humans to virus genotypes that were formerly confined almost exclusively to bat ecologies.

4. The Global Problem of Rabies

Outside the United Kingdom, almost all parts of the world are endemic for classic (genotype 1) rabies (Figure 10.1). Human rabies is undoubtedly underreported in the developing world, but, in 1997, it was estimated that 60,000 human deaths resulted from dog-mediated rabies, and 50 million doses of rabies vaccine were used for post-exposure prophylaxis worldwide (World Health Organization, 1998). Ninety-nine percent of all human rabies deaths occur in developing countries, 90% in Asia. In May 2004, China's Ministry of Health reported the surprising news that, in that

Table 10.1. The Genus Lyssavirus

Genotype virus		Source	Known distribution
Phylogroup I			
1	Classic rabies virus	Dog, fox, raccoon, skunk, bat, etc.	Widespread
4	Duvenhage	Insectivorous bat (e.g., *Nycteris the baica*)	South Africa, Zimbabwe (very rarely identified)
5	European bat lyssavirus-1a	Bats, for example, *E. serotinus*	Northern and Eastern Europe
	European bat lyssavirus-1b	Bats, for example, *E. serotinus*	Western Europe
6	European bat lyssavirus-2a	*M. dasycneme* bat	The Netherlands (rare)
		M. daubentonii bat	United Kingdom (and Ukraine in another bat species)
	European bat lyssavirus-2b	*M. duabentonii* bat	Switzerland (very rare)
7	Australian bat lyssavirus	Flying foxes (*Pteropus* spp)	Australia
?	Aravan virus	Insectivorous bats	Kyrghystan
?	Khujand virus	Insectivorous bat	Tajikistan
?	West Caucasian bat virus		Caucasus
?	Irkut virus		Siberia
Phylogroup II			
3	Mokola	Shrews (*Crocidura* spp), cats	South Africa, Nigeria, Cameroon, Ethiopia (rare)
2	Lagos bat virus	Bats, cat (has *not* been detected in man)	Africa (rare)

month, rabies had been the most important single cause of death from infectious diseases in China (127 out of 477 deaths), ahead of AIDS, infantile tetanus, and epidemic cerebrospinal meningitis, although tuberculosis remained the most commonly reported infectious disease (http://www.moh.gov.cn/zhgl/gzdt/1200406110002.htm). By August 2004, more than 1,000 people had died from rabies in China that year, most of whom had not received any wound treatment or post-exposure vaccination through ignorance of the dangers of rabies.

The epidemiology of rabies varies throughout the world. For example, in South Africa, the principal mammalian vectors and reservoir for rabies are domestic dogs

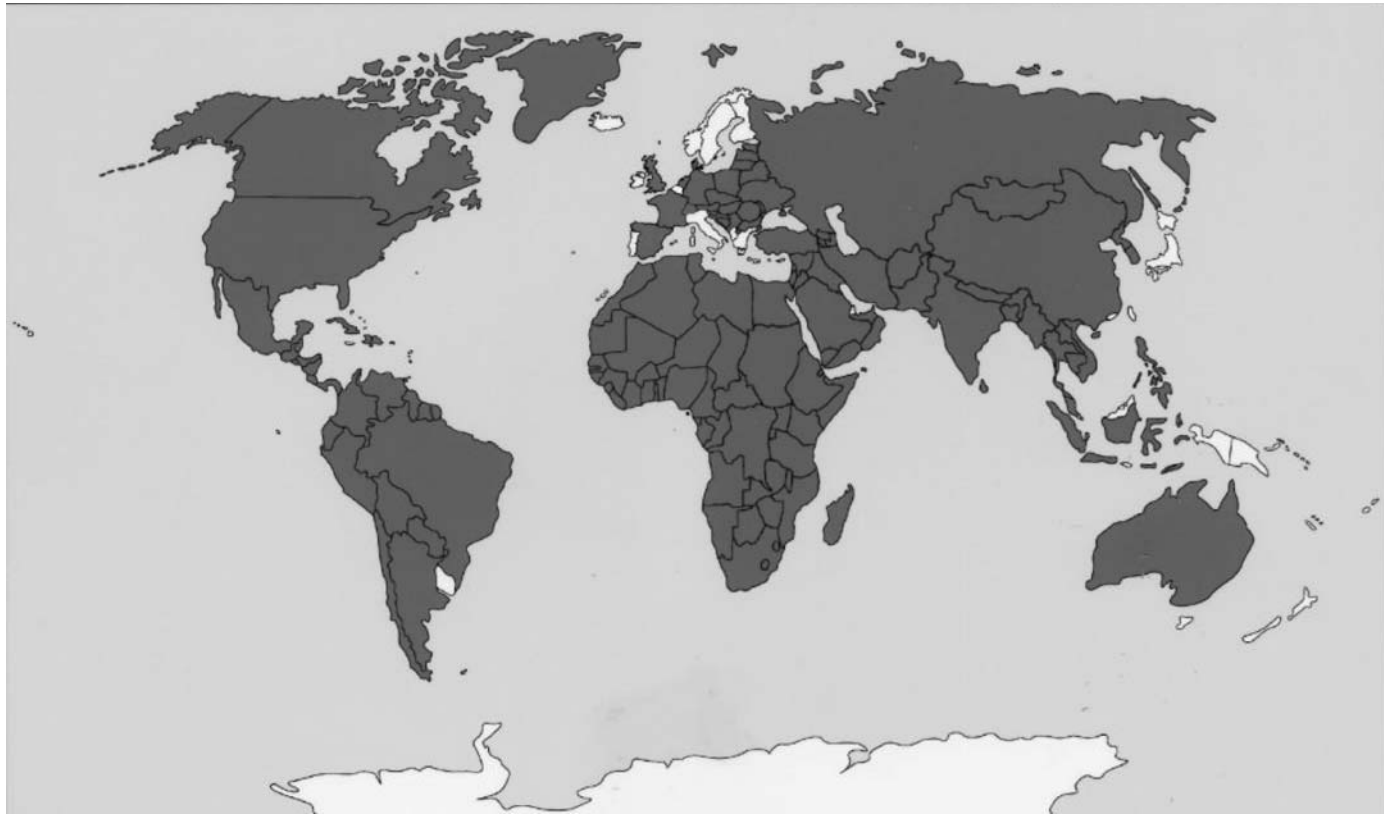

Figure 10.1. World distribution of classic rabies virus (genotype 1) in red and of European bat lyssaviruses (genotypes 5 and 6) and Australian bat lyssavirus (genotype 7) in green.

along the east coast (Kwa-Zulu Natal), yellow mongooses throughout the central area of the country, bat-eared foxes in the west and black-backed jackals in the northern border regions with Botswana and Zimbabwe. Rabies transmitted by hematophagous vampire bats (Desmodontinae) is confined to parts of Central and Southern America and to the islands of Margarita and Trinidad off the coast of Venezuela. Recently, there have been reports of two outbreaks of vampire bat-transmitted rabies resulting in human deaths in Portel and Viseu, Pará, Brazil, and from Chocó on the Pacific coast of Northwestern Colombia. In the Bajo Baudo and Birrinchao (Purricha River) area of Chocó, there were at least 173 vampire bats resulting in the deaths of 13 children from rabies during the period May to June 2004. A mass vaccination day for 2,000 people in this area is being organized.

In the United States of America, rabies is usually found in raccoons (37.2%), skunks (30.7%), bats (17.2%), foxes (5.9%), and other wild animals, including rodents and lagomorphs (0.7%) (data for 2001) (http://www.cdc.gov/ ncidod/dvrd/ rabies/Epidemiology/Epidemiology.htm). Since 1990, 24 of the 32 human cases have been attributed to insectivorous bat strains of classic rabies genotype-1.

In domestic dogs, the furious form of rabies is well known. The animal becomes aggressive, and it wanders away from its home, attacking animate and inanimate objects. However, the commoner clinical form in dogs is the much less easily recognizable paralytic form, characterized by paralysis of the lower jaw, neck muscles, and hind quarters; altered bark; dysphagia; and drooling (Figure 10.2).

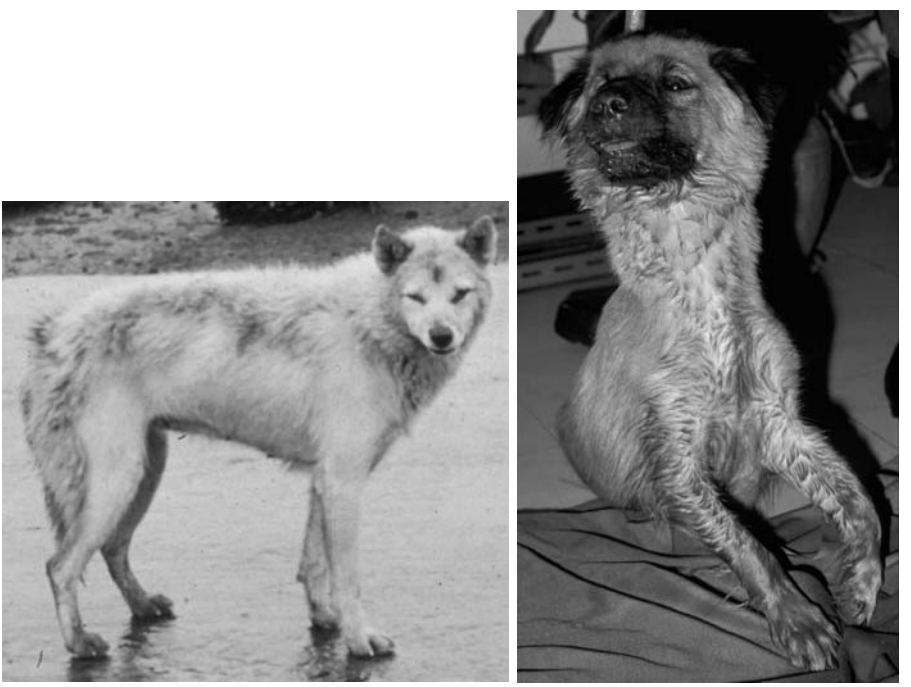

Figure 10.2. Paralytic rabies in a husky in Greenland (copyright B. E. Juel-Jensen) (left) and in a domestic dog in Bangkok, Thailand (copyright D.A. Warrell). Note the paralysis of hind limbs and drooling of saliva.

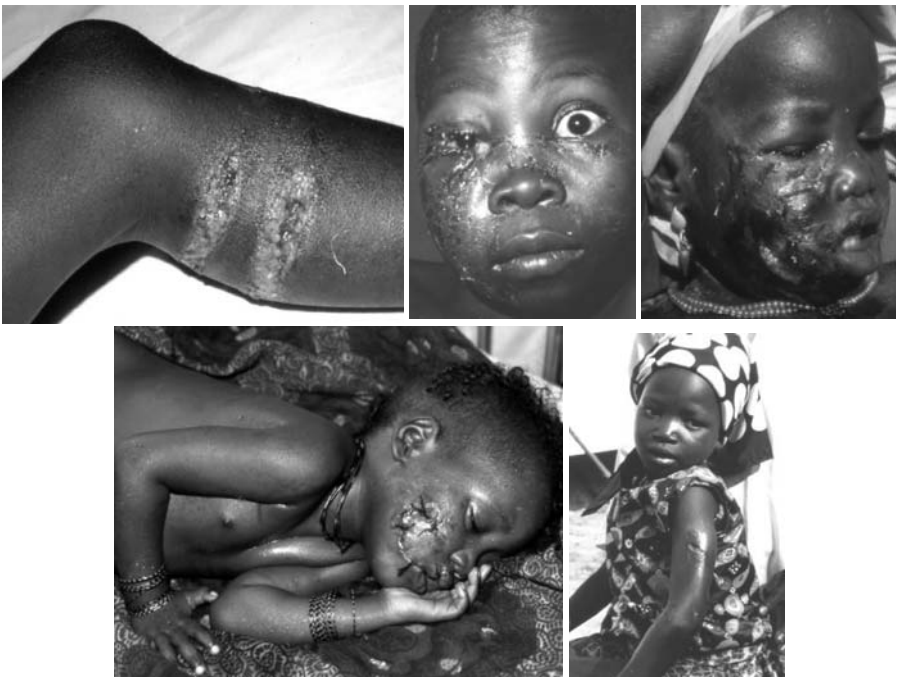

Figure 10.3. Bites inflicted by rabid dog on Nigerian children. Post-exposure prophylaxis was successful in preventing the development of rabies in these patients. As a rule, suturing of suspected-rabid bite wounds is not recommended as it carries the risk of inoculating virus deeper into the tissues (copyright D.A. Warrell).

Bites by domestic dogs are extremely common in developing countries and even in the West (Figure 10.3). In the United States in 1994, 4.7 million bites were recorded, of which 799,700 required medical care. Of 333,700 treated in Accident & Emergency Departments, 1.8% were admitted to hospital. In 2001, 368,245 people were treated in Accident & Emergency Departments for dog bites (an incidence of 130/100,000 population). Forty-two percent of these bite victims were children less than 15-years old. In children less than 5-years old, 64% of the injuries were to the head and neck (Centers for Disease Control, 2003). In the United States, 1% of all Emergency Room visits are for dog bites. Thirty to fifty percent of these cases are in school-age children and 70% of the bites are inflicted by the victim's own pet dog or a dog familiar to them. It appears that about half of all children have been bitten by dogs.

The pathogenesis of rabies involves the introduction of virus through broken skin or intact mucosae, usually by the bite or contamination by the saliva of a rabid animal (but see below); invasion of the peripheral nervous system via peripheral neuromuscular junctions; centripetal transport in the axoplasm to reach the central nervous system; replication in the brain and spinal cord causing an encephalomyelitis; and, finally, centrifugal transport from the central nervous system to salivary glands, lacrimal glands, skin, heart, lung, and a wide variety of other tissues (Warrell and Warrell, 2004).

5. Rabies in Humans

The incubation period between the infecting contact, usually at bite, and the first symptoms of rabies encephalitis is extremely variable. In a series of 319 Thai patients admitted to Bamrat Naradun Hospital in Bangkok, the median incubation period was 2 months, but in 10 cases it was 1–2 years and in 5 cases more than 2 years. The extreme limit in the documented cases is 19 years (Smith et al., 1991).

Unusual routes of infection, other than by the contamination of bite or scratch wounds by infected mammalian saliva, include inhalation, rabies vaccine containing virulent rabies virus, transplacental, and following transplantation of infected tissue grafts (Warrell, 2004). At Baylor University Medical Center in Dallas, Texas, in 2004, the donor of organs and tissues to several recipients was found to have been infected with rabies, probably of bat origin. So far, four of these recipients have died of rabies. In one of the them, the infection was thought to have been acquired through the use of a segment of iliac artery in a liver transplantation operation (Centers for Disease Control, 2004 a, b; Dietzschold & Koprowski, 2004). Rabies due to a bat-related virus strain has also been reported in a Canadian patient who received a renal transplant more than 2 years previously (Parker et al., 2003; Jackson, 2004).

Furious and paralytic ("dumb") clinical manifestations of rabies encephalitis are seen in humans as in other mammals (Warrell & Warrell, 1995). The pathognomic symptom and sign is hydrophobia/aerophobia; jerky spasms of the inspiratory muscles associated with an indescribable feeling of terror (Figure 10.4). This reflex is provoked by attempts to drink water or, through conditioning, by the sight, sound, or the very mention of water. A draft of air on the skin can cause the same reaction

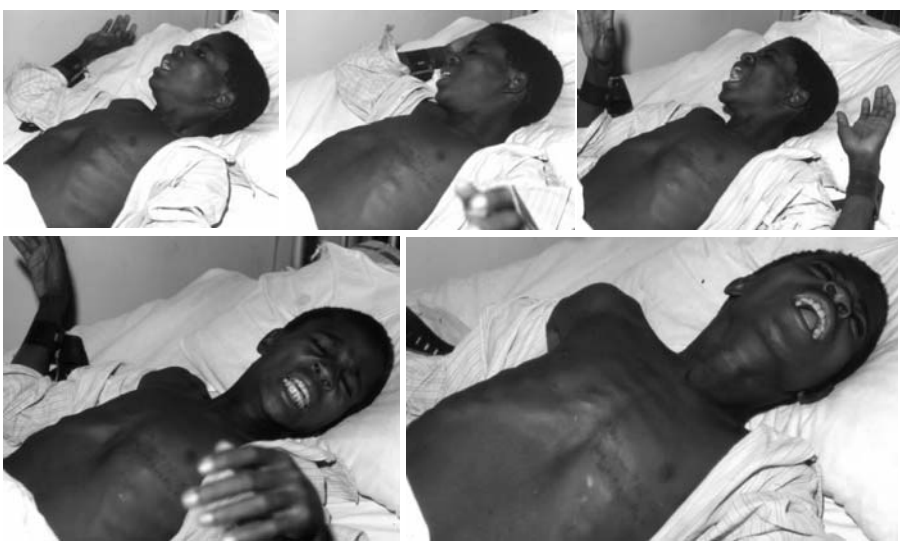

Figure 10.4. Rabies encephalomyelitis in a Nigerian boy showing the progression of a hydrophobic spasm associated with terror. Note the powerful contraction of the diaphragm (depressing the xiphisternum) and sternocleido-mastoid muscles (copyright D.A. Warrell).

(aerophobia). The patient may die from cardio respiratory arrest during one of these spasms and, without supportive care, patients with furious rabies rarely survive for more than a few days. Paralytic rabies is more insidious. There is an ascending flaccid paralysis, often starting in the bitten limb and progressing through flaccid paraparesis with sphincter disturbances to fatal bulbar and respiratory paralysis over a period of days or a few weeks. There may be associated sensory abnormalities and fasciculations. Hydrophobic spasms are rare. The type of clinical presentation may depend on the strain of rabies virus, on genetic characteristics of the host (as has been demonstrated in different strains of laboratory mice), and the level of host immunity. In the famous epidemic of vampire bat rabies in Trinidad between 1929 and 1931, paralytic rabies was the predominant manifestation in human victims (Hurst and Pawan, 1931). Whatever the clinical pattern, rabies encephalomyelitis in humans is almost inevitably fatal and most of the few documented survivors have been left with severe neurological sequelae.

6. Diagnosis of Rabies in Humans

During life, the diagnosis can be confirmed by immunofluorescence of skin punch biopsies taken in hairy areas of the skin such as the nape of the neck; viral isolation; PCR of saliva or CSF and by detection of rabies neutralizing antibodies in serum and CSF. The corneal smear method should be abandoned as it is falsely positive in many cases. Postmortem confirmation can be achieved by testing brain tissue with immunofluorescence or PCR and by viral isolation. It is not necessary to open the skull as brain tissue can be sampled by a needle necropsy though the superior orbital fissure, transnasally through the ethmoid sinus or, in the case of young children, through the fontanelles. The classical method of rabies diagnosis is by demonstrating intra-cytoplasmic Negri bodies using Seller's stain. Used since the beginning of the 20th century, it is less sensitive and specific than immunofluorescence and is no longer recommended.

7. Survival from Rabies

Over the last 30 years, six human patients have been claimed as survivors of rabies encephalomyelitis. The most recently reported case is a 15-year-old girl who was bitten by a bat in a church in Wisconsin, USA, on 12 September 2004 and presented with symptoms of rabies encephalitis on 18 October 2004. She had received no post-exposure prophylaxis. She was treated in the Children's Hospital of Wisconsin at Milwaukee with drug-induced coma and a "cocktail of antivirals." By December 2004, she appeared to be making a good recovery but was still unable to speak. All the five previous cases, including the famous 9-year-old boy in Lima, Ohio, USA, in 1970, had received some rabies vaccine before the onset of symptoms. No virus or viral antigen was detected in any of these patients and so the diagnosis was based on finding high rabies neutralizing antibody levels in the CSF (Warrell, 2004; Warrell & Warrell, 2004).

8. Pre- and Post-exposure Prevention of Rabies by Vaccination

Once rabies virus reaches the central nervous system and the characteristic symptoms of rabies encephalomyelitis develop, death is virtually inevitable. However, this appalling consequence can be prevented by pre- and post-exposure prophylaxis.

8.1. Post-exposure Prophylaxis

Rabies is one of the few infections that can be prevented by vaccination started after the virus has been introduced into the tissues. However, delay in initiating full post-exposure treatment increases the risk of failure. The principles of post-exposure prophylaxis include prompt and thorough cleaning of the bite or scratch wound, active immunization with vaccine, and passive immunization with rabies immune globulin (RIG). Louis Pasteur's original rabies vaccine, first used post-exposure in 1885, consisted of rabbit spinal cord infected with live rabies virus which had been attenuated by desiccation. Examination of Pasteur's laboratory notebooks suggests that he had little evidence to support this revolutionary intervention (Geison, 1995). Pasteur-type nervous tissue vaccines, in which the virus has been killed, are still widely used in India, Pakistan, Bangladesh, Nepal, Viet Nam, Africa, and South America. Semple and Fermi vaccines are raised in sheep or goat brain while Fuenzalida vaccine is raised in suckling mice before their nervous system has become myelinated. Compared to modern tissue culture vaccines, nervous tissue vaccines lack potency and their use is associated with potentially fatal or debilitating neurological reactions. For example, in Pakistan, 40% of patients dying of rabies encephalitis had received full courses of Semple vaccine. Neurological reactions may occur in up to 1 in 200 vaccinees.

The new generation of rabies tissue culture vaccines has been developed since the 1970s by growing vaccine strain rabies virus in a variety of cell lines. Currently, diploid cell vaccine ("Imovax rabies" Sanofi Pasteur), purified chick embryo vaccine ("RABIPUR" Chiron), and purified vero cell vaccine ("VERORAB" Sanofi Pasteur) are widely distributed, at least in Western countries. The standard regimen approved by WHO and CDC Atlanta for post-exposure prophylaxis consists of a full dose (1 ampoule) of vaccine on five occasions (days 0, 3, 7, 14, and 30) with RIG on day 0 (see below). This regimen has proved extremely safe and effective in several million recipients, but its requirement for five full doses of vaccine makes it unaffordable in most developing countries. Yet it is the developing countries that bear 99% of the burden of global human rabies mortality. The pressure to replace nervous tissue vaccines, on the grounds of their unacceptable record of safety and potency, and to deploy tissue culture vaccines worldwide has stimulated the search for economical post-exposure regimens. The aim is to reduce cost while maintaining full immunogenecity. Rapid induction of immunity is specially important in developing countries as RIG is rarely available. It has been estimated that RIG is used in only 1.6% of 7 million post-exposure prophylaxis courses in Asia each year. The

intradermal inoculation of antigens is well known to have a more powerful and sustained immunogenicity than intramuscular or subcutaneous routes. In laboratory animals, multiple site immunization was found to induce immunity more rapidly than single site, perhaps through recruitment into antibody production of several groups of local lymph nodes. This observation led to the idea of dividing a single dose of vaccine between eight different sites, chosen to be drained by different groups of lymph nodes, and by giving the vaccine by intradermal injection. In the initial studies, carried out at Queen Saovabha Memorial Institute and The Hospital for Tropical Diseases, Mahidol University in Bangkok in the 1980s, the rabies neutralizing antibody responses to a variety of regimens were tested in adult volunteers. The eight-site intradermal regimen which proved to be the most rapidly immunogenic (Warrell et al., 1983; Warrell et al., 1984) was compared to the then standard post-exposure regimen of Thai Red Cross Society Semple-type nervous tissue vaccine in a randomized trial in a group of Thai adults (Warrell et al., 1985). These patients' exposure to rabies had been confirmed by demonstrating rabies virus antigen by rapid immunofluorescence in the brains of the dogs which had bitten them. Again, according to the then current practice in Thailand, patients were stratified according to the severity of their bites to receive equine RIG or not. There were no deaths from rabies in any of the treatment groups but the eight-site intradermal regimen using human diploid cell strain vaccine proved superior to Semple vaccine in neutralizing antibody response and safety. Further studies in volunteers demonstrated a substantial "safety factor" with the eight-site intradermal regimen. If, due to faulty technique, up to four of the eight injections given on day 0 were given subcutaneously rather than intradermally, the speed and magnitude of the neutralizing antibody response was not compromised (Suntharasamai et al., 1987). Subsequently, the principle of economical, multi-site intradermal immunization against rabies was accepted by the World Health Organization (World Health Organization, 1997) and by the vaccine producers. The eight-site intradermal regimen reduces the requirement of tissue culture vaccine from five to less than two full doses and the number of hospital visits from five to four.

8.2. Passive Immunization

The dramatic results of an *ad hoc* study in 29 victims of a vicious attack by a rabid wolf in Iran in 1954 (Baltazard and Bahmanyar, 1955) demonstrated the importance of adding passive immunization with RIG to active immunization with rabies vaccine in patients with severe exposure such as head wounds and multiple bites. Infiltrated around the bite wounds, RIG provides immediate rabies neutralizing antibody during the critical 7–14 days before there is a detectable humoral immune response to active rabies vaccination. Although the use of RIG for post-exposure prophylaxis, other than with trivial exposures in which the skin is not broken, is strongly recommended by WHO, CDC, and other authorities, equine RIG and particularly the safer human RIG are scarce, very expensive, and as a result rarely used in the developing world. This demands a greater dependence on meticulous wound cleaning and the most rapid possible induction of active immunity by vaccination to prevent rabies.

In this respect, eight-site intradermal immunization promises some advantage over other regimens. However, continuing efforts should be made to improve the supply of RIG which remains of crucial importance in the post-exposure treatment of severely exposed people.

8.3. Pre-exposure Prophylaxis

A simple regimen of three injections (days 0, 7, and 28) has proved highly effective in protecting people who were subsequently exposed to rabies. There has been no reported death from rabies in recipients of this pre-exposure treatment, provided that they were given booster doses of vaccine after the suspected rabid bite. There is increasing evidence that the three injection initial pre-exposure course confers prolonged protection, if immunity is recalled by boosting after exposure to a suspected rabid bite. However, there is continuing debate about the need to boost routinely after 1–3 years to improve the protection of those people who respond poorly to vaccines. Ideally, non-responders should be detected by serological screening for neutralizing antibodies, but in the United Kingdom this test costs as much as a dose of vaccine and is not widely available. Those people with an increased risk of exposure to rabies, such as some laboratory workers, veterinarians, and conservationists, should receive routine booster doses or have their rabies neutralizing antibody titers checked every year.

In Viet Nam, pre-exposure immunization of children, as part of the Expanded Programme on Immunization (EPI), proved effective in a small pilot study (Lang et al., 1999), but this strategy has never been implemented in any country. Pre-exposure prophylaxis in the community would have the advantage that RIG would not be required for post-exposure prophylaxis in those who had received effective pre-exposure prophylaxis.

9. Control of Rabies

Rabies is predominantly a zoonosis and although pre- and post-exposure prophylaxis are effective in sparing human life from this appalling disease, the most effective and economical way of controlling the infection is by tackling the epizootic and enzootic in its mammalian reservoirs and vectors, notably in the domestic dog. Rabies control starts with establishing the prevalence and host range of rabies in wild and domesticated mammals and continues with public education.

In the case of urban rabies, owned dogs are muzzled, their movements are restricted to their owners' compounds, and there is a campaign of mass vaccination (Figure 10.5). In the period 1969–1982, these methods proved remarkably effective in several massive South American conurbations. In Buenos Aires, dog rabies was eliminated in 9 years and human rabies in 5 years. In Lima and Callao, Peru, dog rabies was eliminated in 4 years and human rabies in 3 years. In Sao Paulo, Brazil, dog rabies was eliminated in 16 years and human rabies in 12 years (Larghi et al., 1988).

Figure 10.5. Rabies control program in Chanchamayo, Peru, involving door-to-door vaccination of owned dogs.

Traditional methods for controlling rabies in stray dogs are poisoning, shooting, or catching the animals. These methods were unpopular in dog-loving and Buddhist communities and proved ineffective because of the biological resilience of animal populations in restoring depleted numbers. A more intelligent policy is to capture stray dogs, vaccinate, sterilize, and then release them. A successful rabies control program in domestic dogs in Jaipur, India, employs Defensor3 rabies vaccine (Pfizer), together with sterilization. A single injection of this vaccine appears to produce prolonged immunity to challenge for the dog's expected life span, which, in the case of stray dogs in several countries, is likely to be only a few years (J. Reece, personal communication) (Coyne et al., 2001). Rabies control breaks down in war-torn countries, as was seen in the civil war in Sierra Leone from 1995 to 2001. There was a significant increase in the incidence of canine-transmitted urban human rabies, particularly among children, during this war, attributed to the large numbers of unvaccinated, unlicensed, and largely unrestrained domestic dogs which were roaming about (Hatch et al., 2004).

In countries where there are important wild life reservoirs of rabies, such as during the fox rabies epizootic in Europe, immunization of wild mammals has proved remarkably effective in controlling or even eliminating rabies. Live attenuated or recombinant vaccines have been distributed in oral baits, appetizing enough to be eaten by mammals such as foxes in Western Europe, raccoons in the United States, and black-backed jackals in Zimbabwe.

Some countries, especially islands such as Hawaii, Japan, Singapore, and Iceland, may be able to protect their rabies-free status by enforcing strict quarantine regulations. However, the difficulty of preventing the smuggling of domestic dogs across international borders was illustrated in August 2004 by an incident in southwest France. A four-month-old dog acquired near Agadir, Morocco, was illegally imported into France on 11 July, via Spain. It was neither registered nor vaccinated. It eventually developed symptoms of rabies on 18 August, died on 21 August, and was confirmed to have been suffering from rabies on 26 August. During the period 2–21 August, when it was infectious, this dog had been in contact with numerous people and other dogs, and had even bitten some of them, in Gironde, Dordogne, Lot, and Garonne. There was a full-scale international alert to identify and vaccinate actual and potential contacts (Health Protection Alerting System, National (UK) Alert 28 October 2004, 2100 hr, http://www.hpa.org.uk/hpa/news/articles/ press_releases/2004/040828_ rabies.htm).

10. Conclusions

1. EBLV, a rabies-related virus of insectivorous bats, is now known to be enzootic in Britain.
2. Rabies remains an important global problem killing tens of thousands of people every year. More than 99% of human deaths from rabies occur in tropical developing countries.
3. Although rabies encephalomyelitis is incurable, it is eminently preventable.
4. Modern, safe, and potent tissue culture vaccines can be used more effectively and economically when administered intradermally at multiple sites.
5. RIG is scarce but essential for post-exposure treatment of severe bites.
6. Control of canine rabies by vaccination is the most effective and economical method for preventing human rabies.

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11

Prevention of Typhoid Fever

Myron M. Levine and Philippe Lepage

1. Introduction

Typhoid fever, the generalized infection of the reticuloendothelial system (spleen, liver, and bone marrow), gut-associated lymphoid tissue, and gall bladder caused by the highly human host restricted pathogen *Salmonella enterica* serovar Typhi (*S*. Typhi), is the quintessential infectious disease associated with inadequate sanitation and lack of protected drinking water. The pediatric (school-age) and young adult populations in endemic areas bear the brunt of the clinical disease burden worldwide. Typhoid fever also represents a risk for pediatric and adult travelers from industrialized countries who visit the developing countries (Steinberg et al., 2004). In endemic areas, chronic gall bladder carriers (usually adult females who excrete large numbers of typhoid bacilli) constitute an important reservoir of infection (Levine et al., 1982). Where sanitation is deficient, fecal contamination from inapparent carriers (chronic or temporary) and clinically ill patients can contaminate water supplies. If treatment of water sources is inadequate or unavailable, water can serve as an important vehicle of transmission (Mermin et al., 1999). Consumption of contaminated water and food vehicles by susceptible subjects leads to clinical or sub-clinical infection, depending on the dose ingested, the precise vehicle conveying the typhoid bacilli, and the host susceptibility factors (Hornick et al., 1970). Depending on the age of the infected patient, the presence of pre-existent gall bladder pathology, and the specific antibiotic treatment administered, up to a few percent of infected persons can become chronic gall bladder carriers, thereby maintaining the reservoir of infection. A fairly long incubation period (8–14 days) follows the ingestion of typhoid bacilli before the onset of clinical disease. The typical general features of typhoid fever include fever (that increases in step-wise fashion and persists for weeks if improperly treated), headache, and abdominal discomfort. Since typhoid bacilli may reach many organs, a wide array of clinical complications can ensue. However, because the gut-associated lymphoid tissue (in particular Peyer's patches in the ileum) constitutes the most overt site of gross pathology, intestinal complications such as perforation or hemorrhage (which occur in ~1–2% of patients) are particularly well recognized and feared.

Hot Topics in Infection and Immunity in Children, edited by Andrew J. Pollard, and Adam Finn. Springer, New York, 2005.

In the pre-antibiotic era, the case fatality of typhoid fever was ~15%. It is against this background that in 1948 a hallmark breakthrough was made in the treatment of typhoid, when Woodward et al. (1948) discovered that chloramphenicol could successfully treat typhoid fever, dropping the case fatality to <1%. Over the next quarter century, this hallmark observation led to the control of mortality due to typhoid fever in many endemic areas. In many settings worldwide, it was confirmed that oral chloramphenicol constituted a simple, practical, and effective treatment for typhoid fever that diminished the case fatality rate to low levels. Nevertheless, over the ensuing decades, typhoid fatality remained a problem when relevant antibiotic therapy was delayed or unavailable, or when inappropriate antibiotics were administered. With this background, one appreciates how the emergence of antibiotic-resistant *S*. Typhi greatly diminishes the role that antibiotics can play as a control measure. Epidemics of chloramphenicol-resistant typhoid fever in the 1970s in Mexico (Gilman et al., 1975) and in Southeast Asia (Butler et al., 1973) led to the identification of alternative oral antibiotic regimens to treat typhoid fever in developing countries (e.g., amoxicillin and trimethoprim-sulfamethoxazole). Within a few years, antibiotic-sensitive *S*. Typhi reappeared in those areas to replace the chloramphenicol-resistant strains. However, since 1990 the world has entered a hallmark era in the history of typhoid fever because of the emergence, dissemination, and persistence in Asia and Africa of *S*. Typhi strains that carry resistance to most of the clinically relevant antibiotics (Mikhail et al., 1989; Gupta, 1994; Rowe et al., 1997; Mermin et al., 1998, 1999).

2. Secondary Prevention of Typhoid Morbidity and Mortality

Secondary prevention aims to mitigate severe clinical typhoid illness, complications, and fatality. If available, the early judicious use of effective antibiotics in patients with acute typhoid fever constitutes the mainstay of secondary prevention. Recent reviews discuss the antibiotics that are useful for treating pediatric typhoid fever patients who acquire infection in geographic areas where multiple-resistant *S*. Typhi are prevalent (Bhutta, 1996; Cao et al., 1999; Stephens and Levine, 2002).

3. Primary Prevention of Typhoid Morbidity and Mortality

The primary prevention of typhoid fever implies measures that prevent infection entirely, or at least prevent the development of overt clinical illness. Three main strategies achieve primary prevention: (1) environmental measures that ensure adequate sanitation and treated water supplies; (2) the identification of chronic carriers responsible for outbreaks and related sporadic cases, and interrupting their role in the chain of transmission; and (3) immunization with typhoid vaccines to make susceptible hosts resistant.

3.1. Environmental Measures

In the mid-19th century in the urban populations of Europe and North America, typhoid fever was hyper-endemic; indeed it was virtually a rite of passage. In most cities at this time human fecal wastes were dumped into nearby rivers which also usually served as the source of drinking water for the urban population. In this way, the transmission of *S*. Typhi was amplified and very high incidence rates ensued (JAMA, 1920; Wolman and Gorman, 1931). Notably, throughout Europe and North America, as treatment of municipal water supplies (by means of chlorination, sand filtration, etc.) became increasingly commonplace, the amplified transmission of typhoid fever was interrupted and was followed by a precipitous fall in the incidence of typhoid fever (Wolman and Gorman, 1931). In those places in the developing world where it has been possible in recent years to institute adequate sanitation and to provide bacteriologically monitored, treated water supplies, amplified transmission of *S*. Typhi has similarly been interrupted. Regrettably, large segments of the developing world remain unserved by adequate sanitation and potable water supplies because the capital investment required for such improvements is enormous.

3.2. Identification, "Epidemiologic Neutralization," and Treatment of Chronic Typhoid Carriers

In specific epidemiologic situations, chronic typhoid carriers, particularly if they are food handlers, can cause localized outbreaks or related sporadic cases of typhoid fever (Lin et al., 1988; Cote et al., 1995). If epidemiologically relevant carriers can be identified, they can be removed from situations where they put others at risk (Lin et al., 1988; Cote et al., 1995). Techniques to screen for chronic typhoid carriers (Lanata et al., 1983, 1990; Losonsky et al., 1987; Ferreccio et al., 1990) and to confirm the carrier state bacteriologically (Gilman et al., 1979) have been developed, and non-surgical methods to treat the carrier state have been discovered (Ferreccio et al., 1988; Gotuzzo et al., 1988).

Approximately, 90% of the chronic typhoid carriers manifest highly elevated titers of serum Vi antibody (Losonsky et al., 1987), exhibiting levels that are observed in no other situation, including following acute disease. Thus, using purified Vi polysaccharide as antigen, assays have been developed to screen serologically for high titers of serum Vi antibody (Lanata et al., 1983; Losonsky et al., 1987), indicative of the chronic carrier state. Bacteriologic confirmation of the carrier state is preferably made by obtaining bile-stained duodenal fluid for culture (Gilman et al., 1979), although repetitive stool cultures following administration of a strong purgative represent another accepted method.

Prior to the advent of ciprofloxacin, norfloxacin, and other fluoroquinolones, it was necessary to perform cholecystectomy followed by many weeks of antibiotic therapy to achieve a high rate of cure among chronic typhoid carriers. However, it has since been shown that several weeks of oral ciprofloxacin or norfloxacin can eradicate the carrier state in 80–90% of instances, without surgery (Ferreccio et al., 1988; Gotuzzo et al., 1988).

3.3. Typhoid Vaccines

Immunization with typhoid vaccines aims to alter the susceptibility of nonimmune hosts to render them resistant to the development of typhoid fever, despite exposure to virulent typhoid bacilli. The first typhoid vaccines consisting of heat-inactivated phenol-preserved whole bacteria administered parenterally were prepared at the end of the 19th century (Pfeiffer and Kolle, 1896; Wright and Semple, 1897). In large-scale, controlled field trials sponsored by the World Health Organization in the 1960s, heat-inactivated killed whole-cell parenteral vaccines exhibited a moderate (~51–67%) level of efficacy (Ivanoff et al., 1994). However, these vaccines were extremely reactogenic, causing fever, malaise, and absenteeism in ~25% of recipients. Thus, they never became popular control measures and were rarely used in programmatic fashion (Ivanoff et al, 1994).

Two modern typhoid vaccines that became licensed in the late 1980s and early 1990s proved to be very well-tolerated, yet are at least as protective as the inactivated whole-cell vaccines (Levine et al., 1989a, b). These vaccines include the attenuated *S*. Typhi strain Ty21a used as a live oral vaccine, and the purified Vi capsular polysaccharide of *S*. Typhi employed as a parenteral vaccine (Levine et al., 1989a, b) (Table 11.1). The salient features of these vaccines will be reviewed below (and summarized in Table 11.1) with respect to their formulations, immunization schedules, target ages, clinical tolerability, efficacy (including duration), practicality in large-scale campaigns, and evidence of indirect protective effects ("herd immunity").

3.3.1. Disease Burden and Target Populations for Immunization

In recent years, there has been much discussion over the age groups that suffer the most significant burden of disease caused by *S*. Typhi and who should be targeted for immunization. The classic clinical syndrome of typhoid fever (and its many complications if inappropriately treated) is mainly observed among children 3–19 years of age, and young adults. Even in large water-borne epidemics that affect the entire population, this age group manifests the highest incidence of typhoid fever. In endemic areas, it is typhoid hospitalizations and deaths in this age group that drive the interest of public health authorities to consider the options for controlling typhoid fever. On the other hand, several systematic blood culture studies among infants and toddlers <24 months of age have generated interesting observations. Whereas the prevalence of *S*. Typhi bacteremia was low among infants <12 months of age, blood cultures of ~2–4% of febrile 12–23-month olds yielded *S*. Typhi (or *S*. Paratyphi) (Ferreccio et al., 1984; Sinha et al., 1999). Since such febrile episodes are so common, this represents a large number of bacteremic typhoid infections in toddlers. In most instances, the clinical syndrome manifested by these young hosts with *S*. Typhi bacteremia is a mild febrile syndrome that is indistinguishable from the other 96–98% of febrile children (a few percent of whom have "occult" pneumococcal bacteremia but most of whom have self-limited viral infections) (Ferreccio et al., 1984; Lepage et al., 1987; Mahle and Levine, 1993).

Two main immunization strategies have been proposed for controlling endemic typhoid fever: (1) school-based mass immunization campaigns (Ferreccio

Table 11.1. Summary of the Salient Features of Ty21a and Vi Typhoid Vaccines

	Ty21a	Vi
Type of vaccine	Live attenuated	Purified subunit (polysaccharide)
Route of administration	Oral	Parenteral (subcutaneous or intramuscular)
Formulations	Enteric-coated capsule or "liquid" (lyophilized vaccine and buffer powder suspended in water)	25 μg of Vi polysaccharide suspended in 0.5 ml of phenolic isotonic buffer
Number of doses; immunization schedule	Three doses (except for the United States, where a four-dose regimen is licensed); every-other-day interval	One dose
Clinical tolerability	Very high (few adverse reactions, non-severe)	Very high (few adverse reactions, non-severe)
Track record of safety	Excellent	Excellent
Immunogenicity in:		
Adults	Good	Good
School children	Good	Good
Pre-school children	Good	Good
Infants	No data	Poor
Immune responses that are presumed to mediate protection	Serum IgG O & H antibodies; intestinal SIgA antibodies; CD4+and CD8+cell-mediated immune responses (including cytotoxic lymphocytes)	Serum Vi antibodies
Level of efficacy:		
17–21 months of follow-up		64–72%
3 years of follow-up	Enteric capsules, 67% Liquid formulation, 77%	55%
5 years of follow-up	Liquid formulation, 78%	
7 years of follow-up	Enteric capsules, 62%	
Target age groups for vaccination	Pre-school-and school-age children, adults	Pre-school-and school-age children, adults
Has been used effectively in mass campaigns	Yes	Yes
Evidence of indirect protection of unvaccinated susceptibles ("herd immunity")	Yes	No

et al., 1989; Levine, 2003) and (2) routine immunization of infants through the Expanded Program on Immunization (EPI) (Levine, 2003). The current licensed typhoid vaccines are well-suited to school-based immunization, but either are not amenable to infant immunization because of poor immunogenicity (Vi), or no infant immunogenicity or efficacy data are available (Ty21a) to support their suitability. A new generation of typhoid vaccines under development, including Vi conjugate and highly immunogenic attenuated strains, may be suitable for use in the EPI.

3.3.2. Ty21a

Germanier and Furer (Germanier and Furer, 1975), who developed attenuated strain Ty21a in the early 1970s as a live oral typhoid vaccine candidate, assumed that the combination of a mutation in *galE* (encoding an isomerase involved in LPS production) and absence of Vi was responsible for the attenuation of this vaccine strain. However, it is now recognized that Ty21a has multiple additional mutations, some combinations of which are responsible for the strain's attenuation.

3.3.2.a. Formulations of Ty21a

There are two commercial formulations of Ty21a produced by Berna Biotech, each containing $\sim$3–5 $\times$ 10^9 colony forming units per dose. One formulation consists of enteric-coated (phthalate-coated) capsules (Levine et al., 1987). The other consists of two sachets, one containing buffer and the other lyophilized vaccine (Levine et al., 1990); the contents of the two powders are suspended together in 100 ml of water to make a liquid vaccine cocktail. The enteric-coated capsule formulation is licensed in more countries than the "liquid" formulation.

3.3.2.b. Immunization Schedules

Ty21a in either formulation is administered in a three-dose, every-other-day schedule. The one exception is the United States, where four doses are given on an every-other-day schedule (Levine et al., 1989a, b). Interestingly, large-scale field trials showed that separating the doses by several weeks did not enhance efficacy over that achieved with an every-other-day regimen (Levine et al., 1987). Re-immunization is recommended every 3–5 years, depending on the country.

3.3.2.c. Target Age Groups for Immunization

The immunogenicity and efficacy of Ty21a have been documented in subjects aged 3 years to $>$60 years (Wahdan et al., 1982; Levine et al., 1987, 1989a, b, 1990; Black et al., 1990; Simanjuntak et al., 1991; Olanratmanee et al., 1992; Cryz et al., 1993). Although the liquid formulation would likely be practical for use in infants, heretofore, no immunogenicity results have been reported with the commercial liquid formulation in subjects $<$12 months of age. On the other hand, oral Ty21a is very well-suited for immunizing school-age children and young adults. In a large comparative trial, a small proportion ($\sim$5%) of young children, 5–7 years of age, had difficulty in swallowing enteric-coated capsules (Levine et al., 1990), whereas the liquid formulation was readily consumed by all subjects of this age (Levine et al., 1990).

3.3.2.d. Clinical Tolerability

Ty21a has an extraordinary track record of safety and clinical acceptability, both pre-licensure (Wahdan et al., 1980; Levine et al., 1987, 1990; Black et al., 1990; Simanjuntak et al., 1991; Olanratmanee et al., 1992; Cryz et al., 1993) and post-licensure (Cryz, 1993; Begier et al., 2004; Steinberg et al., 2004).

3.3.2.e. Efficacy

In a large-scale, randomized, placebo-controlled, double-blind field trial in school children, 6–19 years of age in Santiago, Chile, three doses of Ty21a in enteric-coated capsules (every-other-day regimen) conferred 67% efficacy (95% confidence interval (CI), 47–79%) against bacteriologically confirmed typhoid fever during 3 years of follow-up (Levine et al., 1987); over 7 years of follow-up, this regimen conferred 62% protection efficacy (95% CI, 48–73%) (Levine et al., 1999).

In another field trial in school children in Santiago, Chile, three doses (every-other-day regimen) of the liquid formulation of Ty21a conferred 77% efficacy (95% CI, 60–87%) over 3 years of follow-up (Levine et al., 1990) and 78% efficacy (95% CI, 65–87%) over 5 years of follow-up (Levine et al., 1999). The long-term efficacy conferred by Ty21a makes it a cost-effective intervention to control endemic typhoid in high incidence areas.

3.3.2.f. Practicality for Mass Campaigns

A large-scale effectiveness trial of Ty21a was carried out in which 189,819 Chilean schoolchildren were randomly allocated to receive two, three, or four doses of Ty21a in enteric-coated capsules (Ferreccio et al., 1989). The enteric-coated capsule formulation proved to be highly practical for immunizing school-age children in this school-based mass campaign (Ferreccio et al., 1989). The lowest incidence of typhoid in that study was observed in recipients of the four-dose regimen (Ferreccio et al., 1989), resulting in that regimen being licensed by the US Food and Drug Administration.

3.3.2.g. Evidence of Indirect Protection

Continued surveillance of the placebo control group in the first Ty21a field trial in Santiago, Chile, provided convincing evidence of an indirect protective effect that followed mass use of Ty21a in other areas of Santiago (Levine et al., 1989a, b). Each time another large-scale field trial was carried out in another area of Santiago, the incidence rate in the placebo group in the first trial fell notably. Thus, the benefit to the population at risk that follows mass use of Ty21a extends beyond the individual protection bestowed upon vaccinated subjects.

3.3.3. Vi Polysaccharide Vaccine

S. Typhi bears a capsular polysaccharide on its surface that is a recognized virulence property, hence its name "Vi." The polysaccharide consists of highly polymerized *N*-acetyl-D-galactosaminuronic acid (Robbins and Robbins, 1984). Early attempts to purify Vi from typhoid bacilli resulted in a denatured product that was only modestly protective (Hornick et al., 1970; Robbins and Robbins, 1984). Modern methods to purify bacterial capsular polysaccharides allowed non-denatured purified Vi to be prepared on large-scale (Wong et al., 1974; Robbins and Robbins, 1984).

3.3.3.a. Formulations of Vi

There are two major suppliers of Vi vaccine among industrialized country vaccine manufacturers (Sanofi Pasteur and GSK), each producing a formulation containing 25 μg of Vi per dose for subcutaneous or intramuscular administration (Plotkin and Bouveret-Le Cam, 1995; Lebacq, 2001). There are also multiple producers of Vi vaccine among manufacturers in the developing and middle-income countries, including China (Yang et al., 2001), India (Sabitha et al., 2004), Cuba (Azze et al., 2003), and South Korea (Sabitha et al., 2004).

3.3.3.b. Immunization Schedules

Vi is administered as a single subcutaneous or intramuscular immunization. Booster doses are not useful since this T-independent antigen does not elicit immunologic memory and titers are not increased by administering a second dose of vaccine (Keitel et al., 1994; Plotkin and Bouveret-Le Cam, 1995). Re-immunization is recommended every 2–3 years, depending on the country.

3.3.3.c. Target Age Groups for Immunization

The immunogenicity and efficacy of Vi have been documented in subjects aged 3 years to >60 years (Acharya et al., 1987; Klugman et al., 1987; Plotkin and Bouveret-Le Cam, 1995; Lebacq, 2001; Yang et al., 2001). As with most other polysaccharide vaccines, Vi is poorly immunogenic in infants and toddlers (Plotkin and Bouveret-Le Cam, 1995) and, therefore, is not recommended for children <2 years of age. The primary targets for use of this vaccine are school-age children and young adults. One specific advantage of Vi vaccine is that as a non-living antigen, Vi can be safely administered to pregnant women and to immunocompromised hosts, without concern.

3.3.3.d. Clinical Tolerability

Vi has an excellent track record of safety and clinical acceptability, both prelicensure and post-licensure (Acharya et al., 1987; Klugman et al., 1987; Plotkin and Bouveret-Le Cam, 1995; Lebacq, 2001; Yang et al., 2001). Only low-grade fever in a few percent of vaccinees and local discomfort at the injection site are adverse reactions of any consequence and are similar to what is seen with other parenteral polysaccharide vaccines.

3.3.3.e. Efficacy

Controlled field trials in Nepal (Acharya et al., 1987) and South Africa (Klugman et al., 1987) established that a single dose of Vi vaccine confers significant protection against typhoid fever. In the Nepal trial, in subjects 5–44 years of age, Vi vaccine conferred 72% efficacy during a follow-up of 17 months (Acharya et al., 1987). In the South Africa field trial, a single dose of Vi provided school children with 64% protection (95% CI, 36–79%) against confirmed typhoid fever over 21 months of surveillance (Klugman et al., 1987) and 55% protection over 3 years

of follow-up (Klugman et al., 1996). In a placebo-controlled field trial in China (Yang et al., 2001), a locally produced Vi vaccine administered as a single 30 μg subcutaneous dose provided vaccinees (mostly schoolchildren) 69% efficacy (95% CI, 28–95%) against bacteriologically confirmed typhoid fever.

3.3.3.f. Practicality for Mass Campaigns

Vi suffers the drawbacks of any parenteral vaccine used in mass campaigns, with respect to concerns over ensuring injection safety for vaccinated subjects and for the health personnel who must administer the injections if needles and syringes are used. On the positive side, the fact that only a single dose is required makes campaigns shorter.

3.3.3.g. Evidence of Indirect Protection

At present there are no data that address whether parenteral Vi vaccine provides indirect protection to un-immunized individuals following its large-scale use.

3.4. New Live Oral Typhoid Vaccine in Development

Recognizing that the main drawback of Ty21a is the requirement to administer at least three oral doses, researchers have undertaken to engineer a new generation of attenuated *S*. Typhi live oral vaccine that requires only a single dose. Four candidate vaccines live oral vaccines have completed Phase 1 or Phase 2 clinical trials. These include CVD 908-*htrA* (Tacket et al., 1997; Tacket et al., 2000), CVD 909 (a further derivative of CVD 908-*htrA* that constitutively expresses Vi) (Wang et al., 2000; Tacket et al., 2004), Ty800 (Hohmann et al., 1996), and ZH9 (Hindle et al., 2002). In Phase 1 or 2 clinical trials, each of these vaccines was well-tolerated and robustly immunogenic following administration of a single oral dose. In the clinical trials with CVD 908-*htrA* and CVD 909, cell-mediated immune responses were measured (Salerno-Goncalves et al., 2003), as well as serum and mucosal antibody responses. CVD 908-*htrA* (Salerno-Goncalves et al., 2003) and CVD 909 elicited strong cell-mediated immune responses, including cytotoxic lymphocytes, in addition to strong serum IgG and mucosal IgA antibody responses.

As single-dose oral vaccines, the new generation of attenuated typhoid vaccine strains would be ideal tools for school-based mass immunization campaigns. There is also eagerness to investigate their immunogenicity in young infants at an age when other (EPI) vaccines are routinely administered (Levine, 2003).

3.5. Vi Conjugate Vaccine

The main drawback of Vi vaccine is that it acts as a T-independent antigen; consequently, it does not elicit immunologic memory, the serologic response is not boostable (Keitel et al, 1994), and Vi is poorly immunogenic for infants (Plotkin and Bouveret-Le Cam, 1995). By conjugating Vi to a carrier protein, it becomes a T-dependent antigen that elicits immunologic memory, higher antibody titers than Vi, and the serum titers are boostable (Kossaczka et al., 1999; Canh et al., 2004). A

conjugate consisting of Vi conjugated to exotoxin A of *Pseudomonas aeruginosa* was shown to be well-tolerated and immunogenic in pre-school children age 2–5 years in Vietnam (Kossaczka et al., 1999; Canh et al., 2004). In a controlled field trial in 2–5-year olds in Vietnam, two doses of Vi conjugate conferred 91% efficacy against confirmed typhoid fever over 27 months of follow-up (Lin et al., 2001). Surveillance was continued for a total of 46 months; over the entire 46 months of follow-up the vaccine efficacy was 89% (95% CI, 76–97%) (Mai et al., 2003). This Vi conjugate vaccine is likely to be immunogenic in infants immunized in early infancy through the EPI. If so, it will be necessary to demonstrate that protection continues throughout childhood and adolescence, the age period when clinical typhoid fever is most commonly seen.

4. Commentary

The emergence, dissemination, and persistence in Asia of *S*. Typhi strains that carry resistance to most of the clinically relevant antibiotics used to treat typhoid fever in developing countries has led to a resurgence of interest in the expanded use of vaccines to control the burden of typhoid fever. Two vaccines licensed in the late 1980s and early 1990s, oral Ty21a and parenteral Vi polysaccharide, are well-tolerated, effective vaccines that constitute a vast improvement over the earlier killed whole-cell parenteral vaccines (that were highly reactogenic). However, Ty21a and Vi suffer from drawbacks. A new generation of improved live oral vaccine candidates and a Vi conjugate vaccine offer great promise to serve as even better control measures, should they become licensed and be employed by public health authorities.

Achieving control of typhoid fever by means of vaccines will require a significant financial investment. Accordingly, if immunization is restricted to high incidence populations where antibiotic-resistant strains are prevalent, and if the vaccine is highly effective and confers long-term protection, it is likely that vaccination will be a sound public health investment.

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12

Dexamethasone in the Treatment of Pediatric Bacterial Meningitis in Developing Countries: Is it Beneficial?

Elizabeth Molyneux

1. Introduction

Acute bacterial meningitis causes many deaths and substantial long-term morbidity in many parts of the world (Murray and Lopez, 1996). The incidence in resource constrained countries is 10 times that of developed countries, the mortality is 12–50% compared with less than 5% and sequelae are under-reported and probably greatly exceed the 15–20% reported from the West (Cadoz et al., 1991; Salih et al., 1991; Tefaurini and Vince., 1992; Carroll and Carroll., 1994; Chotpitayasunondh., 1994; Daoud et al., 1995 Schuchat et al., 1995 Wanyoike et al., 1995; Qazi et al., 1996; Campagne et al., 1999; Molyneux et al., 2000a Palmer et al., 1999 Goetghebauer et al., 2000 Hakim et al., 2000; Duke et al., 2003).

Many different therapeutic interventions have been used to improve the outcome for these children. In the late 19th and early 20th century, the illness was recognized and treated with intrathecal "meningitis" antiserum injections, or regular aspirations of cerebrospinal fluid (Flexner, 1906, 1913). The mortality was 90–100%. It was not until the 1930s when sulphonamides were introduced that a drop in mortality (to 30% for meningococcal meningitis) occurred (Scheld and Mandell, 1984). The mortality was further reduced when penicillin and chloramphenicol were discovered and used in 1940s and 1950s (Fothergill, 1937; Alexander, 1944). The understanding that these antibiotics need not be given intrathecally, but were effective when given intravenously improved management and compliance (Tauber and Sande, 1984).

2. Context of Care

In the last two decades, new bactericidal drugs with rapid and effective CSF penetration have been discovered with further improvement in outcome. At the same

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time, the supportive care for critically ill children has improved dramatically. Better pediatric intensive care, fluid management, ventilatory support, and seizures control have all led to better outcomes for the critically ill. Public awareness of meningitis, early referral, diagnosis, and treatment all lead to a further reduction in mortality to less than 8% in well resourced countries (Mcdermott et al., 1982; Schuchat et al., 1997).

It is in this setting that steroids have been used as an adjuvant to antibiotic therapy in the hope that mortality and sequelae could be further reduced and, especially, hearing loss be prevented.

The context of care is rather different in developing countries. In many countries, penicillin and chloramphenicol remain the first line antibiotics with few alternatives. Intensive care units are scarce and not always well staffed. Laboratories may be ill equipped. Patients present late and often after having received inadequate amounts of inappropriate antibiotics.

3. Why Steroids?

Most of the harm caused in pyogenic meningitis is thought to be through the host-mediated inflammatory response to the invading bacteria (Mustafa et al., 1990). When bacteria invade the central nervous system an intense inflammatory response occurs in the subarachnoid space. This reaction increases the permeability of the blood–brain barrier that allows increased transfer of neutrophils and protein into the subarachnoid space. Cerebral edema, increased intracranial pressure, and altered cerebral blood flow ensue. The rationale for adding steroids to the treatment is an attempt to attenuate the host's inflammatory response and thus reduce mortality and morbidity. The inflammatory response is activated especially by the breakdown of bacterial cell walls and release of toxic products, and it seems appropriate to try to forestall this reaction by giving steroids a few minutes before the antibiotic therapy.

Results of several studies and meta analyses of randomized control trials in children in developed countries suggest that the addition of steroids to antimicrobial therapy may reduce hearing loss in cases caused by *Haemophilus influenzae* type b and possibly reduce deaths from *Streptococcus pneumoniae* (Schaad et al., 1993; Kennedy et al., 1991; Coyle, 1999; Geiman et al., 1992; Quagliarello and Scheld, 1997; Lebel et al., 1998; Havens, 1992).

The results from studies in developing countries are less persuasive.

4. Causes of Bacterial Meningitis in Children in Developing Countries

Table 12.1 shows the causes of bacterial meningitis in various parts of the world. In the developed, but in only a few resource-constrained countries, *H. influenzae* type b (Hib) immunization has been introduced which has reduced invasive Hib infection by 98%. In countries where malaria and consequently post malarial anemia are common, non-typhoidal salmonella species (NTS) are the most commonly

Table 12.1. Bacterial Causes of Meningitis in various developing countries

Place [year of study]	Bacterial cause as (%)						Comments
	H.infl	S.pneum	NM	Salmonellae	GNR	Nogrowth	
Dhaka[87–94]	47	32	8.1		8.1	31	n = 852. All <5 years of age. In no growth, CSF WBC >100, glucose <30 g/L
Kigali[83–90]	31	36.5	11.5	13		21	n = 681, 75% <5 years of age
Bangkok[80–94]	42	22.2		12.4			n = 618, 0–15 years
Recife[91–92]	33	10	27			25	n = 179, age 1 month–16 years
Islamabad[90–92]	22.4	6.7	8.9			44.9	n = 89. Age 2 months–12 years. No growth in CSF 3 of WBC >100, glucose <1.66 mmol/L, Protein >1g/L
Blantyre[96–97]	16.9	23	2.6	7.9	3.7	28.8	n = Aged 0 months <13 years
Maputo[91–92]	37	8	22.3			26.8	n = 70 1 month–16 years. No growth, CSF WBC >100, glucose <2 mmol/L
Cape Town[91–92]	36.8	12.9	50.2				n = 201. Age 2 months–14 years
Goroka[97–2000]	32	36.8	0.05		2.3		n = >1 month–<12 years
Dakar[70–79]	19.6	28.7	10.7			26.5	n = 3422 >1 month
Gambia[90–95]	27	26.4	4			35.9	n = 420 Aged 0 months–14 years

Countries in the meningococcal belt will have very different prevalence rates in different years

H. infl = *Haemophilus influenzae* type b, S.pneumo = *Streptococcus pneumoniae*, NM = *Neisseria meningitidis*, Salm = salmonella spp, GNR = Gram negative rods, NG = No growth CSF = cerebrospinal fluid, WBC = white cell count /mm^3,

isolated blood-borne bacteria, and salmonella meningitis is frequently seen. Malarial anemia occurs in the young child, aged 6–18 months, and the age that children develop salmonella meningitis is the same.

Streptococcus pneumoniae (and to a lesser degree in children, NTS) invasive disease is strongly associated with HIV/AIDS in both adults and children. Pneumococcal meningitis is now the most common bacterial cause of meningitis in countries where the prevalence of HIV infection is high and rising (Molyneux et al., 2002a, b; Madhi et al., 2000; Hakim et al., 2000).

In the meningococcal belt of Africa, the prevalence rate of various bacteria will vary by year.

4.1. Outcome

The case fatality and sequelae rates for acute bacterial meningitis are high in many countries (Table 12.2). There are several reasons for this. Patients present late in the illness with a large number in coma and having had seizures. Many children have underlying malnutrition and/or anemia and may be infected with HIV/AIDS (Table 12.3).

5. Studies of Adjuvant Steroid Therapy in Children with Bacterial Meningitis in Developing Countries

Studies of the use of steroids in children with bacterial meningitis in this setting have been few (Table 12.4). Until the study from Blantyre, Malawi (Molyneux et al., 2002a) carried out in 1997–2001, most studies enrolled small numbers. One in Recife (Macaluso et al., 1996) used retrospective data. The ages of the children were not comparable, though all the studies enrolled children who were much sicker (comatose and having a history of, or having seizures) and had been ill for longer than those reported in the developed countries. Definitions of coma varied from using a score such as the Blantyre Coma Score to using the terms drowsy, stupor, unrousable, moderate, and light coma. Nutritional state was variously described as mild, moderate, severe malnutrition, or as weight for age. The definitions of meningitis in cases in which no bacteria were isolated also differed (Table 12.1). The bacteria causing disease were similar but varied as to which were most prevalent. The steroid/non-steroid arms of the study were not always strictly comparable, for example in Recife (Macaluso et al., 1999) and Islamabad (Qazi et al., 1996). Hearing tests varied in quality and follow-up varied from none to 6 months. The conclusions differed. Girgis et al. (1989) in Egypt, Ciana et al. (1995) in Mozambique, and Macaluso et al. (1996) in Recife showed a reduced mortality in certain groups who had been given steroids, namely pneumococcal infections, in Egypt and early mortality in 6–59-month-old children in Mozambique. Qazi (1996) on the other hand found an increased risk of both morbidity and death in the steroid-treated group. In this last study, 48% of the patients had received pre-enrollment antibiotic therapy.

None of the studies had statistically significant differences in the outcome.

Table 12.2. Case fatality and sequalae in bacterial meningtis in some developing countries

Place [year of study]	Bacterial as % of total Sequelae (%)							Comments
	H.infl	S.pneum	NM	Salmonellae	GNR	Nogrowth	Overall	
Kigali[1983–90]	52			39			38	n = 681, 75% <5 years of age
Bangkok[80–94]							45.5 (17.3)[1] 26.3 (11.4)[2]	n = 618, 0–15 years
Recife[91–92]	37.4	9	22			26.8	24 (44)	n = 179 Age 1 month–16 years
Islamabad[90–92]	22	6	8.9			55	12 (26)	n = 89 Age 2 months–12 years
Blantyre[96–97]	38.9 (18)	43.5 (13)		57 (5)	30 (20)	37.3 (4)	40 (9)	n = 598 Aged 2 months–13
Maputo[91–1992]	17	35.7	15.7			27	36 (29.1)	n = 70 Age 1 month–16 years
Cape Town[91–92]	5.3 (20.7)	19.2 (37.5)	1 (8.6)				5[3]	n = 201 Age 2 months–14 years
*Goroka[97–2000]	26.7 18.5						18.7 (8.6)	n = 346 Age >1 month–<12 years
Dakar[70–79]	33.5	59.5	13.9	40			44.2	n = 108
Gambia[90–95]	27 (25)	48 (58)	13?					N = Age 0–12 years

H. infl = *Haemophilus influenzae* type b, S.pneumo = *Streptococcus pneumoniae,* NM = *Neisseria meningitidis*, Salm = salmonella spp, GNR = Gram-negative rods, NG = No growth, CSF = cerebrospinal fluid.

[1]1980–87, [2]1987–90, [3]all deaths in *Strep. pneumoniae.*

*Combines death and sequelae. First row treated with chloramphenicol and second with ceftriaxone as first line treatment.

Table 12.3. Confounding Factors in the Management, Course and Outcome for Children with Bacterial Meningitis in Resource Poor Countries

	At presentation					
Place[year of study]	Fever (days)	Coma	Seizures	Nutrition	Anaemia	Pre-antibiotics MPs
Dhaka[87–94]	5.7	Drowsy 43% Coma 12%	47%			
Recife[91–92]	>3 (51%)	Moderate 50% Severe 6.7%	30.1%	Mild 15% Moderate 7%		
Islamabad[90–92]						48%
Blantyre[95–96]	4.6	BCS <5 57% BCS <2 19%	39.5%	WFA <80% 48% WFA <60% 38%	<10mg/dl 49% >10mg/dl 14%	23%
Maputo[91–1992]	2.47	21.9%	64%	23% <80% WFA	53% <10g/L	33%
Cape Town[91–1992]	2.29	Stupor/coma 13.9% 20.4%				
Port Moresby[89–90]	>3 (44%)	16.6%	16.6%	25.9% <80% WFA 3.7% <60% WFA	45% <10g/L 21% <8g/L	
Goroka[97–2000]	5.6		66.7	27% <80% WFA		49%
Cairo[1989]	3–5	60%				

Table 12.4. Summary of Some Trials of Adjuvant Steroids in the Management of Acute Childhood Bacterial Meningitis in Developing Countries

Study	Number and ages	Antibiotics given	Results			Comments
				Dexamethasone	Placebo	
Odio et al Costa Rica 1991	101 Infants and children	Cefotaxime	Hearing loss	5%	16%	OR 0.0 [0.0–0.39]
			Neuro sequelae	14%	38%	OR 0.43 [0.12–1.52]
Girgis et al Egypt 1989	429 Adults and children	Ampicillin + chloramphenicol	Hearing loss	0%	12%	Dex given IM 12 hourly for 3 days
			Mortality	13%	40%	Mortality only affected in *S. pneumoniae*
Kanra et al Turkey 1995	56 children >2 years	Amp–sulbactam + chloramphenicol	Hearing loss	7.4%	26.9%	OR 0.0 [0.0–5.09]
			Neuro sequelae	7.4%	23%	OR 0.96 [0.06–16.2] Coma score > in Dex
Ciana et al Mozambique 1988	70 children 2 –72 months	Ampicillin + chloramphenicol	Mortality	23.5%	36.3%	RR 0.71 [0.33–1.51]
			Mortality in <1 yr	17%	57%	P = 0.05 RR 0.29 [0.08–1.12]
			Neuro sequelae	15%	29%	RR 0.76 [0.27–2.16] No steroid group were younger, more coma, more *H. influenzae* and less *N. meningitides* No follow after discharge
Qazi et al Pakistan'90	89 children 2–144 months	Ampicillin + chloramphenicol	Mortality	25%	12%	RR 2.05 [0.79–5.33]
			Neuro sequelae	26%	24%	
			Hearing loss	42%	30%	P = 0.99 RR 1.42 [0.8–2.55] ↑no: of *N meningitides* in Dex (15% v 2%) 43% Pre treated with antibiotics

(*continued*)

Table 12.4. Summary of Some Trials of Adjuvant Steroids in the Management of Acute Childhood Bacterial Meningitis in Developing Countries (*Continue*)

Study	Number and ages	Antibiotics given	Results	Comments		
				Dexamethasone	Placebo	
Macaluso et al Recife '91–'92	179 children 1 month–16 yrs	Penicillin + chloramphenicol	Mortality	14%	24%	RR 1.7 [0.91–3.2] Retrospective
			Sequelae	16%	20%	RR 0.81 [0.64.1.02]
			In 6–59 months			
			Mortality	11% v No steroid 25%		
			Sequelae	27% v No steroid 48%		
Molyneux et al Malawi 97–01	598 children 2 months–13 yrs	Penicillin + chloramphenicol	Mortality	30%	30%	P = 0.93 RR 1.0 [0.8–1.25]
			Neuro sequelae	23%	19%	P = 0.97 RR 0.99 [0.78–1.27]
			Hearing loss	27%	32%	P =0.29 RR 0.84 [0.63–1.13]

No study has shown a significant improvement in outcome with the use of steroids in meningitis management.

5.1. The Blantyre Study

This is the largest double-blind, placebo-controlled, randomized study of the use of adjuvant steroid therapy for children with acute bacterial meningitis in a developing country. Children, aged 2 months to 13 years, were enrolled over a period of 42 months (July 1997–March 2001). A total of 598 children with meningitis were enrolled and assigned to receive either intravenous dexamethasone 0.4 mg/kg 12 hourly for 48 hr, each dose preceding the antibiotic medication by 5–10 minutes, or a similar volume of water for injection from an identical looking vial. The antibiotics used were chloramphenicol and penicillin. The primary outcome measure was overall death or sequelae. Neurological, developmental, and physical assessments were made at discharge from hospital and at 1 and 6 months post discharge.

Table 12.5 shows the bacterial findings in the enrolled cases.

The dexamethasone and placebo groups were comparable on presentation except that the dexamethasone group had received less antibiotic prior to admission ($p = 0.02$).

Progress in hospital was similar but fever resolution was quicker in the steroid-treated group than in placebo group (18 hr vs. 36 hr $p = 0.001$).

The outcome on discharge from hospital was similar as was that of the survivors to 6 months post-discharge (Table 12.6).

Table 12.5. Bacterial Causes of Meninigtis in the Blantyre Study

598 enrolled	
307 (51%) assigned to dexamethasone group, 295 (49%) assigned to the placebo group	
S. pneumoniae	238 (40%)
H. influenzae	170 (28%)
N. meningitides	66 (11%)
Salmonella *spp*	29 (5%)
No growth on culture	78 (13%)

Table 12.6. Outcome in Blantyre Study at 6 Months Post-discharge

Outcome	Steroids ($n = 305$) (%)	No steroids ($n = 293$) (%)
Absconded	3 (1)	3 (1)
Died in hospital	92 (30)	89 (30)
Died after discharge		
Meningitis related	10 (4)	5 (2)
Not meningitis related	10 (4)	9 (4)
Total sequelae	84 (38)	81 (39)
Hearing loss	61 (27)	66 (32)
Full recovery	119 (53)	114 (55)

5.1.1. Conclusion

In this study, dexamethasone had no beneficial effect on overall outcome. Mortality in the steroid versus placebo group had a relative risk of 1.00 (0.8–1.25). The relative risk for sequelae was 0.9 (0.78–1.27).

5.1.1.a. Did the Results Differ by Causative Agent?

(a) *Pneumococcal meningitis*

In pneumococcal meningitis ($n = 238$), those who recovered completely were 35 of 91 (38%) in the dexamethasone arm and 34 of 65 (38%) in the placebo treated arm.

Thirty-nine percent ($n = 93$ of 238) died, 37% ($n = 49$ of 132) in the steroid arm, and 42% ($n = 44$ of 106) in the placebo arm.

Hearing loss occurred in 60 of 156 survivors (29%), 18% of these losses were profound. In the dexamethasone group, 34 of 91 (37%), and in the placebo group 26 of 65 (40%) had hearing loss of equal severity.

Neurological sequelae were found in 29% of the survivors ($n = 46$ of 156). This occurred in 42% ($n = 33$ of 80) of the survivors in the dexamethasone-treated group and 21% ($n = 13$ of 62) in the placebo group; $p = 0.01$.

Conclusion. In pneumococcal meningitis, in this study, dexamethasone caused a significant increase in neurological sequelae.

(b) *Haemophilus influenzae type b meningitis*

Seventy of 123 (59.3%) survivors of *H. influenzae* meningitis made a full recovery; 39 of 61 (64%) had received steroids; and 31 of 62 (50%) had received placebo; $p = 0.1$. Forty-nine of a total of 170 (28.8%) had died, of whom 22 of 81 (27%) had received steroids, and 27 of 87 (30%) had received placebo. Hearing-loss occurred in 29 of 123 survivors (23%), and in 7.3% this loss was profound. Twelve of 61 (20%) cases treated with steroids and 17 of 62 (27%) treated with placebo were affected; $p = 0.64$.

Neurological sequelae were found in 16 of 59 (26%) survivors given steroids and 25 of 62 (40%) given placebo; $p = 0.1$. If sequelae and mortality are combined, in the steroid group 36 of 57 (69%) and in the placebo group 52 of 78 (66%) children were affected.

Conclusion. Steroids had no significant benefit on outcome in *H. influenzae* type b meningitis.

(c) *Meningococcal meningitis*

The numbers of meningococcal meningitis in the study were few. Malawi is below the meningitis belt of Africa. It tended to affect older children (mean age 9.1 years vs. 13.5 months; $p = 0.0001$) and had a better outcome. Sixty-six children made a full recovery, 78% ($n = 25$ of 32) in the steroid group and 68% ($n = 23$ of 34) in the placebo group. Overall 3 of 69 (4.3%) died. Hearing loss occurred in 15 children, 5 of 32 (17%) in the steroid group, and 10 of 34 (30%) in the placebo group. Four children had profound hearing loss, 3 in the steroid group and 1 in the placebo group.

Hearing loss in the survivors was not influenced significantly by the use of steroids. Table 12.7 shows the effect of various factors on hearing loss.

Table 12.7. Confounders for Hearing Loss on Survivors

HIV positive	19/43 (44.2%)	HIV negative	59/159 (37.1%)	$p = 0.5$
History ≤3 days	63/174 (36%)	History >3 days	38/92 (41%)	$p = 0.49$
Blantyre Coma score ≤2	29/56 (52%)	Blantyre Coma Score >2	72/211 (34%)	$p = 0.006$
Neurological sequelae	23/31 (74%)	No neurological sequelae	71/223 (31.8%)	$p = 0.0004$
Seizures history or on arrival	55/158 (42%)	No seizures	45/108 (34.8%)	$p = 0.31$

Age, sex, prior antibiotics had no significant effect on hearing. A high CSF protein, WBC $< 1000/cm^3$, CSF positive gram stain all correlated with increased hearing loss.

6. What Affected Mortality?

Mortality was affected by several factors—namely the bacterial cause—salmonella spp had the poorest outcome (58.6% mortality) and meningococcal had the best outcome (4.3% mortality). The age—the younger the child the greater the risk (OR 0.82; $p = 0.053$). Malnutrition (OR 0.97; $p = 0.0001$), coma score ≤2 (OR 5.47; $p = 0.0001$), and HIV positivity (OR 2.01; $p = 0.0029$) adversely affected the outcome.

7. What Affected Hearing Loss?

Hearing loss was affected by the level of coma on presentation, age, CSF findings, and additional neurological sequelae at discharge (Table 12.7).

8. What was the Role of HIV in Outcome in the Steroid Treated and Placebo Group?

In practice, the HIV status of children is seldom known before treatment is initiated for bacterial meningitis. But on analysis, it appeared that steroids had a detrimental effect on HIV-negative children and was of benefit to those infected with HIV (Table 12.8).

Table 12.8. Role of HIV Status on Outcome in Steroid and Placebo Groups

	HIV	Total (%)	Steroids (%)	No steroids (%)	
Total	+	157	73 (46)	84 (53.5)	
Alive and well	+	27 (17)	13 (48)	14 (50)	
Died	+	94 (60)	38 (52)	56 (67)	$p = 0.08$
Sequelae	+	30 (19)	18 (60)	12 (40)	
Total	−	302	159 (53)	143 (47)	
Alive and well	−	92 (30)	43 (27)	49 (59)	
Died	−	103 (34)	62 (61)	40 (39)	$p = 0.05$
Sequelae	−	88 (29)	39 (44)	49 (56)	

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The first line antibiotics used in this study were chloramphenicol and penicillin. These are the first line therapy in many developing countries. Resistance is developing to these antibiotics. Perhaps this influenced the outcome in the study. To examine this, we looked at only those children who had a history of less than 4 days fever and no antibiotic resistance to penicillin or chloramphenicol. The number was 124. The only significant difference in outcome was that in the HIV-negative group 7 of 33 (21.2%) died who had received placebo compared with 16 of 44 (36.4%) of those receiving steroids (OR 2.12 (0.68–6.82); $p = 0.2$). But in the HIV-positive group, 11 of 26 (42.3%) died in the steroid group compared with 20 of 28 (71.4%) of the placebo-treated group (OR 0.29 (0.08–1.04); $p = 0.059$).

In this small number of children, it did appear that steroids were beneficial to the HIV-positive children.

9. Overall Conclusion

This study shows no benefit in the use of adjuvant steroids in the management of bacterial meningitis in settings where presentation is delayed, children are very ill on arrival, there is significant underlying illness and infection, and the first line antibiotic therapy is chloramphenicol and penicillin.

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13

Should Dexamethasone be Part of Routine Therapy of Bacterial Meningitis in Industrialised Countries?

Peter McIntyre

1. Introduction

Although the first study of adjuvant corticosteroid therapy in bacterial meningitis was published over 40 y ears ago, (1) its value remains controversial in 2004. There was minimal evidence of reduction in mortality or severe sequelae in early trials. (2) Interest was rekindled following the publication of results of two trials conducted in Dallas by Dr George McCracken and colleagues, which demonstrated for the first time a reduction in hearing loss as a specific benefit of corticosteroid therapy in childhood meningitis. (3) However, almost 80% of participants in the Dallas trials had meningitis due to *Haemophilus influenzae* type b (Hib) which became a rare cause of childhood bacterial meningitis in most industrialised countries by the mid 1990s following the success of Hib immunisation programs. (4) Numerous commentators opined that adjunctive corticosteroid therapy could not be generally recommended on three main grounds—uncertain value for organisms other than Hib, the possibility of adverse effects especially on non-hearing deficits and the possibility that benefit was confined to certain types or timing of antibiotic therapy. (5)

A meta-analysis in 1997 found suggestive evidence of benefit in pneumococcal meningitis, but only when dexamethasone was commenced prior to or with antibiotic therapy. (5) Subsequently, a Cochrane review has been published (6) which includes a larger number of trials in both adults and children. The Cochrane review found significant reduction in hearing loss in children, both for all organisms and organisms other than *Haemophilus influenzae*, and a significant reduction in mortality in adults but not in children. Since completion of the Cochrane review, two further important randomised controlled trials have been published, one including 598 children in Malawi (7) and the other 301 adults in the Netherlands, (8) with apparently contradictory findings. What are practitioners to make of this bewildering

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mass of information in the era of evidence-based decision making? This review seeks to put the available evidence into context and derive the most appropriate recommendations for protocols for management of putative bacterial meningitis in developed countries.

2. Steroids and Infection

The use of adjunctive corticosteroid therapy has been considered in a wide range of infections since corticosteroids became available in the 1950s. In 1992, the Infectious Disease Society of America published guidelines regarding their use. (9) The guidelines found good evidence for benefit from steroid therapy in only 3 conditions – typhoid fever with associated shock, tuberculous pericarditis and pneumocystis carinii pneumonia with hypoxia. There was judged to be good evidence against use (that is steroids were contraindicated, at least routinely) in a further 6 conditions (gram-negative shock, cerebral malaria and a number of viral infections—herpes zoster, hantaan virus, viral broncholiotis and acute viral hepatitis). However, it was noteworthy that in the other 33 infections considered by the guidelines no conclusive recommendations could be made.

3. Steroids and Bacterial Meningitis

The plethora of recommendations over time, the particular risk in central nervous system infections that the diagnoses other than bacterial meningitis could be masked (10) and the changing epidemiology of bacterial meningitis as a result of vaccination campaigns (4) have all undoubtedly contributed to the caution of physicians in industrialised countries in adopting recommendations for steroid use. Indeed, the role of steroids in meningitis has been the subject of editorial pieces in the journals of at least 9 countries and the deliberations of working parties in the United States, Canada and the UK.

I will address the state of the evidence by looking at 4 sources relevant to industrialised countries—the 1997 meta-analysis, (5) the 2003 Cochrane review, (6) the Netherlands adult trial (8) and an study of impact of steroids in practice in pneumococcal meningitis from Australia. (11) I will not discuss the Malawi trial (7) further, as this is comprehensively presented in the context of meningitis in developing countries elsewhere in this volume, but will identify and distinguish data from developing countries and industrialised countries in the evidence discussed.

3.1. The 1997 Meta-Analysis (5)

3.1.1. Included Trials

The meta–analysis published in 1997 included 11 eligible trials reported since the original Dallas report; the 9 in which all organisms were reported included 848 children. There were 526 cases due to *Haemophilus influenzae* type b (Hib) , (62%),

122 due to *Streptococcus pneumoniae* (14%) and 125 (15%) due to *Neisseria meningitidis* leaving 75 due to other organisms or culture-negative purulent meningitis. There were an additional two trials with assessable subjects with pneumococcal meningitis only, increasing the total from 122 to 178 for this organism. Three trials were conducted in non-industrialised countries—Egypt, Mozambique and Turkey. The weighted mortality rate among control subjects was 20.5% (95% CI 15–27%) in Egypt and Mozambique but 1.4% (95% CI 0.6%–3.3%) in other studies combined, including Turkey, suggesting that the study setting in Egypt and Mozambique differed significantly from others. However, the latter two trials contributed data only to the outcomes of hearing loss in pneumococcal meningitis (Egypt) and mortality due to all organisms (Mozambique). Thus this analysis is largely applicable to industrialised country settings.

3.1.2. Results

A major finding from the studies with available data was a significantly lower incidence of severe hearing loss in meningococcal cases. Among control subjects, 11.6% of 233 Hib cases, 19.6% of *S. pneumoniae* cases but 0/50 meningococcal cases had severe hearing loss. This would clearly make it difficult to detect any effect on hearing in meningococcal meningitis. There was convincing evidence of reduction in hearing loss in Hib meningitis with dexamethasone (OR 0.31, 95% CI 0.14–0.69) which was similar in studies where all subjects were given dexamethasone before or with antibiotics (early) and others. However, for pneumococcal meningitis, there was evidence of benefit only with early dexamethasone for both for severe hearing loss (OR 0.09, 95% CI 0–0.71) and for any neurologic or hearing deficit (OR 0.23, 95% CI 0.04–1.05). Adverse effects of dexamethasone were examined; only secondary fever was significantly increased in dexamethasone recipients, by 11% (95% CI 2–21%). There was a non-significant reduction in late seizures and reactive arthritis. Clinically evident gastrointestinal bleeding, previously raised as an issue for dexamethasone use, was reported in 2.8% of 466 patients who received 4 days of dexamethasone compared with 0.8% of 122 receiving 2 days and 0.4% of 450 controls.

3.1.3. Summary and Conclusions

This analysis was the first to provide some information about differential effects by causative organism. It confirmed prior suggestions that hearing deficits were more common in pneumococcal meningitis and showed that timing of dexamethasone was less important in Hib meningitis than pneumococcal meningitis, where benefit was only demonstrated when dexamethasone was commenced prior to or with parenteral antibiotics, though no detrimental effects were evident with delayed therapy. As few or no included subjects had antibiotic resistant organisms, no information on dexamethasone in this situation was available, but older data showing a reduction of CSF penetration of penicillin with dexamethasone (12) suggested that a third generation cephalosporin should be preferred when dexamethasone was given.

3.2. The 2003 Cochrane Review (6)

3.2.1. Included Trials

The Cochrane review had a different design to the 1997 meta-analysis. First, all potentially eligible studies were included, rather than only those since 1988, second it included studies with adult patients and third there was a specific focus on mortality as an outcome. An extensive literature search revealed 28 trials, with 18 meeting methodological quality criteria and 4 of these including adult subjects. In the 18 eligible trials, with 1853 subjects, there were 7 where all subjects had been given steroid therapy before or with parenteral antibiotics. Six studies were conducted in developing country settings (Nigeria, Mozambique, Egypt, Turkey, Pakistan and India) and included 726 subjects (39%). With the exception of the Turkish study where it was 5%, there was a substantially higher mortality (13–44%) in these developing country studies than in the 12 studies from industrialised countries (1–3%), the exception being two studies including adults, one conducted in 1963 (45%) and one in 1999 (13%). Neither of the latter studies was included in the 1997 meta-analysis. Dexamethasone was the steroid used in all except the 3 earliest studies, conducted prior to 1980. Hearing was adequately assessed in 1013 subjects.

3.2.2. Results

Mortality was significantly reduced overall (8.5% vs 11.6%, OR 0.8, 95% CI 0.6–0.97) but this was mostly due to the contribution from studies including adults, where mortality was 17.8 % in controls and 8% in corticosteroid treated (OR 0.38, 95% CI 0.2–0.8), with no effect on mortality demonstrated in paediatric studies (OR 0.95, 95% CI 0.65–1.37). Data on severe hearing loss were available only for children, but showed significant protection in the steroid group both for Hib meningitis (OR 0.31, 95% CI 0.15–0.62) and in meningitis due to other organisms (OR 0.42, 95% CI 0.2–0.9). The number of subjects with long-term neurologic sequelae was also significantly less in the corticosteroid group (6% vs 9%, OR 0.7, 95% CI 0.45–1.00), although the reduction was less impressive than for death or hearing loss. Similar point estimates were seen in both children and adults but were not statistically significant in these sub-group analyses. Adverse events were grouped and included clinically evident gastrointestinal bleeding, reactive arthritis, pericarditis, herpes zoster or herpes simplex infection, fungal infection and secondary or persistent fever. There were 146 adverse events in 599 treated subjects (24%) and 131 in 584 controls (22.4%), a non-significant increase (OR 1.06, 95% CI of 0.9 to 1.3).

3.2.3. Summary and Conclusions

The strength of the Cochrane review process is that clear and comprehensive guidelines are followed for conduct of reviews and conclusions from the evidence presented are arrived at by independent reviewers. There was substantial heterogeneity in the studies included in the Cochrane review, which covered a period of almost 35 years and a range of settings, but all were of high methodologic quality. A substantial number of subjects were included from 5 countries with a high mortality among controls. Although the results from the Egyptian study numerically dominated

this group, with a total of 429 subjects and the only independently statistically significant result, the point estimate of mortality was lower in treated subjects in 4/5 of the developing country studies, with a non-significant increase reported only from Pakistan. It was notable that mortality was significantly different only in adults. Whether this is simply due to higher mortality in adults, making it easier to detect an effect, or a real biologic difference, was unclear. Importantly, there was no evidence of any increase in mortality in paediatric or adult studies or of a significant increase in any other of a wide range of potential adverse effects. Other significant findings were that there was evidence of a beneficial effect on severe hearing loss in children with meningitis due to organisms other than Hib, and an independent reduction in long-term non-hearing deficits. The independent reviewers concluded that the consistency and degree of benefit identified in the analysis merits a recommendation for general use of dexamethasone in industrialised countries with good access to medical services.

3.3. The Netherlands Adult Trial (8)

3.3.1. Patients

In November 2002, the results of a trial of dexamethasone therapy in adults with bacterial meningitis were published in the New England Journal of Medicine. (8) This study was of high methodologic quality and required many years of recruitment; 301 patients were included, 157 in the dexamethasone group and 144 in the placebo group. All were over 17 years of age, with a mean age of 44yrs. *Streptococcus pneumoniae* and *Neisseria meningitidis* together accounted for 49 and 48% respectively in treatment and control groups (all susceptible to penicillin), with 23 and 21% of cases in treatment and control groups respectively being purulent meningitis with negative bacterial culture.

3.2.2. Outcomes

An unfavourable outcome (defined as anything other than apparently normal or mild disability) was significantly less common in the treatment group (15%) than controls (25%, RR 0.6, 95% CI 0.4–0.94). Among organism subgroups, there was a significant reduction only in pneumococcal meningitis (26% vs 52% in controls), (8) but point estimates were in the direction of benefit in all except the culture-negative group. Hearing loss and focal neurologic deficits were also in the direction of benefit but did not reach significance overall or in any subgroup, while mortality was significantly reduced. This was almost entirely attributable to pneumococcal cases (14% vs 34% in controls), with other bacteria combined having a mortality of 3% (3/99) in the treatment group and 4% (4/94) in controls. There was no increase in adverse events with dexamethasone treatment and in particular gastrointestinal bleeding occurred in 3% of the controls and 1% of dexamethasone-treated patients.

The accompanying editorial recommended that “routine use of adjunctive dexamethasone therapy is warranted in most adults with suspected bacterial meningitis.....Vancomycin should not be used as the sole antimicrobial agent.” (13)

3.4. Dexamethasone in Practice—Pneumococcal Meningitis, Sydney 1994–99 (11)

Randomised controlled trials may differ from clinical practice in a number of ways. In particular, with dexamethasone therapy of meningitis, the most severely ill patients may not be recruited given their parlous clinical condition (as suggested by the low mortality rate in trials relative to unselected patients in industrialised countries) (5,6). In addition, protocols calling for dexamethasone prior to parenteral antibiotics or the lack of a protocol, with differing policies followed by various treating clinicians, present practical difficulties. Two previous studies had examined the impact of dexamethasone in childhood bacterial meningitis in clinical practice, one finding no significant benefit (14) and the other a reduction in admissions to intensive care and in prolonged fevers. (15) However, the Sydney study is the first to include all cases in a geographic area and to use multivariate analysis to adjust for differing prognostic factors potentially confounding the assessment of outcomes in an observational study. (11)

3.4.1. Patients

A population-based register was used to identify all cases of pneumococcal meningitis in children in Sydney, Australia and surrounding areas from 1994–1999. A total of 120 cases were identified, almost half of whom (46%) were under the age of 12 months and 89% under 5 years. Cases were treated at 21 hospitals, with only 42% initially admitted to hospitals with a tertiary referral role. The first presentation was to general practice in 72 (60%) and of these 23 (32%) had two or more presentations before admission to hospital, while 6/48 (12%) of patients whose first presentation was to hospital had more than one attendance. A lumbar puncture was performed in 103 (85%), but only after initiation of parenteral antibiotics in 43 of them (42%). The cerebrospinal fluid Gram stain showed Gram-positive cocci in 90 (87%) of cases. Dexamethasone was used in 58 (48%) cases, with timing of the first corticosteroid dose known in 57; in 31/57 (54%), this was after parenteral antibiotics. Most cases (64%) treated with corticosteroids received at least 4 doses, but 9 (16%) received only one dose. A total of 45 cases (37.5%) were admitted to an intensive care unit (ICU), of whom 64% (29/45) required intubation.

3.4.2. Outcomes

Not having a lumbar puncture done ($p = 0.002$) and admission to intensive care ($p=0.01$) were independently associated with a significantly increased risk of death, whilst receipt of antibiotic prior to admission ($p = 0.05$) and early parenteral corticosteroids ($p = 0.05$) tended to lower the risk of death, but did not reach statistical significance. Children who were intubated were significantly more likely to have received corticosteroids, suggesting that disease severity was greater in the steroid-treated group. Among cases who did not receive dexamethasone, 23/62 (37%) either died or had severe morbidity, compared with 11/27 (41%) of those receiving dexamethasone after antibiotics and 4/31 (13%) of those receiving early dexamethasone ($P=0.01$). When entered into a multivariate model controlling for other prognostic

factors such as intubation, not having a lumbar puncture done and delayed hospitalisation, early dexamethasone remained associated with significant protection against death or severe morbidity (OR 0.21, 95% CI 0.05–0.77).

4. Summary

Two issues are clear from the data available regarding the current place of dexamethasone in routine management of suspected bacterial meningitis in industrialised countries.

First, there is now good evidence of benefit from adjunctive dexamethasone therapy which is not confined to Hib meningitis, but in the case of pneumococcal meningitis probably requires that dexamethasone is given with or before, rather than after, parenteral antibiotics. In meningococcal meningitis, statistically significant benefit has not been demonstrated for any outcome, even in meta-analyses, but the point estimate is in the direction of benefit and failure to demonstrate an effect is more likely to be due to limited power from low event rates rather than no benefit; certainly there is no evidence of a detrimental effect. Most culture-negative cases of presumptive bacterial meningitis outside the neonatal period are likely to be due to one of the above 3 organisms. In the neonatal period or in some settings in developing countries, the spectrum of organisms is very different and extrapolation of these findings cannot be assumed.

Second, the suggestion that dexamethasone is not applicable to certain subgroups in industrialised countries (such as cases not treated with cefuroxime, or some other sub-optimal therapy, or cases treated with vancomycin) (5) or that benefit only applies to hearing loss or Hib cases, either do not stand up to scrutiny or are not answerable from available data.

What does this mean for clinical practice? The results of randomised controlled trials may not readily translate to clinical practice, particularly with respect to early commencement of steroids. The Sydney data show that in a representative developed country population with good access to services, after controlling for other prognostic variables, early corticosteroid therapy is associated with improved outcome. These data also show that deferred lumbar puncture is frequent and so criteria requiring presumptive identification of an organism are not practical to guide dexamethasone use, indeed those patients in whom lumbar puncture is deferred are more likely to have severe disease. This experience is an important addition to the findings from clinical trials of dexamethasone in pneumococcal meningitis in industrialised countries (5,6) as it demonstrates that adjunctive steroid therapy is beneficial in a ‘real world’ situation. In addition, the prospect of clinical trials in children, already limited by small case numbers, will be further reduced when the use of the conjugate pneumococcal vaccines is widespread. In Canada, a trend to decreasing use of corticosteroids was noted between 1991 and 1999, probably reflecting conflicting evidence. (16). Unless clear protocols are in place, the commencement of steroids before or with antibiotics will be difficult to implement in emergency situations, as illustrated by the data from Sydney.

5. Conclusions

Although not applicable in settings with delayed presentation to hospital, significant co-morbidities, a wide range of organisms and sub-optimal antibiotic choice, such as Malawi (7), these data add to the previous evidence from meta-analysis of childhood trials (5,6) and the recent randomised controlled trial in adults (8) that adjunctive steroid therapy is beneficial in industrialised countries. The lack of any evidence of adverse effects from steroid therapy is important to reassure clinicians that early, routine use of dexamethasone in presumptive bacterial meningitis will not disadvantage their patients. The variation in clinical practice and evidence of rapidly declining steroid use in developed countries (16) suggests the need for protocols to ensure that adjunctive corticosteroids are used in presumptive childhood bacterial meningitis in emergency practice. Given the low rates of adverse events, such a routine policy will continue to be justified even with further falls in bacterial meningitis due to vaccination against the three most common childhood bacterial pathogens, especially as the clinical diagnosis is usually clear within 24 hours and dexamethasone can be discontinued if bacterial meningitis has been ruled out.

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14

How to Interpret a CSF—The Art and the Science

Tom Connell and Nigel Curtis

1. Introduction

Cerebrospinal fluid (CSF) was described by Galen as early as the second century as a vaporous humor produced in the ventricles that provided energy to the rest of the body. Interestingly, such theories, although humorous, have not been universally replaced by scientific rationale (http://www.healtouch.com/csft/csf.html).

CSF is metabolically active and has many functions. By providing buoyancy, it reduces the effective weight of the brain from 1,500 to 50 g (Conly and Ronald, 1983) and provides a buffer against the effects of trauma. CSF is predominantly produced by the choroid plexuses of the lateral, third, and fourth ventricles and flows within the subarachnoid space, with approximately one-fifth lying in the ventricles (Davson et al., 1987; Blatter et al., 1995). Daily CSF production varies between 400 and 500 ml in adults and the entire CSF volume is replenished every 5–7 hr (Cutler et al., 1968; Wood, 1983). The CSF volume varies with age: from 170 ml in an adult (Blatter et al., 1995; Fujimaki et al., 2003); to between 65 and 140 ml from 4 to 13 years (Cutler et al., 1968); to 40 ml in a healthy full-term neonate; and to between 10 and 30 ml in a premature neonate (Otilia, 1948). CSF flow rate differs in the cervical, thoracic, and lumbar spinal canal (Freund et al., 2001), and CSF microscopy and biochemistry values may differ between lumbar and ventricular CSF (Freemont-Smith and Dailey, 1925; Goodwin and Shelley, 1925; Stewart, 1928; Marks, 1960; Fishman, 1980; Herndon and Brumback, 1989; Greenlee, 1990). Normal CSF comprises 99% water and contains various plasma components including glucose, proteins, electrolytes, minerals, and enzymes, as well as antibacterial components. The specific gravity ranges from 1.004 to 1.007. CSF is typically described as crystal or "gin" clear and colorless.

Lumbar puncture is frequently performed in the evaluation of the febrile infant or child to confirm or exclude a diagnosis of bacterial meningitis. A positive CSF culture ultimately provides the gold standard for diagnosing bacterial meningitis but is

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unavailable at the time of initial evaluation. Physicians therefore frequently make decisions based on initial CSF microscopy and biochemical analysis, including CSF pressure, white cell count, protein concentration, glucose concentration, and Gram stain. This requires knowledge of the normal ranges of these indices across different age groups and other factors that may influence them. Debate about the interpretation of CSF findings dates back nearly a century (Sidbury, 1920; Levinson, 1928; Munro, 1928; Stewart, 1928).

2. Variation in CSF Normal Values with Age

2.1. Pressure

In a series of 1,000 normotensive adults, CSF pressure ranged from 70 to 180 mm of water in 94% of cases (Meritt and Fremont-Smith, 1937). Although pressures up to 280 mm have been recorded in normal children (Fishman, 1980; Ellis, 1994), the generally accepted upper limit for the normal opening pressure during lumbar puncture with the patient in the lateral decubitus position is 150 mm of water in older children and lower (85–110 mm) in younger children (Bonadio et al., 1992; Meritt and Fremont-Smith, 1937). In newborns, a normal range between 0 and 60 mm of water has been reported (Sidbury, 1920; Levinson, 1928; Munro, 1928; Stewart, 1928). The CSF pressure is higher in the sitting position (up to 400 mm of water in adults) due to the hydrostatic effect of the column of CSF (Fishman, 1980). CSF pressure changes with respiration, typically increasing with expiration and decreasing with inspiration by 5–10 mm of water (Meritt and Fremont-Smith, 1937; Conly and Ronald, 1983; Seehusen et al., 2003). Hyperventilation is associated with a lower opening pressure, whereas obese and uncooperative patients may have higher opening pressures (Fishman, 1980; Ellis, 1994).

2.2. White Cell Count

The normal CSF white cell count in infants over 2 months of age is the same as for older children and adults, that is, less than 5×10^6 lymphocytes per liter (L). In contrast, as a consequence of increased permeability of the blood brain barrier (Adinolfi, 1985; Anagnostakis et al., 1992), it is quite common for CSF from normal neonates to contain more than 5×10^6/L white cells. This leads to difficulty in interpreting CSF white cell count in the newborn and early neonatal period. Normal ranges for CSF white cell count in non-infected neonates have been provided in several studies, but these differ in the age ranges evaluated as well as methodology (Stewart, 1928; Wolf, 1961; Sarff et al., 1976; Naidoo, 1982; Pappu et al., 1982; Portnoy and Olson, 1985; Rodriguez et al., 1990; Bonadio, 1992; Ahmed et al., 1996). In addition, viral CNS disease was not reliably excluded in most of these studies. Bonadio et al. compared normal CSF white cell counts in 75 infants in two age groups in the first 8 weeks of life (Bonadio et al., 1992). In 90% of cases, the total cell count was less than 22×10^6/L in infants in the first 4 weeks of life and less than 15×10^6/L in infants aged 4–8 weeks. In contrast, Ahmed et al. analyzed CSF from 108 normal infants from 0 to 30 days of age and found a lower normal CSF white

cell count: 90% had a CSF white cell count of less than 11×10^6/L (Ahmed et al., 1996). This was attributed to tighter exclusion criteria including the use of PCR to exclude enteroviral meningitis. This study found that there was no significant difference in CSF white cell counts in each of the first 4 weeks although there was a trend towards increased number of cells in the first week.

The CSF white cell count does not differ remarkably between the term babies and premature or low birth weight infants. Statz reported mean CSF white cell counts in infants at different gestational ages (Statz and Felgenhauer, 1983). The mean numbers of CSF white cells were 7.6, 6.5, and 7.3×10^6/L at 27–32, 32–36, and 30–40 weeks, respectively, with a range from 0 to 20×10^6/L. Other studies have reported similar findings in premature and low birth weight infants (Otila, 1948; Gyllesward.A and Malmstrom, 1962; Rodriguez et al., 1990).

In summary, whilst there is wide variation in the reported 'normal' upper limit for CSF white cell count in neonates, the most robust studies suggest that CSF white cell counts over $11–22 \times 10^6$/L in the first four weeks of life are unusual in normal infants (Table 14.1).

2.3. Neutrophil Count

The presence of neutrophils in the CSF has been deemed as always abnormal (Conly and Ronald, 1983). Some authorities, however, accept that a single neutrophil may rarely be seen in normal CSF and is not necessarily abnormal if the CSF white cell count is 4×10^6/L or less and the protein and glucose are normal (Fishman, 1980; Bonadio, 1988b; Herndon and Brumback, 1989; Greenlee, 1990; Scheld et al., 1997).

In contrast, the presence of neutrophils in the CSF from neonates may not be so abnormal. There is disagreement in the literature about the differential white cell composition in normal neonatal CSF. Whilst Sarff et al. reported that up to 61% of the total CSF white cell count in healthy neonates may be neutrophils (Sarff et al., 1976), Bonadio et al. found substantially lower proportions of neutrophils (mean 2–3%) (Bonadio et al., 1992). In 90% of cases reported by Bonadio et al., the percentage of neutrophils was less than 6% in infants in the first 4 weeks of life and less than 9% in infants aged 4–8 weeks. This lower proportion was confirmed by Ahmed et al. who found a median CSF neutrophil count across all age ranges of 0×10^6/L with only 5% of infants having a neutrophil count greater than 1×10^6/L at any age (Ahmed et al., 1996).

There are fewer studies in low birth weight and premature infants but one study found the percentage of neutrophils to be as high as 11% in this group (Rodriguez et al., 1990).

In summary, neutrophils are absent in the CSF of the majority of normal neonates and their presence, particularly if more than 5% of the CSF white cell count should raise concern.

2.4. Protein

CSF protein concentration depends on serum protein concentration and the permeability of the blood brain barrier, which varies with age (Adinolfi, 1985). For this reason, CSF protein concentration is age dependent, being greatest at birth and decreasing

Table 14.1. Normal Values for CSF White Cell Count ($\times 10^6$/L) from Selected Studies

Study reference (n= number of patients)		1 to 7 days	8 to 30 days	1 to 2 mo	2 to 3 mo	3 to 4 mo	4 to 6 mo	6 mo to 10 y	10 to 14 y	14 to 16 y
(Ahmed et al., 1996) (n=108)	Median (90^{th} centile)	6 (18)	4 (8–13)							
		4 (11)								
(Bonadio et al., 1992) (n=75)	Median (90^{th} centile)	8.5 (22)		4.5 (15)						
(Rodriguez et al., 1990) (n=71)	Mean ±SD	3±3	4±4	4±3		For infants of birth weight ≤1,000 g				
	Mean ±SD	4±4	7±11	8±8		For infants of birth weight 1,001–1,500 g				

Table 14.2. Normal Values for CSF Protein Concentration (g/L) from Selected Studies

Study reference (n= number of patients)		1 to 7 days	8 to 30 days	1 to 2 mo	2 to 3 mo	3 to 4 mo	4 to 6 mo	6 mo to 10 y	10 to 14 y	14 to 16 y
(Biou et al., 2000) (n=1074)	95^{th} centile	1.08*	0.90	0.77	0.60	0.40		0.32	0.41	
(Wong et al., 2000) (n=225)	90^{th} centile	0.87			0.55	0.25			0.33	
(Ahmed et al., 1996) (n=108)	Mean ±SD	0.81±0.31	0.69±0.23 (8 to 14 days) 0.60±0.23 (15 to 21 days) 0.54±0.16 (22 to 30 days)							
		0.64±0.24								
(Bonadio et al., 1992) (n=75)	Mean ±SD	0.84±0.45		0.59±0.25						
(Rodriguez et al., 1990) (n=71)	Mean ±SD	1.62±0.37	1.59±0.77	1.37±0.61		For infants of birth weight ≤1,000 g				
	Mean ±SD	1.36±0.35	1.37±0.46	1.22±0.47		For infants of birth weight 1,001–1,500 g				

*1 to 8 days

during the first few weeks of life, reaching a minimum value at 6 months of age (Tibbling et al., 1977; Statz and Felgenhauer, 1983; Lott and Warren, 1989; Rodriguez et al., 1990; Bonadio, 1992; Biou et al., 2000; Wong et al., 2000). Thereafter, the normal CSF protein range remains low, not increasing to adult values until at least after adolescence (Tibbling et al., 1977; Wong et al., 2000) (Table 14.2). Therefore, the use of adult reference ranges is inappropriate as values at the higher end of this range may

be abnormal in preadolescent children. This was also illustrated in a study of 1,074 CSF protein concentrations by Biou et al. that showed CSF protein concentration rarely exceeds 0.35 g/L between 6 months and 10 years of age (Biou et al., 2000).

The study by Biou et al. also addressed the normal range for CSF protein in neonates. In the first week of life, the 95th centile for CSF protein in this study was 1.08 g/L, which is less than that documented in some previous studies for the early neonatal period, such as the often quoted upper value of 1.7 g/L reported by Sarff et al. (Sarff et al., 1976). Other authors have also suggested the upper limit for CSF protein in neonates is lower than previously suggested (Anagnostakis et al., 1992; Ahmed et al., 1996; Biou et al., 2000). In contrast, higher normal limits for CSF protein have been suggested for premature and low birth weight infants (Rodriguez et al., 1990).

In summary, CSF protein concentrations greater than around 1.0 g/L in neonates and greater than 0.35 g/L in children less than 10 years of age should be considered potentially abnormal.

2.5. Glucose

CSF glucose is derived from serum and as a general rule has a concentration approximately two-thirds of the serum level (Stewart, 1928; Powers, 1981; Donald et al., 1983; Greenlee, 1990). CSF glucose should therefore ideally always be interpreted in relation to the serum glucose concentration, taking into account that it takes approximately 4 hr for the CSF glucose to equilibrate with changes in the serum glucose (Powers, 1981). Like CSF protein, the CSF glucose normal range is age dependent. It is lower in neonates compared to older children reflecting their lower blood glucose levels, immature glucose exchange mechanisms, and the increased permeability of the blood brain barrier (Fishman, 1980; Remington and Klein, 2001). Bonadio et al. reported a mean CSF glucose of 2.55 $\pm$ 0.57 mmol/l in the first 8 weeks of life (Bonadio et al., 1992). There was no significant difference between values in the first 4 weeks of life and weeks 4–8. Ahmed et al.'s findings were similar though this study found a slightly higher mean value in the first 4 weeks (2.84 $\pm$ 0.71 mmol/l) (Ahmed et al., 1996).

3. Can CSF Abnormalities be Caused by Convulsions?

Whether convulsions cause changes in the CSF is frequently debated as there is inconsistent information about this in the literature (Aminoff and Simon, 1980; Schmidley and Simon, 1981; Edwards et al., 1983; Simon, 1985; Thompson and Salinsky, 1988; Woody et al., 1988). The reported frequency of seizure-induced CSF abnormalities has been estimated to be as high as 30% (Prokesch et al., 1983). The mechanism by which seizure activity might induce a CSF pleocytosis is unclear but a transient disruption of the blood brain barrier has been demonstrated (Meldrum and Breirley, 1973; Petito et al., 1977; Wenzel et al., 1980). Physiological responses, including the release of noradrenaline, have been proposed to be responsible for a transient rise in CSF glucose following an episode of status epilepticus.

A number of studies have reported postictal CSF pleocytosis in patients with epilepsy following an episode of status epilepticus (Aminoff and Simon, 1980; Woody et al., 1988; Barry and Hauser, 1994; Rider et al., 1995) or prolonged seizure (Schmidley and Simon, 1981; Edwards et al., 1983; Prokesch et al., 1983; Devinsky et al., 1988; Rider et al., 1995). However, in most of these studies, observed changes were minimal. Moreover, it was difficult to exclude the possibility of undiagnosed infection or other cause for CSF changes in some of these studies.

Recently, there have been studies suggesting that it is inappropriate to attribute CSF changes to convulsions. Wong et al. retrospectively reviewed the results of CSF examinations on 62 patients who had had a convulsion within the previous 24 hr (in whom infection or other causes of CSF changes had been excluded) (Wong et al., 2001). Although CSF changes were found in a few patients (pleocytosis in 5%, neutrophilia in 6%, and increased protein in 10%), these were minimal; the maximum observed white cell count was 8×10^6/L and maximum protein 0.52 g/L. There was no significant difference between those with short or prolonged seizures. This and other studies (Portnoy and Olson, 1985) suggest that seizure-induced CSF abnormalities are rare in children.

4. CSF Eosinophilia

4.1. Eosinophilic Meningitis

Eosinophilic meningitis, defined by the presence of more than 10 eosinophils $\times 10^6$/L in the CSF, is rare (Bosch and Oehmichen, 1978). There are both infectious (most commonly parasitic) and non-infectious causes. The nematode infections angiostronglyliasis, gnathostomiasis, and baylisascaris are the commonest infections associated with eosinophilic meningitis (Lo Re and Gluckman, 2001; Lo Re and Gluckman, 2002; Lo Re and Gluckman, 2003). They are seen worldwide, but predominantly in the Pacific Basin including Hawaii, Indonesia, the Philippines, Japan, and Papua New Guinea. Clinical manifestations differ depending on the parasite but generally occur between 2 and 35 days following ingestion of larvae and can range from headaches and neck stiffness to delirium and seizures. Peripheral blood eosinophilia may also be present depending on the parasite (Schulte et al., 2002). The CSF is usually mildly turbid with a white cell count between 150 and 2,000 cells $\times 10^6$/L and eosinophils representing more than 10% of the total in the majority of patients (Kuberski et al., 1979). Patients with gnathostomiasis can have a CSF eosinophilia as high as 95% of the total CSF white cell count. Protein concentration is usually elevated with glucose being low or normal.

4.2. Shunt Pathology

CSF analysis is often used to help diagnose shunt pathology and to distinguish obstruction and infection. CSF eosinophilia has been reported to be a strong indicator of shunt pathology (Bosch and Oehmichen, 1978; Tzvetanova and Tzekoc, 1986; Vinchon et al., 1992; Wiersbitzky et al., 1998). Although specific, the finding of CSF eosinophilia has low sensitivity. In one study, hypereosinophilia

(defined as greater than 5% of CSF white cells) was present in only 15 of 94 shunt infections (68 culture positive) (Vinchon et al., 1992). Recurrence of infection occurred earlier and more frequently in the group of patients with eosinophilia. Additionally, subsequent shunt complications, appeared to be associated with a higher level of eosinophilia at the end of treatment of the initial infection, a finding supported by Tung et al. (1991).

According to at least one study, the finding of CSF eosinophilia is less helpful in distinguishing between an infected and obstructed shunt. McClinton et al. reported CSF eosinophilia in 2 of 12 patients with infection and 11 of 13 patients with malfunction in a series of 81 patients concluding only that CSF eosinophilia greater than 5% is a good predictor of shunt pathology, rather than helpful in distinguishing between infection and obstruction (McClinton et al., 2001). However, this study did find that a combination of fever and greater than 10% ventricular CSF neutrophils had a 99% specificity, a 93% positive predictive value, a 95% negative predictive value, and a posttest probability of 92% for infection.

5. Interpretation of Blood-Contaminated CSF ("Traumatic Taps")

Traumatic lumbar punctures occur in up to 20% of cases undertaken in children and neonates (Yogev, 2002). It takes between 5,000 and 6,000 red cells $\times 10^6$/L for the CSF to appear bloody to the naked eye (Conly and Ronald, 1983). Interpreting the results of CSF analysis with a "bloody tap" is notoriously difficult.

Calculations to determine the corrected number of white cells in the CSF of blood-contaminated samples frequently underestimate the true CSF white cell count (Osborne and Pizer, 1981; Novak, 1984). Simple calculations to correct for blood contamination are based on the assumption that in a normal person the ratio of white to red cells in the CSF attributable to blood contamination is 1:500 to 750 (Solomon et al., 1934; Meritt and Fremont-Smith, 1937; Fishman, 1980; Feigin, 1981; Novak, 1984; Fuchs, 1997). These ratios are based predominantly on *in vitro* studies. Others have suggested calculations based on the peripheral blood white to red cell ratio. Rubenstein et al. used an equation to calculate the expected (predicted) CSF white cell count based on the ratio of red to white cells in the peripheral blood (CSF WBC (predicted) = CSF RBC $\times$ Blood WBC/Blood RBC) and concluded that CSF pleocytosis could go undetected using this calculation (Rubenstein and Yogev, 1985). This was highlighted by three patients with viral meningitis who had both a nontraumatic and traumatic lumbar puncture, in whom the calculation underestimated the number of CSF white cells in the traumatic sample, resulting in falsely normal corrected CSF results.

In contrast, the observed to predicted *ratio* of CSF white cells has been found to be helpful by some authors. Mayefesky et al. analyzed 720 traumatic lumbar puncture samples and confirmed a high proportion (55%) of false positive results by simply calculating the *adjusted* (observed—predicted) CSF white cell count to predict the presence of CSF pleocytosis (Mayefsky and Roghmann, 1987). In this study, an observed to predicted CSF white cell ratio greater than 10 was claimed to be both

a sensitive (88%) and specific (90%) indicator of bacterial meningitis. Bonadio et al. reported an observed to predicted CSF white cell ratio greater than 1 as 100% sensitive but only 62% specific for predicting meningitis (Bonadio et al., 1990). In a study of 57 patients, 12 of whom had culture proven meningitis, the observed to predicted ratio was significantly higher in the meningitis group (Mazor et al., 2003). Mazor et al. reported that the observed to predicted CSF white cell ratio in a non-infected group was less than 1 in 98% of cases and concluded that an observed to predicted ratio less than or equal to 0.01, a CSF white to red cell ratio less than or equal to 1:100, and the absence of pleocytosis were each highly specific with a high positive predictive value for predicating the absence of meningitis (Mazor et al., 2003).

It has been suggested that contamination of the CSF by less than 10,000 red cells $\times 10^6$/L does not influence the CSF white cell count (Osborne and Pizer, 1981). Moreover, although the observed to predicted CSF white cell ratio appears to offer better sensitivity and specificity for the diagnosis of bacterial meningitis than using the CSF white to red cell ratio alone, many authorities still believe that it is safer to interpret blood-contaminated CSF using the same criteria as a non-contaminated CSF (Mehl, 1986; Bonadio, 1988a; Bonadio et al., 1990).

6. Distinguishing Viral and Bacterial Meningitis on the Basis of CSF Findings

The "typical" CSF findings in meningitis described in many textbooks make it seem deceptively easy to distinguish viral, bacterial, and tuberculous meningitis (Figure 14.1.). These indicate that in viral meningitis, the CSF is clear, contains up to a few hundred lymphocytes with a normal to slightly elevated protein concentration. In contrast, in bacterial meningitis the CSF is turbid, contains thousands of neutrophils, with substantially increased protein and decreased glucose concentrations, together with a positive Gram stain. In tuberculous meningitis, the CSF contains hundreds of lymphocytes, with substantially increased protein and markedly decreased glucose concentrations, together with a positive Ziehl–Neelsen stain. However, these CSF profiles relate to established meningitis in the absence of prior antibiotics. In reality, although many studies demonstrate a statistically significant higher CSF white cell count and protein concentration, and lower glucose concentration in bacterial compared with aseptic meningitis, there is considerable overlap in these values, particularly between the CSF findings in viral and early bacterial meningitis.

6.1. "Scary Facts" About CSF Findings in Meningitis

The macroscopic appearance of CSF does not appear cloudy until there are between 200 and 500 white cells $\times 10^6$/L, and turbid until there are greater than 500 white cells $\times 10^6$/L.

Delays in the laboratory analysis of CSF can alter the cell count as a result of lysis in CSF, which is hypotonic. There is a progressive reduction in both neutrophils

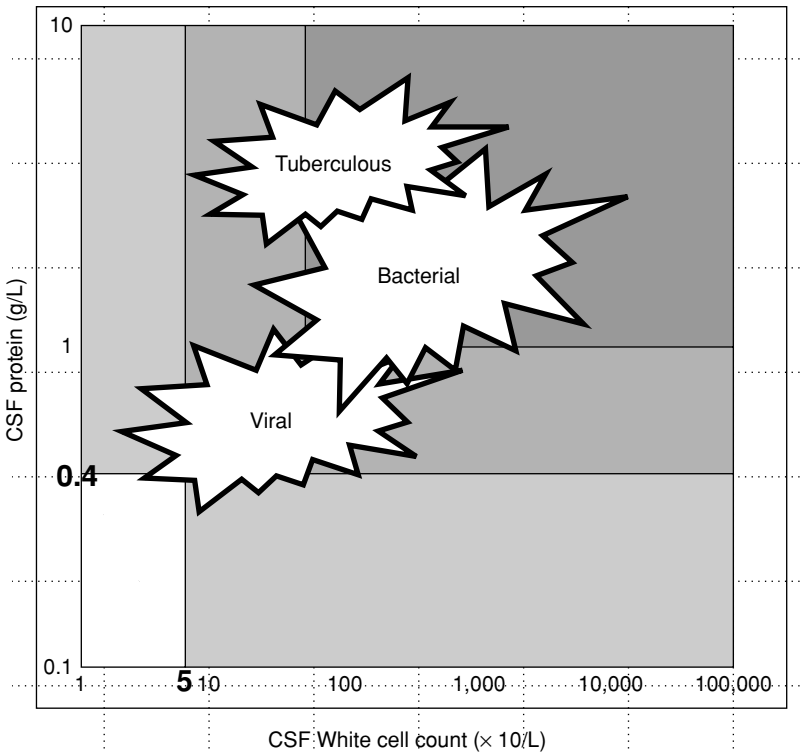

Figure 14.1. The "typical" CSF findings in viral, bacterial, and tuberculous meningitis.

and lymphocytes over a 4-hour period (Steele et al., 1986). The neutrophil count decreases by 33% at 1 hr and by 50% at 2 hr. For lymphocytes, the decrease is somewhat less with a 10% decrease at 2 hr.

CSF lymphocyte predominance in viral meningitis and neutrophil predominance in bacterial meningitis is not a constant finding. Whilst several studies have documented a predominance of neutrophils in early viral (particularly enteroviral) meningitis (Nye, 1983; Harrison and Risser, 1988; Greenlee, 1990), it is generally believed that there is a rapid shift from a neutrophil to a lymphocyte predominance over several hours (Feigin and Shackelford, 1973). A recent study of CSF findings during the enteroviral season over a 6-year period found that the majority of children with viral meningitis (57%) had a predominance of neutrophils in the CSF as expected (Negrini et al., 2000). However, in many cases a neutrophil predominance was still observed even when a lumbar puncture was performed up to 96 hr after the onset of symptoms, a finding supported by some earlier studies (Quick et al., 1965; Harrison and Risser, 1988). To add to the confusion, studies have demonstrated CSF lymphocytosis on initial evaluation in patients with bacterial meningitis (Powers, 1985; Bonadio, 1988a, b), although Bonadio reported this to be a rare event

(Bonadio, 1988a). CSF lymphocytosis is also recognized to occur in bacterial meningitis after antibiotic treatment (Dalton and Allison, 1968; Converse et al., 1973; Blazer et al., 1983).

An initial normal CSF does not exclude bacterial meningitis. CSF culture may subsequently be positive even when CSF white cell count, protein, glucose, and Gram stain are negative, particularly in the early stages of bacterial meningitis (Scheld et al.). A repeat lumbar puncture may therefore be needed (Moore and Ross, 1973; Bonadio, 1989).

Bacterial meningitis can occur in the absence of significant CSF pleocytosis (Moore and Ross, 1973; Onorato et al., 1980; Fishbein et al., 1981; Michael et al., 1986; Polk and Steele, 1987; Wong et al., 1989; Coll et al., 1994; Freedman et al., 2001), particularly in patients with sickle cell disease (Seeler et al., 1972; Rao et al., 1983).

The CSF glucose concentration is normal in 9% of cases of bacterial meningitis (Greenlee, 1990).

The CSF Gram stain is negative in up to 40% of cases of bacterial meningitis even without prior antibiotics (Marton and Gean, 1986; Hristeva et al., 1993).

Prior treatment with antibiotics decreases the sensitivity of CSF Gram stain by 20% and of culture by 30% (Dalton and Allison, 1968; Converse et al., 1973; Blazer et al., 1983). CSF culture becomes sterile in 90–100% of patients within 24–36 hr of antibiotic treatment (Dalton and Allison, 1968; Converse et al., 1973; Blazer et al., 1983; Bonadio et al., 1992).

6.2. CSF Scores and Algorithms

Given the overlap in CSF findings in viral and bacterial meningitis, several authors have recently added to the many previous attempts to create meningitis scores (Nigrovic et al., 2002), diagnostic models, and algorithms to differentiate between viral and bacterial meningitis based on CSF white cell count, protein, and glucose. Some of these scoring systems also include features such as age, season, peripheral blood neutrophil count, and glucose (Spanos et al., 1989; Hoen et al., 1995; Leblebicioglu et al., 1996). These models are designed to identify a group of patients who are at low risk of bacterial meningitis who do not need admission and antibiotics. The most recent attempt at using a model to discriminate patients with bacterial meningitis illustrates the difficulties with this strategy (Bonsu and Harper, 2004a, b). Firstly, all such models are a trade off between sensitivity and specificity. Inevitably, any incremental increase in specificity (i.e., a reduction in the number of patients with viral meningitis who are allocated incorrectly to the high-risk group ("false positives")) is associated with a decrease in sensitivity (i.e., an increase in the number of patients with bacterial meningitis who are allocated incorrectly to the low-risk group ("false negatives")). These models are able to improve upon the specificity and sensitivity offered by the use of one parameter alone but ultimately suffer the same problem that there is overlap between cases of viral and (usually early) bacterial meningitis. Secondly, these models are sometimes complex and require calibration, if not revalidation, in each setting in which they are to be used (Bonsu and Harper, 2004a, b).

6.3. Approach for Patients with Mild CSF Pleocytosis Only

The issue of bacterial meningitis in children with mild or no CSF pleocytosis has been addressed in a number of studies (Moore and Ross, 1973; Onorato et al., 1980; Fishbein et al., 1981; Michael et al., 1986; Polk and Steele, 1987; Wong et al., 1989; Coll et al., 1994; Freedman et al., 2001). Freedman et al. reported that 11% of 44 cases of bacterial meningitis had a CSF white cell count less than 3×10^6/L (Freedman et al., 2001). All of these children, however, had at least one other factor that indicated an increased risk of bacterial meningitis including age less than 1 year, increased FBE band count, increased CSF protein, or decreased CSF glucose. Freedman et al. offered an interesting and clear-thinking approach to the issue of CSF white cell count in influencing the probability of bacterial versus viral meningitis (Freedman et al., 2001). His study highlighted that, although bacterial meningitis can occur in patients with low CSF white cell counts, likelihood ratios can be determined that quantify the effect of CSF white cell magnitude on increasing or decreasing the post-test probability of bacterial meningitis. Of 1,469 patients (2 months–17 years) with a CSF white cell count less than or equal to 30×10^6/L, only 11 had bacterial meningitis. This was associated with a decrease in probability of bacterial meningitis from a pretest probability of 2.7% to a post-test probability of 0.7%. In contrast, bacterial meningitis was present in 33 out of the 148 patients with CSF white cell counts above 30, increasing the post-test probability to 22.2% (Frohna et al., 2001). The negative predictive value for a CSF white cell count of 30×10^6/L or less was 99.3%, and Freedman et al. concluded that children older than 6 months of age with CSF white cell counts in this range have a low risk of bacterial meningitis, provided other CSF parameters (protein, glucose, Gram stain) and peripheral blood band count are normal.

Interpretation of CSF results, whether evidence-based or statistically manipulated into algorithms, provides only one piece of information. Ultimately, distinguishing aseptic and bacterial meningitis depends on clinical evaluation in combination with CSF findings. The clinical picture should always remain foremost in the physician's decision process.

7. Alternative Methods for Distinguishing Bacterial and Viral Meningitis

7.1. Rapid Antigen Tests

Much has been reported in the literature about the detection of bacterial antigens by latex agglutination. This technique developed in an era when infection with *Haemophilus influenza* was common. Recent reports have highlighted the shortcomings of latex agglutination tests and many authorities believe that their low sensitivity and specificity preclude their use in clinical management decisions (Freedman et al., 2001; Tarafdar et al., 2001). More recently, it has been suggested that ultrasound-enhanced latex immunoagglutination has superior sensitivity and specificity, but this technique has yet to find widespread acceptance or use (Gray et al., 1999; Sobanski et al., 2002; Porritt et al., 2003).

7.2. Inflammatory Cytokines and Other Marker

Most markers of inflammation in the peripheral blood or CSF have proved unhelpful in distinguishing bacterial from viral meningitis. Peripheral blood C-reactive protein (CRP) has been suggested to be useful in distinguishing bacterial from viral meningitis (Clarke and Cost, 1983; De Beer et al., 1984; Sabel and Hanson, 1974). In a recent study, CRP was shown to have high sensitivity and specificity in a group of 55 Finnish children with an initial negative Gram stain (Sormunen et al., 1999). Two children, however, with culture positive meningitis had a normal CRP level at presentation, a finding reported in other studies (Petola and Jaakola.M, 1988; Gendrel et al., 1997; Gendrel et al., 1998; Gendrel et al., 1999).

Procalcitonin (PCT), a glycopeptide that is normally undetectable in the blood of healthy children, has been shown to increase in a variety of bacterial infections with minimal elevation in viral disease (Schwarz et al., 2000; Marc et al., 2002). Gendrel reported that an initial blood PCT level greater than 5 μg/L had a sensitivity of 94% and specificity of 100% for the diagnosis of bacterial meningitis (Gendrel et al., 1997). False negative results (normal PCT in confirmed bacterial meningitis) limit the usefulness of this marker as with the other markers described above.

Although the mean value of a wide range of other peripheral blood and CSF markers (e.g., lactate, lactic dehydrogenase, creatine phosphokinase, glutamic oxaloacetic transaminase, elastase-α proteinase inhibitor, defensin, lactoferrin, TNF-α, IL-1β, IL-6, IL-8, S-100β) are generally higher in patients with bacterial when compared with viral meningitis, there is sufficient overlap in the values between the groups for these tests to be of limited value in clinical practice (Rutledge et al., 1981; Lorino et al., 2000; Paul et al., 2003; Sato et al., 2003).

7.3. The Future: Molecular Testing

Molecular tests hold much greater promise. The use of PCR for the diagnosis of enterovirus (Ramers et al., 2000; Stellrecht et al., 2002; Guney et al., 2003; Archimbaud et al., 2004; Mohamed et al., 2004; Mulford et al., 2004), meningococcus (Saunders et al., 1997; Kotilainen et al., 1998; Seward and Towner, 2000; Taha, 2000; Bryant et al., 2004), and pneumococcus (Cherian et al., 1998; Corless et al., 2001) are already routine in many hospitals (Hall et al., 1995; Backman et al., 1999; Dicuonzo et al., 1999; Lu et al., 2000; Rantakokko-Jalava et al., 2000; Saravolatz et al., 2003). More recently, the reported high sensitivity and specificity (regardless of prior antibiotics) offered by the use of broad range 16S ribosomal DNA PCR points to the future for CSF analysis (Schuurman et al., 2004).

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15

Reverse Vaccinology and Vaccines for Serogroup B *Neisseria meningitidis*

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Abstract

Whole genome sequence data are increasingly available for a wide range of human pathogens. The use of bioinformatic tools allows the comprehensive *in silico* screening of genome data for surface-expressed proteins, in order to identify candidate vaccine antigens. *In vitro* confirmation of surface location and the use of animal models to test immunogenicity further refine the list of proteins likely to be of use as vaccine antigens. This process, first applied to serogroup B *Neisseria meningitidis*, has been termed as reverse vaccinology. Reverse vaccinology offers the ability to undertake a rapid and comprehensive assessment of a micro-organism's surface protein repertoire, and has advantages over conventional approaches to identifying candidate antigens. Despite the advantages of the approach, development in conventional areas of vaccinology remains important to support the process of producing vaccines from genome-derived antigens.

1. Reverse Vaccinology—An Introduction

In 1995, the genome sequencing of a single strain of *Haemophilus influenzae* type b was completed (Fleischmann et al., 1995). Nine years later, there are more than 170 bacterial genome sequences completed, many of which are human pathogens (www.genomesonline.org). The availability of large amounts of sequence data from pathogenic micro-organisms has been rapidly followed by attempts to determine the complete range and the function of proteins encoded within each genome. In the field of vaccinology, these efforts have focused on the surface-expressed proteins of micro-organisms, which are the targets of the immune response.

All vaccines in current use rely on the induction of antibody to protect against disease. Except for diphtheria and tetanus vaccines, where antibody is directed against pathogenic toxoid, vaccine-induced protective antibodies are directed against

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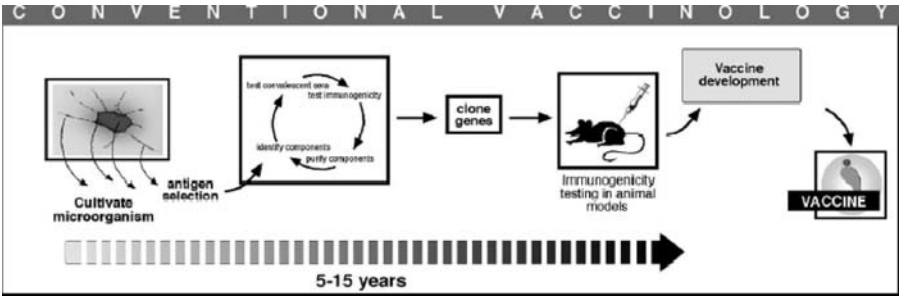

Figure 15.1. Schematic representation of the conventional approach to vaccine development. (Adu-Bobie et al. (2003). *Vaccine* **21**, 605–610.)

surface structures. Previous approaches to determining vaccine candidate antigens have been slow and, even after decades of research, known surface proteins represent a small fraction of those likely to be present. The identification of all the proteins potentially accessible to an antibody-mediated immune response is made possible by the availability of whole genomes of human pathogens. Reverse vaccinology is a process of deriving vaccines from the starting point of genomic sequence data (Capecchi et al., 2004; De Groot and Rappuoli, 2004). The process involves the following steps: bioinformatic software to screen genomes for surface-expressed proteins; high-throughput expression of these proteins and *in vitro* confirmation of their surface location; animal-based immunogenicity testing; finally, conventional human vaccine trials (Figures 15.1 and 15.2). This review outlines a practical example of reverse vaccinology, and discusses the advantages and limitations of the approach.

2. Serogroup B *N. meningitidis*

In 2000, the sequence of the genome of MC58, a serogroup B meningococcus, was completed (Tettelin et al., 2000). This was the first genome available for *N. meningitidis*, an important cause of invasive bacterial disease in childhood and adolescence. Whereas, effective polysaccharide-based vaccines are available for

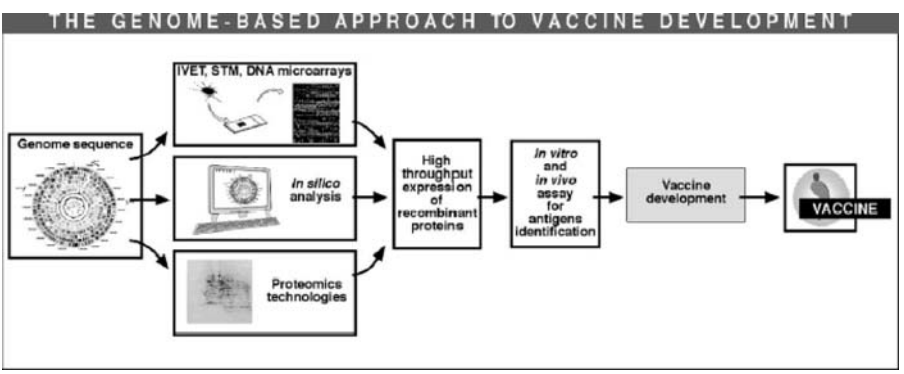

Figure 15.2. The genome-based approach to vaccine development.

pathogenic meningococci with serogroup A, C, Y, and W135 capsules, no such vaccine exists for serogroup B organisms (Morley and Pollard, 2001). Serogroup B meningococci are unusual in that their enveloping capsular polysaccharide is poorly immunogenic. The completed MC58 genome was rapidly followed by the first demonstration of the reverse vaccinology paradigm (Pizza et al., 2000). An initial screening of the MC58 genome identified open reading frames (ORFs) which potentially code for individual genes. Genes coding for known proteins with a cytoplasmic location was excluded from further analysis. The remaining ORFs were further analyzed with a variety of software tools for features predictive of typical surface proteins. Five hundred and seventy ORFs with predicted surface localization were identified from a total of over 2000 (Tettelin et al., 2000). PCR amplification of these ORFs, cloning, and expression in *Escherichia coli* were used to produce recombinant proteins which incorporated modifications such as His-tagging or glutathione S-transferase fusion. These modifications enabled subsequent recombinant protein purification. Three hundred and fifty proteins were produced from a total of 570 ORFs, and used to immunize mice, to produce sera with antibody specific for each protein. These sera were then used in enzyme-linked immunosorbent assays (ELISA) and fluorescence-activated cell sorting (FACS) with encapsulated and non-encapsulated meningococcal strains as antigen. The presence of specific antibody as determined by ELISA assay or fluorescent staining of organisms in FACS confirmed that a particular protein was exposed to interaction with the immune system on the meningococcal surface. In addition, the sera were tested for bactericidal activity. Seven proteins that gave positive results in ELISA, FACS, and bactericidal assays, and whose sequence was not predictive of phase variability, were chosen for further evaluation (see Table 15.1). None of these proteins had been identified prior to this study. Other candidate antigens continue to be identified by similar processes. These include NadA, an adhesin, and NarE, an ADP-ribosyltransferase with homology to bacterial toxins (Comanducci et al., 2002; Masignani et al., 2003). The latter was identified by its structural homology to previously described bacterial toxins.

Subsequent experimental work has further defined the potential utility of these antigens as vaccines. For example, GNA33 is a murein hydrolase, and present in all *Neisseria*. GNA33 deficient meningococcal mutants are unable to cause bacteraemia in animal models (Adu-Bobie et al., 2004). GNA33 antisera are bactericidal, and confer passive protection in infant rats (Granoff et al., 2001). NadA was the only one of the 94 genome-derived antigens to which there was an antibody response only in convalescent sera from patients who had had invasive meningococcal disease (Litt et al., 2004), while most of the other antigens had antibody also in healthy people, suggesting that normal colonization may induce the antibodies. An increased level of NadA antibody in adults and teens carrying meningococcus, as compared to children, suggests that meningococcal carriage may sustain specific NadA antibody levels in the population. However, NadA was present in only 50% of strains from cases of meningococcal disease, limiting its ability to provide comprehensive protection as an isolated vaccine antigen. It is hoped that further investigation of genome-derived antigens will lead to an efficacious vaccine against serogroup B meningococci.

Table 15.1. Properties of Seven Genome-Derived Antigens (GNA)

Antigen (length, amino acids)	Remarks/similarities[a]	FACS	ELISA	Serum bactericidal activity (SBA)
GNA33 (441)	Lipoprotein/similar to *E. coli* membrane-bound lytic transglycosylase A (MltA) of *E. coli* and of *Synechocystis* sp. (22)	++++[b]	13,000	1/16,000[c]
GNA992 (591)	Outer membrane protein/similar to Hsf and Hia of *Haemophilus influenzae* and FhaB of *Bordetella pertussis* (26)	+++	2,750	1/256
GNA1162 (215)	Lipoprotein/no significant similarities	++	1,270	1/4
GNA1220 (315)	Membrane protein/contains a stomatin-like domain	+++	1,000	1/256
GNA1946 (287)	Lipoprotein/similar to HlpA of *H. influenzae*, belongs to the NlpA family of lipoproteins (27)	+++	13,100	1/32
GNA2001 (251)	Outer membrane protein/similar to P60 invasion-associated extracellular proteins (28)	++	500	1/512
GNA2132 (488)	Lipoprotein/low similarity to transferrin binding proteins	++	1,700	1/16,000
GST[d]	–	−	<50	<1/4
OMV[d]	Mixture of proteins containing mainly PorA	++++	260,000	1/32,000

Source: Pizza, M., Scarlato, V., Masignani, V. et al. (2000). *Science* **287**(5459), 1816–1820.

[a]Homology searches have been performed against non-redundant protein databases by means of the Φ-BLAST algorithm (National Center for Biotechnology Information). Hits with an *E* score of $<10^{-20}$ and an assigned biological role are reported.

[b]Titers −, +, ++, +++, and ++++ indicate no difference with pre-immune serum or differences in fluorescence of 0.5, 1, 1.5, and 2 or more orders of magnitude, respectively.

[c]Serum bactericidal activity (SBA) was evaluated with pooled baby rabbit serum as complement source. Titers were expressed as the reciprocal of the serum dilution, yielding ≥50% bacterial killing.

[d]Sera against GST and OMV were used as negative and positive controls, respectively.

3. Advantages and Limitations

The reverse vaccinology approach provides an extremely rapid and relatively comprehensive method for the identification of large numbers of candidate vaccine antigens, once genomic sequence data are available. Application of this approach to the MC58 genome has already identified a significant number of candidate antigens, whose amino acid sequence appears relatively conserved across different meningococcal clones, in contrast to the variable nature of most antigens identified in 30 years of previous research (Pizza et al., 2000). Because of this success, reverse vaccinology is now being applied to the identification of candidate vaccine antigens in other bacteria including *Streptococcus pneumoniae* (Adamou et al., 2001; Wizemann et al., 2001), *Streptococcus agalactiae* (group B streptococcus; Tettelin et al., 2002), and *Staphylococcus aureus* (Etz et al., 2002). Reverse vaccinology approaches rely on

conventional vaccinology techniques, to progress from candidate antigen sequences toward vaccines based on these antigens. Given the large amount of data available from genome analysis, rapid progress from candidate antigens to vaccines will rely on developments in areas of vaccinology, such as recombinant protein expression, serological correlates of protection, molecular pathogenesis, and molecular epidemiology. These areas and their importance in reverse vaccinology are discussed below.

At present, the process of obtaining purified and refolded recombinant proteins from cloned PCR sequences is inefficient. For example, only 350 of 570 ORFs in *N meningitidis* MC58 were successfully produced, due to problems with protein folding and the toxicity of some proteins to the expression system. Limitations in the ability to express proteins result in potential candidate antigens being overlooked. Alternative expression systems have been considered (Poolman and Berthet, 2001).

As a large number of candidate vaccine antigens are generated, reverse vaccinology relies upon high-throughput methods for assessing antigen immunogenicity. For many organisms, there are no well-validated serological correlates of protective efficacy for protein antigens. Meningococcus is one of the few exceptions where bactericidal antibodies are known to correlate with protection. However, it is increasingly recognized that even sera with low bactericidal titers may confer protection in animal models, possibly due to opsonophagocytosis (Oliver et al., 2002; Welsch et al., 2003; Welsch and Granoff, 2004). A better understanding of the correlates of protection is needed, and multiple assays need to be considered, particularly for novel antigens.

The large quantity of information generated by the reverse vaccinology approach, in terms of genomic and proteomic data, together with high-throughput screening assays, can obscure the importance of understanding the *in vivo* biology of the organism, and the role of genome-derived antigens in natural immunity. A protein whose expression is limited to part of an organism's life-cycle that is irrelevant to human carriage or invasive disease, may be immunogenic and surface expressed but of little use as a vaccine. Alternatively, a protein expressed transiently at a stage of the life-cycle, critical for the process of invasion, may be an ideal target for a vaccine. Thus, studies of pathogenesis remain important to the interpretation of genomic data. In one example, micro-array gene expression studies of meningococci during contact with human epithelial cells identified 12 adhesion-induced surface antigens of which five elicited bactericidal antibodies when purified. These proteins had not been identified as vaccine candidates in the previous reverse vaccinology project (Grifantini et al., 2002).

Reverse vaccinology is predicated on the assumption that a protein surface antigen will provide an effective vaccine candidate. There are, however, no currently licensed vaccines for bacteria involving single-protein antigens. Immune-driven antigenic variation is one factor underlying the lack of vaccines based on this approach. A full understanding of a pathogen's population biology and comprehensive molecular epidemiological analyses are essential to assess the extent of antigenic diversity in a representative sample of isolates (Spratt and Maiden, 1999). For the MC58 genome-derived antigens, the extent of population diversity has been investigated. Of the seven proteins identified from the MC58 study, all were present, and five were $>99\%$ conserved amongst 22 serogroup B meningococci chosen to

represent the diversity of disease-causing strains. This is in contrast to the highly variable nature of many previously described meningococcal antigens such as PorA and FetA. The advent of MLST for the meningococcus has allowed the resolution of population diversity into a limited number of clonal complexes associated with invasive disease. This has been an important factor in permitting a rapid and comprehensive consideration of antigenic diversity for meningococcal genome-derived antigens (Maiden et al., 1998). For many other organisms to which the reverse vaccinology approach may be applied, the molecular epidemiology is not as well characterized. This will limit the ability to assess candidate vaccine antigens for these species. A recent development has been the observation that non-overlapping *combinations* of variable antigens structure the antigenic diversity within invasive clonal complexes of meningococci (Urwin et al., 2004). This provides an opportunity for the rational design of multiepitope vaccines when combined with the ability to screen a genome for large numbers of vaccine candidate antigens.

Finally, non-protein antigens (e.g., polysaccharide and lipo-polysaccharide (LPS)) are an important group of potential vaccine antigens not identified by reverse vaccinology. Their importance is emphasized by the fact that polysaccharide-based vaccines against serogroup A, C, Y, and W135 meningococci provide the only globally efficacious meningococcal vaccines. The genome of an organism encodes the enzymes necessary for the synthesis and transport of non-protein antigens. Homologues of enzymes known to synthesize non-protein antigens can be identified, and whilst not revealing antigenic structure directly, can inform structural biological investigation of these antigens (Cox et al., 2003). However, *de novo* identification of novel non-protein antigens via genomic methods would be more difficult, given the complexity of determining the nature and relationships of substrates solely from the sequences of their enzymes.

4. Conclusion

Reverse vaccinology has been a significant advance in the ability to rapidly identify novel candidate vaccine antigens for any organism for which there is a significant amount of genome sequence available. The data generated from this approach are providing a stimulus to development in more conventional areas of vaccinology. These areas include recombinant protein expression, serological correlates of protection, molecular pathogenesis, and molecular epidemiology. The full potential of reverse vaccinology will only be realized with increasing knowledge in these areas of research.

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