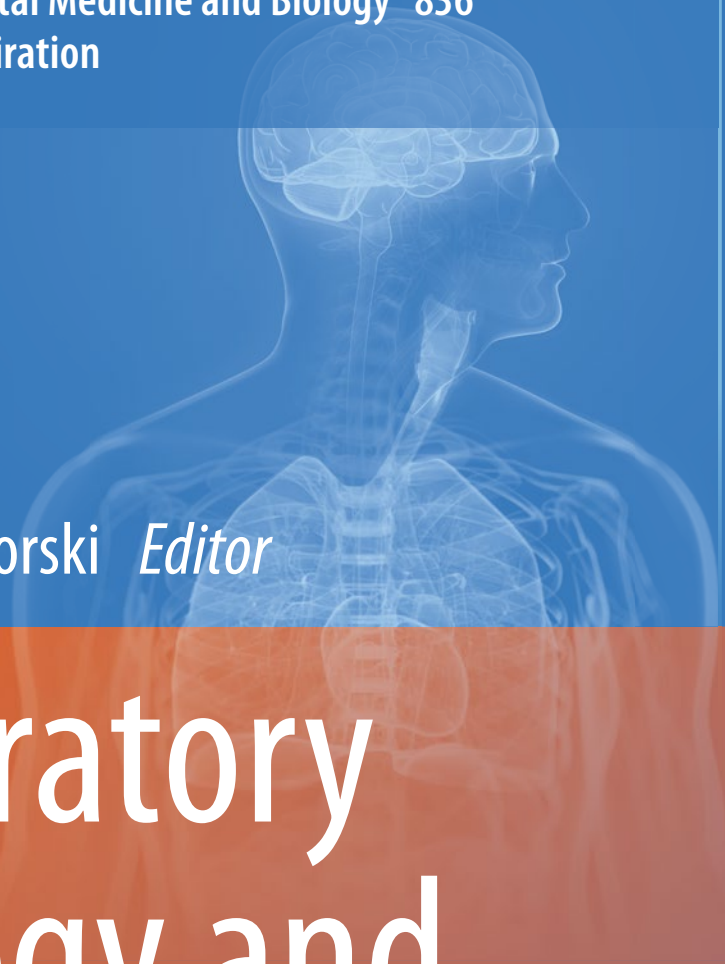
Advances in Experimental Medicine and Biology 836
Neuroscience and Respiration



Mieczyslaw Pokorski *Editor*

Respiratory Virology and Immunogenicity

Springer

Advances in Experimental Medicine and Biology

Neuroscience and Respiration

Volume 836

For further volumes:
http://www.springer.com/series/13457

Mieczyslaw Pokorski
Editor

Respiratory Virology and Immunogenicity

Springer

Editor
Mieczyslaw Pokorski
Institute of Psychology
University of Opole
Poland

ISSN 0065-2598 ISSN 2214-8019 (electronic)
ISBN 978-3-319-10017-3 ISBN 978-3-319-10018-0 (eBook)
DOI 10.1007/978-3-319-10018-0
Springer Cham Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014957141

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

This is a new book series entitled Neuroscience and Respiration, a subseries of Springer's renowned Advances in Experimental Medicine and Biology. The book volumes present contributions by expert researchers and clinicians in the field of pulmonary disorders. The chapters provide timely overviews of contentious issues or recent advances in the diagnosis, classification, and treatment of the entire range of pulmonary disorders, both acute and chronic. The texts are thought as a merger of basic and clinical research dealing with respiratory medicine, neural and chemical regulation of respiration, and the interactive relationship between respiration and other neurobiological systems such as cardiovascular function or the mind-to-body connection. In detail, topics include lung function, hypoxic lung pathologies, epidemiology of respiratory ailments, sleep-disordered breathing, imaging, and biomarkers. Other needful areas of interest are acute respiratory infections or chronic inflammatory conditions of the respiratory tract, exemplified by asthma and chronic obstructive pulmonary disease (COPD), or those underlain by still unknown factors, such as sarcoidosis, respiratory allergies, lung cancer, and autoimmune disorders involving the respiratory system.

The prominent experts will focus their presentations on the leading-edge therapeutic concepts, methodologies, and innovative treatments. Pharmacotherapy is always in the focus of respiratory research. The action and pharmacology of existing drugs and the development and evaluation of new agents are the heady area of research. Practical, data-driven options to manage patients will be considered. The chapters will present new research regarding older drugs, performed from a modern perspective or from a different pharmacotherapeutic angle. The introduction of new drugs and treatment approaches in both adults and children will be discussed. The problem of drug resistance, its spread, and deleterious consequences will be dealt with as well.

Lung ventilation is ultimately driven by the brain. However, neuropsychological aspects of respiratory disorders are still mostly a matter of conjecture. After decades of misunderstanding and neglect, emotions have been rediscovered as a powerful modifier or even the probable cause of various somatic disorders. Today, the link between stress and respiratory health is undeniable. Scientists accept a powerful psychological connection that can directly affect our quality of life and health span. Psychological approaches,

by decreasing stress, can play a major role in the development and course of respiratory disease, and the mind-body techniques can aid in their treatment.

Neuromolecular aspects relating to gene polymorphism and epigenesis, involving both heritable changes in the nucleotide sequence and functionally relevant changes to the genome that do not involve a change in the nucleotide sequence, leading to respiratory disorders will also be tackled. Clinical advances stemming from basic molecular and biochemical research are but possible if the research findings are "translated" into diagnostic tools, therapeutic procedures, and education, effectively reaching physicians and patients. All that cannot be achieved without a multidisciplinary, collaborative, "bench-to-bedside" approach involving both researchers and clinicians, which is the essence of the book series Neuroscience and Respiration.

The societal and economic burden of respiratory ailments has been on the rise worldwide leading to disabilities and shortening of life span. COPD alone causes more than three million deaths globally each year. Concerted efforts are required to improve this situation, and part of those efforts are gaining insights into the underlying mechanisms of disease and staying abreast with the latest developments in diagnosis and treatment regimens. It is hoped that the books published in this series will fulfill such a role by assuming a leading role in the field of respiratory medicine and research and will become a source of reference and inspiration for future research ideas.

Titles appearing in Neuroscience and Respiration will be assembled in a novel way in that chapters will first be published online to enhance their speedy visibility. Once there are enough chapters to form a book, the chapters will be assembled into complete volumes. At the end, I would like to express my deep gratitude to Mr. Martijn Roelandse and Ms. Tanja Koppejan from Springer's Life Sciences Department for their genuine interest in making this scientific endeavor come through and in the expert management of the production of this novel book series.

Opole, Poland Mieczyslaw Pokorski

Volume 5: Respiratory Virology and Immunogenicity

This volume provides a modern look on the age-old influenza infection and the preventive role of anti-influenza shots. Influenza pandemic outbreaks are unrelenting despite the growing understanding of the molecular basis of viral infection and its spreads. A leap in medical technologies has revolutionized the design of new influenza vaccines. The chapters cover vaccination strategies in various age groups of people and provide extensive information on the immune response to influenza vaccination in a spectrum of disease conditions.

Contents

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 5: 1–7
DOI 10.1007/5584_2014_12

Published online: 14 October 2014

Reactive Oxygen Species, Granulocytes, and NETosis

Magdalena Araźna, Michał P. Pruchniak, and Urszula Demkow

Abstract

When pathogens invade the body, neutrophils create the first line of defense. Basic weaponry consists of phagocytosis and degranulation, but these cells have yet another ace in the sleeve, a unique strategy in which invading microorganisms are being destroyed. These cellular warriors are able to release nuclear chromatin and form extracellular structure, known as neutrophil extracellular traps (NET). NET formation is connected with the presence of free radicals. Research has shown that inhibition of free radical formation leads to suppression of NET release. The exact mechanisms controlling cooperation of free radicals with NET still remain unclear. New investigations in this field may contribute to discovery of NET etiology and put a new light on related disorders.

Keywords

Free radicals • Myeloperoxidase • NADPH oxidase • Neutrophil extracellular traps • Reactive oxygen species

1 Introduction

Polymorphonuclear leukocytes have long been considered as the first line defense of the immune system. They are the most abundant fraction of white blood cells in mammals. Moreover, they possess enormous repertoire of abilities to be used against invading pathogens. Many studies have shown a contribution of these cells to inflammation and host defense. Especially, the mechanisms of neutrophil recruitment, phagocytosis, nicotinamide adenine dinucleotide phosphate (NADPH) oxidative burst, and toxic granule-dependent microbial killing have been elucidated (Pruchniak et al. 2013; Yipp and Kubes 2013). Recent years have brought new discoveries in the field of innate immunity. It has been described that neutrophils are able to generate neutrophil extracellular traps (NETs) from their nuclear DNA (Brinkmann et al. 2004; Takei et al. 1996; Tsan 1980). NETs kill pathogens with antimicrobial proteins such as neutrophil elastase and histones that are bound to the frame of this structure.

Recently, attention has been focused on the relation between reactive oxygen species (ROS)

M. Araźna (✉), M.P. Pruchniak, and U. Demkow
Department of Laboratory Diagnostics and Clinical Immunology of Developmental Age, 24 Marszalkowska St, 00-576 Warsaw, Poland
e-mail: magdaarazna@gmail.com

and NET's mechanisms. A consequence of NET formation is cell death called NETosis. NET formation and NETosis have been shown to involve NADPH oxidase (Nox2) activity and oxidative burst (Remijsen et al. 2011). Nox2 mediates superoxide generation by one electron reduction of molecular oxygen. Subsequently, superoxide is dismutated into hydrogen peroxide, which can be converted to hydroxyl radical by either Harber-Weis or Fenton reaction. Hydrogen peroxide is also metabolized to hypochlorite by myeloperoxidase (Imada et al. 1999). All of these mediators as well as enzymatic mechanisms have been examined in connection with the NET signaling cascade.

2 Influence of ROS on Proteins, Nucleic Acids, and Carbohydrates

Being highly active, ROS interact with virtually all cellular components, modifying their properties. They are able of cleavage of polypeptide chains and oxidation of side chains of amino acids. Therefore, ROS can modify proteins by formation of additional carbonyl groups (carbonylation), which are the most frequently used markers of free radical-induced modifications of polypeptide chains. Functional consequences of protein oxidation are involved with aging, adaptation to hypoxia, heat shock, and a spate of other pathologies (Lushchak 2007). Nucleic acids are more resistant to oxidation compared with lipids and proteins. Moreover, damage to nucleic acids caused by ROS is rapidly repaired. Nevertheless, hydroxyl radicals can affect double bonds of heterocyclic DNA bases and remove a hydrogen atom from the methyl group of thymine and from each of the C–H bonds of 2-deoxyribose.

The influence of ROS on lipids, proteins, nucleic acids, and carbohydrates develops an array of free radicals, which can generate new free radical and interact with cell signaling pathways. When a cell or tissue is bombarded with an excess of free radicals, oxidative stress occurs. This event is provoked by endogenous overproduction of free radicals, weakness of the cleansing system or another dysfunction of oxidative homeostasis. Oxidative stress often brings several harmful consequences to a cell or even entire tissue. The main consequences are the following:

- decreased ATP in a cell due to free radical-evoked inactivation of glycolysis by inhibition of glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$);
- elevation of cytosolic Ca^{2+} due to inactivation of the calcium pump;
- DNA damage as a result of hydroxyl radical performance or calcium-dependent nuclease overactivation. As a consequence of massive nucleic damage, NAD^+ ADP-ribosyltransferase is activated and the cellular pools of NAD^+ and adenine nucleotides are diminished;
- greater permeability of lipid bilayer caused by membrane depolarization induced by oxidative inhibition of the potassium pump;
- downregulation of intracellular glutathione and changes in the proportion of reduced to oxidized glutathione (GSH/GSSG).

As a final result of prolonged exposure to oxidative stress, cells undergo apoptosis or necrosis. There is also evidence that this process can affect NETosis of granulocytes (Nakaya et al. 1992).

3 ROS and Cell Death

Scheel-Toellner et al. (2004) showed a correlation between ROS formation and neutrophil apoptosis. Apoptosis, also known as programed cell death, occurs naturally in multicellular organisms and can be initiated by one of two distinct ways: extrinsic path, due to death receptor cascade or intrinsic path, connected with mitochondria. The apoptotic process is executed to get rid of unneeded, senescent, or abnormal cells. This event is especially important for neutrophils, because their apoptosis is part of a fundamental mechanism in which an adequate level of circulating neutrophilic granulocytes is maintained. In consequence, control upon the inflammatory response and prevention of chronic inflammatory diseases are maintained undisturbed. An altered redox state, accompanying reduction of glutathione, initiates ligand-independent death receptor signaling

leading to programed death of neutrophils (Scheel-Toellner et al. 2004). The assessment of cells morphology, detection of caspase-3 activity and the presence of bound annexin V may be used to estimate the frequency of apoptotic events in neutrophils. Programed cell death may occur spontaneously within 24–36 h *in vitro*. Some data show that activation of caspase-3 and caspase-8 plays a role in neutrophil apoptosis. An attempt has been undertaken to trace the kinetics of caspase-8 activation more precisely. The results of this investigation confirmed the role of caspase-8 in neutrophils apoptosis and showed that its activation is an early event preceding activation of caspase-3. Some authors highlighted that caspase-8 activation, followed by CD95 ligation, requires the translocation of acid sphingomyelinase to the cell membrane. That leads to the generation of ceramides; a rich lipid rafts which induce clustering of the death receptor signaling complex (DISC) and amplification of CD95 on lipid bilayer. In the end, caspase-8 is activated and the cell dies (Scheel-Toellner et al. 2004).

Sawada et al. (2001) have shown that ROS are responsible for neutral sphingomyelinase dislocation in cells undergoing apoptosis. Moreover, Qui et al. (2003) have proposed that oxidation of a C-terminal of cysteine residue can induce acid sphingomyelinase activation. Successful inhibition of neutrophil apoptosis after addition of N-acetylcysteine (NAC) or desferrioxamine (an inhibitor of hydroxyl radical generation, acting *via* Fenton reaction) confirms a crucial role of hydroxyl radical in the programed neutrophils death (Scheel-Toellner et al. 2004).

4 Free Radicals and Their Influence on NET Formation

Phorbol 12-myristate 13-acetate (PMA) is as well-known NETosis promoting agent which acts by protein kinase C activation. PMA cooperates with Nox2 during NETosis induction. This collaboration is imperative, as it has been shown by inhibition of Nox2 complex with diphenylene iodonium (DPI). In this experiment, DPI use resulted in complete inhibition of NET formation (Remijsen et al. 2011). Furthermore, neutrophils from individuals with chronic granulomatous disease, a hereditary dysfunction of NADPH oxidase and respiratory burst impairment, are unable to form NET. This observation may help explain increased susceptibility of these patients to recurrent life threatening bacterial and fungal infections. However, neutrophils from patients with chronic granulomatous disease undergo massive vacuolization and lose their mitochondrial potential after PMA stimulation. On the other hand, no intracellular chromatin decondensation is observed. This means that NET inhibition is associated with the lack of NADPH oxidase activity. Remijsen et al. (2011) have tried to explain neutrophils vacuolization after PMA treatment. The authors have noticed vesicles separated by double membranes; a feature not observed in the resting form. The presence of premature autophagosome structures was detectable within 15 min after stimulation. Fusion of autophagosomes with endosomes/lysosomes in the autophagolysosome has been observed after longer incubation of cells. Finally, about 80 min after PMA administration, a different type of vesicle with double phospholipid bilayer appeared. Analysis by electron microscopy show that these structures differed from autophagosomes. To compare them with autophagic vesicles, the authors checked the localization of microtubule-associated protein 1A/1B-light chain 3 (LC3), a marker of autophagy blebs. The distribution of LC3 in normal neutrophils and those from chronic granulomatous disease stimulated with PMA corresponded with the standard pattern of typical LC3 array during autophagy. The authors conclude that PMA may induce autophagy in a superoxide-independent manner. They have further shown that inhibition of both Nox2 activity and autophagy leads to a reduction of PMA-induced NET formation and promotes apoptosis just at the last stage. An inhibitor of autophagy, wortmannin (PI3K), did not affect superoxide production, but the chromatin of cells pretreated with PI3K did not decondense after PMA stimulation. The results show that inhibition of

autophagy or Nox2 activity prevents chromatin decondensation and leads to the inhibition of NET formation. The presence of apoptotic hallmarks in cells treated by these specific mediators may suggest that apoptosis is a backup program for NETosis, when autophagy or Nox2 activity is prevented (Remijsen et al. 2011).

Myeloperoxidase (MPO) is another important mediator for ROS production. It is a hemoprotein found in azurophilic granules of neutrophils. It promotes oxidative stress during inflammation by mechanisms involving production of hypochlorous acid (HOCl). Inhibition of this enzymatic complex takes place due to mutations and deletions in the gene encoding MPO. These changes are responsible for partial or complete deficiency of MPO (Hansson et al. 2006; Petrides 1998). So far, it is established that patients with chronic granulomatous disease fail to make NET. This dysfunction is caused by improper function of NADPH oxidase. Neutrophils from those patients do not generate superoxide, which dismutates to hydrogen peroxide, and becomes the substrate for MPO (Metzler et al. 2011). Nevertheless, NET formation can be restored by addition of exogenous hydrogen peroxide to the cell environment. Metzler et al. (2011) have studied whether alterations in the subsequent steps of superoxide generation can influence the NET release. Neutrophils from unrelated MPO-deficient donors were collected. These cells, along with cells isolated from healthy volunteers, were stimulated with PMA. After incubation, neutrophils from healthy donors released NETs. Neutrophils from partially deficient MPO donors also made these structures. However, neutrophilic granulocytes from patients completely deficient in MPO did not form the traps. When aminobenzoic acid hydrazide (ABAH), an inhibitor of the MPO complex, was used, NET formation was blocked in most cells. The proportion of ABAH-treated cells forming NETs was higher after a longer incubation time. These results show that ABAH did not completely abrogate the MPO activity and even a low level of MPO is sufficient for NET formation. Therefore, partially MPO-deficient neutrophils are able to release NETs, while completely MPO-deficient cells do not make NETs in response to PMA. The authors have also tried to rescue the NET formation in the MPO-deficient neutrophils by addition of extracellular products of this enzyme, but none of the tested: histamines, monochloramine, or dichloramine promoted the NET formation.

Papayannopoulos et al. (2010) have also analyzed the role of MPO in the NET formation. The experiment was designed to test whether MPO promotes nuclear decondensation. The results show that MPO encouraged chromatin relaxation in a dose-dependent manner and this effect was not inhibited by ABAH. MPO alone had an insignificant effect on chromatin decondensation, but the effect was amplified by neutrophil elastase. In the presence of both enzymes, NET formation increases dramatically.

Neutrophil elastase is another enzymatic protein which is abundant in the NET structure. The enzyme is stored in azurophilic granules and contributes to the pathogens destruction. Papayannopoulos et al. (2010) have observed blockade of chromatin decondensation after inhibition of neutrophil elastase by two inhibitors (GW311616A, an neutrophil elastase and serum leukocyte protease inhibitor, and SLPI, a secretory leukocyte protease inhibitor). The authors suggest that neutrophil elastase can degrade histones and in this way it promotes nuclear decondensation. They also show that MPO alone does not affect histone degradation. The influence of NADPH and mitochondria-derived ROS, including the roles of superoxide dismutase (SOD) and MPO on the NET formation, has been demonstrated by Kirchner et al. (2012). To inhibit the ROS production at various steps, they used DPI (Nox2 inhibitor), dipyrone hydrate, 4–dimethylaminoantipyrine (aminopyrine, an inhibitor of MPO), diethyldithiocarbamic acid (DETC), and aroclor (an inhibitor of SOD). To inhibit the ROS production in mitochondria, the electron transport inhibitor rotenone and the uncoupling chain reagents 2,4-dinitrophenol, carbonyl cynunide p-[trifluoromethoxy], and phenyl-hydrosome (FCCP) were used. The intracellular ROS formation, assessed by flow cytometry, and the sum of

intra- and extracellular ROS, assessed by luminal-amplified chemiluminescence, and NET generation, assessed by the fluorescence measurement of DNA, were evaluated. The authors have examined the influence on neutrophils of the inhibitors. None have appeared toxic or induced apoptosis. The cells did not demonstrate the hallmarks of apoptosis or necrosis, even after 18 h of incubation (Kirchner et al. 2012). All the inhibitors were tested for their efficiency to reduce the PMA-induced ROS production and NET formation. DPI, aipyrone, and aminopyrine (MPO inhibitors), decreased the intracellular ROS production. The inhibitors of SOD did not show any such effect. Moreover, FCCP and dinitrophenol significantly reduced ROS generation. The other inhibitors did not exert an appreciable effect on ROS production. Finally, using the luminol-amplified chemiluminescence, the authors confirmed that DPI nearly completely blocks the ROS production. The MPO inhibitors did not inhibit lucigenin oxidation, which is consistent with the lack of effect of these substances on Nox2 function. FCCP and dinitrophenol did not inhibit PMA-induced ROS generation either. From the two tested SOD inhibitors, only aroclor showed a significant inhibition of ROS. Taken together, the above-mentioned study confirms that mitochondrial ROS do not play a major role in the PMA-induced NET release.

Enzymes involved in the ROS production have a pivotal role in the process of NET formation. Activation of NADPH oxidase and superoxide constitutes the first step in the machinery producing ROS. Nishinaka et al. (2011) have shown that PMA-stimulated neutrophils isolated from healthy volunteers, but not from chronic granulomatous disease patients, produce oxygen and superoxide anion. Addition of singlet oxygen scavengers, like edaravone or α-phenyl-N-tert-butyl nitrone (PBN), reduced the singlet oxygen production, but the superoxide and NET formation was not inhibited. The study shows that singlet oxygen directly induces the NET release also in neutrophils from patients with chronic granulomatous disease. It is probable that singlet oxygen donors, independent of Nox2 activation, may induce the NET formation in these patients (Nishinaka et al. 2011). Some authors argue that HOCl stimulates the NET release. Palmer et al. (2012) have confirmed that inhibition of MPO by ABAH reduces the NET release, but when HOCl is added to the culture medium, neutrophils are able to form NET. This effect is observed in both healthy subjects and in patients with chronic granulomatous disease. The authors show that direct neutrophil exposure to 0.75 mM HOCl results in the formation of NET structures between 30 and 70 min. Akong-Moore et al. (2012) have also indicated that HOCl is a key ROS involved in human NETosis. These discoveries are helpful in the understanding why innate immune functions are disturbed in the course of a disease with irregular homeostasis of chloride ion, such as cystic fibrosis.

Extensive studies have been dedicated to ROS participation in NET formation; both these elements being present in the circulation to limit the spread of infection. Additionally, nitric oxide (NO) is a molecule, playing an important role in inflammatory conditions. NO is a modulator of free radical generation in human neutrophils. Patel et al. (2010) have investigated the role of NO in NET production. To stimulate neutrophils they used NO donors, such as sodium nitroprusside (SNP), S-nitroso-N-acetyl-penicillamine (SNAP), or PMA. The effect of various inhibitors (NAC, DPI, ABAH, or 7-nitroindazole (7-NI)) also was examined. NET formation was monitored by confocal and scanning electron microscopy, and by a quantitative estimation of extracellular DNA. Generation of free radicals after addition of NO donors was assessed by confocal microscopy, flow cytometry, MPO activity fluorescent measurement, and by Western blots. Neutrophils incubated with SNP and SNAP generated NETs in a concentration- and time-dependent manner. NETs release induced by SNAP was blocked by NAC suggesting the involvement of free radicals in this process. To investigate the role of NADPH oxidase, migration of $p47^{phox}$, a cytosolic component of this enzyme, was monitored. After PMA, migration of $p47^{phox}$ was observed within 1–3 h. The SNAP-treated cells also exhibited the migration of this particle up to

3 h. These results show that NADPH oxidase is involved in NO-mediated free radical generation and formation of neutrophil extracellular traps.

Production of free radicals and their influence on NET formation may have a significant clinical value. Keshari et al. (2012) have investigated ROS and NET formation in the systemic inflammatory response syndrome (SIRS) patients. Due to high expressions of inflammatory cytokines, like IL-1β, TNF-α, and IL-8, production of ROS in these patients was enhanced. Moreover, a significant augmentation in the circulating MPO activity and DNA content was observed. The above outlined mechanisms may enhance the generation of free radicals and NETs in SIRS patients.

Marcos et al. (2010) have described NET formation independent of NADPH oxidase in cystic fibrosis airway inflammation. They explored participation of G protein-coupled receptor CXCR2 in the mediation of NET release. Pulmonary blockade of CXCR2 by airway delivery of small molecule antagonists inhibited the NET formation and improved lung function; the process required the involvement of Src family kinases.

5 Conclusions

Despite recent progress in studies on NET formation, the exact mechanisms of this form of the innate immune response, including the role of reactive oxygen species, are far from being fully elucidated. The resolution of the NET issue would undoubtedly lead to a better understanding of, and therapeutic advances in combating, NET-related diseases.

Conflicts of Interest The authors declare no conflicts of interest in relation this article.

References

Akong-Moore K, Chow OA, von Köckritz-Blickwede M, Nizet V (2012) Influences of chloride and hypochlorite on neutrophil extracellular trap formation. PLoS One 7:e42984. doi:10.1371/journal.pone.0042984

Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A (2004) Neutrophil extracellular traps kill bacteria. Science 303:1532–1535

Hansson M, Olsson I, Nauseef WM (2006) Biosynthesis, processing, and sorting of human myeloperoxidase. Arch Biochem Biophys 445:214–224

Imada I, Sato EF, Miyamoto M, Ichimori Y, Minamiyama Y, Konaka R, Inoue M (1999) Analysis of reactive oxygen species generated by neutrophils using a chemiluminescence probe L-012. Anal Biochem 271:53–58

Keshari RS, Jyoti A, Dubey M, Kothari N, Kohli M, Bogra J, Barthwal MK, Dikshit M (2012) Cytokines induced neutrophil extracellular traps formation: implication for the inflammatory disease condition. Clin Exp Immunol 168:153–163

Kirchner T, Möller S, Klinger M, Solbach W, Laskay T, Behnen M (2012) The impact of various reactive oxygen species on the formation of neutrophil extracellular traps. Mediators Inflamm 2012:1615–1623

Lushchak VI (2007) Free radical oxidation of proteins and its relationship with functional state of organisms. Biochem Mosc 72:809–827

Marcos V, Zhou Z, Yildirim AO, Bohla A, Hector A, Vitkov L, Wiedenbauer EM, Krautgartner WD, Stoiber W, Belohradsky BH, Reiber N, Kormann M, Koller B, Roscher A, Roos D, Griese M, Eickelberg O, Doring G, Mall MA, Harti D (2010) CXCR2 mediates NADPH oxidase-independent neutrophil extracellular trap formation in cystic fibrosis airway inflammation. Nat Med 16:1018–1023

Metzler KD, Fuchs TA, Nauseef WM, Reumaux D, Roesler J, Schulze I, Wahn V, Papayannopoulos V, Zychlinsky A (2011) Myeloperoxidae is required for neutrophil extracellular trap formation: implications for innate immunity. Blood 117:953–959

Nakaya H, Takeda Y, Tohse N, Kanno M (1992) Mechanism of the membrane depolarization induced by oxidative stress in guinea-pig ventricular cells. J Mol Cell Cardiol 24:523–534

Nishinaka Y, Arai T, Adachi S, Takaori–Konodo A, Yamashita K (2011) Singlet oxygen is essential for neutrophil extracellular trap formation. Biochem Biophys Res Commun 413:75–79

Palmer LJ, Cooper PR, Ling MR, Wright HJ, Huissoon A, Chapple IL (2012) Hypochlorous acid regulates neutrophil extracellular trap release in humans. Clin Exp Immunol 167:261–268

Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A (2010) Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J Cell Biol 191:677–691

Patel S, Kumar S, Jyoti A, Srinag BS, Keshari RS, Saluja R, Verma A, Mitra K, Barthwal MK, Krishnamurthy H, Bajpai VK, Dikshit M (2010) Nitric

oxide donors release extracellular traps from human neutrophils by augmenting free radical generation. Nitric Oxide 22:226–234

Petrides PE (1998) Molecular genetics of peroxidase deficiency. J Mol Med 76:688–698

Pruchniak MP, Arazna M, Demkow U (2013) Life of neutrophil: from stem cell to neutrophil extracellular trap. Respir Physiol Neurobiol 187:68–73

Qui H, Edmunds T, Baker-Malcolm J, Karey KP, Estes S (2003) Activation of human acid sphingomyelinase through modification or deletion of C-terminal cysteine. J Biol Chem 278:32744–32752

Remijsen Q, Berghe TV, Wirawan E, Asselbergh B, Parthoens E, De Rycke R, Noppen S, Delforge M, Willems J, Vandenabeele P (2011) Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. Cell Res 21:290–304

Sawada M, Nakashima S, Kiyono T, Nakagawa M, Yamada J, Yamakawa H, Banno Y, Shinoda J, Nishimura Y, Nozawa Y, Sakai N (2001) p53 regulates ceramide formation by neutral sphingomyelinase through reactive oxygen species in human glioma cells. Cell Death Differ 11:853–861

Scheel-Toellner D, Wang K, Craddock R, Webb PR, McGettrick HM, Assi LK, Parkes N, Clough LE, Gulbins E, Salmon M, Lord JM (2004) Reactive oxygen species limit neutrophil life span by activating death receptor signaling. Blood 104:2557–2564

Takei H, Araki A, Watanabe H, Ichinose A, Sendo F (1996) Rapid killing of human neutrophils by the potent activator phorbol 12-myristate 13-acetate (PMA) accompanied by changes different from typical apoptosis or necrosis. J Leukoc Biol 59:229–240

Tsan MF (1980) Phorbol myristate acetate induced neutrophil autotoxicity. J Cell Physiol 105:327–334

Yipp BG, Kubes P (2013) Netosis: how vital is it? Blood 122:2784–2794

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 5: 9–17
DOI 10.1007/5584_2014_37

Published online: 25 September 2014

Immunizations Against Respiratory Infections in Children in Primary Health Care in Poland: Coverage and Delays

K. Miśkiewicz, E. Kuchar, A. Nitsch-Osuch, K. Preisner, and L. Szenborn

Abstract

Pneumococcal infections, pertussis, and influenza are vaccine-preventable diseases. The aim of this study was to determine vaccine coverage and compliance with the dosage regimen among children in Poland. We performed a retrospective chart analysis of 1,356 children in a large primary healthcare establishment. The complete primary pertussis vaccination, 3 doses in the first year of life, was administered to 1,310/1,356 patients (96.6 %). The self-paid combined acellular vaccine was given in 55.2 % of children. The first dose of the pertussis vaccine was administered in a timely manner to 67.1 % of children. The self–paid pneumococcal vaccine was administered in 499/1,356 (36.8 %) children. In 46.1 % of them immunization started within the first 6 months of life; in 12.6 % aged 7–11 months, in 12.6 % aged 12–23 months, and in 28.7 % aged over 24 months. The dosage regimen was compliant in 49.2 % of patients. Only 3.5 % of patients were immunized against both *pneumococci* and influenza. Compliance with the Polish immunization program should be increased by reducing the number of injections and the cost of vaccines. Education is essential to facilitate simultaneous administration of vaccines during one visit and to prepare the parents for judicious decision-making when it comes to vaccinations.

Keywords

Combination vaccines • Flu • Prevention • Public health • Risk

K. Miśkiewicz, E. Kuchar (✉), K. Preisner, and L. Szenborn
Department of Pediatric Infectious Diseases, Medical University of Wrocław, 44 Bujwida St., Wroclaw, Poland
e-mail: ernest.kuchar@gmail.com

A. Nitsch-Osuch
Department of Family Medicine, Warsaw Medical University, Warsaw, Poland

1 Introduction

Pertussis, influenza, and pneumococcal disease are vaccine-preventable respiratory tract infections in children. Pertussis is a respiratory illness caused by Gram negative bacteria *Bordetella pertussis* and characterized by

paroxysmal coughing, inspiratory dyspnea (whooping cough in children), and a prolonged clinical course. The disease in most serious for newborns and young babies. The mortality rate peaks in infants younger than 6 months old, especially newborns. Infants too young to be fully immunized (<6 months old) are the most affected. Immunization-related risk factors include lack of vaccination, incomplete course of vaccinations, delayed vaccination, and increased time since last pertussis vaccination, because immunity following natural infection and vaccination wanes over time. Pertussis occurs six times more often in children exempt from the vaccination than in immunized children (Feikin et al. 2000). An incomplete course of vaccinations is a risk factor since at least 2 doses of the vaccine are needed for protection. Next, delayed immunization is associated with an increased risk of hospital admission for pertussis in infants (Grant et al. 2003). Another risk factor is exposure to contact with *Bordetella pertussis* and household members appear to be most common source of pertussis infection for infants (Wendelboe et al. 2007).

Children under five are the population group most likely to contract influenza, with an incidence rate greater than in the elderly. Influenza is a serious infectious disease for children especially for those under 2 years of age, who are at most risk (Poehling et al. 2006). Healthy infants and children under 2 years are hospitalized for influenza at similar rates to adults in high-risk groups (Neuzil et al. 2000). Infants are the most likely to develop serious complications such as pneumonia and secondary bacterial infections (Neuzil et al. 2002). Universal vaccination of healthy children is not widespread in Europe, despite clear demonstration of the benefits of vaccination in reducing the large health and economic burden of influenza (Cohen et al. 2011). The inactivated seasonal influenza vaccine, the only one available in Poland, can be used for any person >6 months old without contraindications (e.g., history of severe allergic reaction to any element in the vaccine). According to the updated recommendations on immunization of the Advisory Committee on Immunization Practices of the National Center for Immunization and Respiratory Diseases (ACIP 2011), all children aged between 6 months and 8 years should receive 2 doses (≥4 weeks apart), instead of 1 dose, of the seasonal influenza vaccine. The seasonal influenza vaccine was proven to be highly effective for reducing laboratory-confirmed influenza in healthy children over 2 years old, but modestly effective for reducing flu-like illness (Jefferson et al. 2012). Immunization of children in daycare may reduce morbidity among household contacts (Hurwitz et al. 2000).

Streptococcus pneumoniae or *pneumococci* are Gram-positive, anaerobic bacteria recognized as a major cause of community-acquired pneumonia, otitis media, and a significant proportion of bacteremia and bacterial meningitis in humans (Verma and Khanna 2012). As *pneumococci* are responsible for 17–37 % of pneumonia, they are the most common causes of pneumonia in otherwise healthy children aged 2–59 months (Grant et al. 2009). The highest pneumococcal disease hospitalization rates (>200 per 100,000 person-years) were reported in children under 2 years following a preterm birth, a low birth weight, a low 5-min Apgar score, or birth defects (Mahon et al. 2007). *Pneumococci* lead to death of approximately one million children under the age of five every year: the vast majority in developing countries (Verma and Khanna 2012).

All children aged 2–59 months should be immunized with the pneumococcal conjugated vaccine. The World Health Organization recommends worldwide vaccination against childhood pneumococcal disease with a 13-valent (PCV13) or 10-valent (PCV10) pneumococcal conjugate vaccine. The dosage regimen is age-related with the preferred regimen consisting of 3 primary doses (3 + 0 schedule) for optimal protection. The pneumococcal immunization should begin at 6 weeks of age and be given with an interval of 4–8 weeks between doses. Alternatively, 2 primary doses plus booster (2 + 1 schedule) can begin at age 6 weeks with a second dose after at least 8 weeks for younger infants or 4–8 weeks later for infants aged ≥7 months; a booster should be given between the ages of 9–15 months.

The aim of our study was to present the coverage of vaccination against the most significant respiratory tract infections (pertussis, pneumococcal infections, and influenza) in children in Poland to assess the use of these feasible interventions to protect children and the impact of the cost of vaccines – the mandatory pertussis immunization is available free of charge, while influenza and conjugated pneumococcal immunizations are self-paid.

2 Methods

The study was approved by a local Ethics Committee. We analyzed the immunization charts of 1,356 children aged between 1 month and 18 years. All children were patients at a large primary healthcare establishment in a southern city of Wroclaw, Lower Silesia, Poland. Vaccinations against pertussis, pneumococcal infections, and influenza were taken into account. In the case of the pertussis vaccination, we assessed the coverage of the complete primary vaccination (3 doses in the first year of life) and booster doses (in the second and sixth year of life). We assessed the age at which each dose was given and the time delay between the doses. Based on these results, we calculated the percentage of doses given in a timely manner according to the Polish National Immunization Program (Chief Sanitary Inspector 2012). We also analyzed the type of vaccine (whole-cellular or acellular). In the case of vaccination against *Streptococcus pneumoniae*, we analyzed the immunization coverage and the number of doses given. We also assessed the time the vaccination program started and compliance of the dosage regimen with the Polish National Immunization Program and guidelines from the vaccine's producers. Patients immunized against pneumococcal infections were assigned to one of the four groups according to the age at which they were immunized (first 6 months of life, 7–11th month of life, 12–23rd month of life, and 24th month of life or later). A correct schedule means the proper dosage regimen, number of doses, age at which each dose is given and intervals between the doses. In the case of the influenza vaccination, we assessed the number of patients immunized once and more than once in their lifetime. Adherence to the correct schedule, which means the administration of two doses separated by 4 weeks in previously non-immunized children, was also analyzed. To evaluate the age and number of patients immunized each year, the patients were assigned to the following age groups: 0 to <12 months, ≥ 12 to <24 months, ≥ 2 to <5 years, and >5 years. We also assessed the number of children immunized against both influenza and pneumococcal infections. Data were presented as means $\pm$ SD and 95 % confidence intervals (CI). Calculations were performed using Statistica ver. 10.

3 Results

3.1 Pertussis Vaccination

Pertussis immunization coverage was high in our study group, 1,337/1,356 patients (98.6 %) received at least one dose of a vaccine. The complete primary vaccination (3 doses in the first year of life) was administered to 1,310/1,356 patients (96.6 %). Three doses in the first year of life and a fourth, a booster dose, in the second year, was administered to 1,152 patients (87.9 %), but in 91/1,310 children (7.0 %) the booster dose was not given due to a young age, so vaccine coverage might have approached 94.9 %. In the remaining 67 patients, the fourth dose was not given or delayed for an unknown reason. The first dose of pertussis vaccine was administered in a timely manner (i.e., within the second month of life) in only 910/1,337 of children (67.1 %). The mean age at which the first dose was given amounted to 2.1 $\pm$ 1.5 months (range: 0.8–24.5 months). The average delay in the first dose was 1.5 $\pm$ 2.4 months (range: 0.1–24.3 months), median: 0.8 months. The second dose was administered at the 3–4th month of life, according to the Polish National Immunization Program, in 988/1,330 of children (72.9 %), with the mean age being 3.9 $\pm$ 2.1 months (range:

2.0–49.7 months). The mean delay of the second dose was 1.8 ± 3.5 months, ranging 0.03–45.7 months, median: 0.8 months. One of the patients took the second dose of the vaccine earlier than recommended. The third dose was given at the 5–6th month of life in 985/1,310 children (72.6 %). The mean age at which this dose was given was 5.9 ± 2.7 months (range: 3.6–52.7 months) and the average delay of the dose was 2.6 ± 4.4 months (range: 0.7–46.7 months), median: 1.1 months. The third dose was given earlier than recommended in 7/1,310 children (0.5 %). The fourth dose was given at the 16–18th month of life in 408/1,152 patients (35.4 %) and the mean age at which the dose was given was 20.1 ± 5.4 months (range: 5.4–90.6 months). The mean delay of the fourth dose was 3.8 ± 6.1 months (range: 0.3–6.1 months), median: 2.0 months, while in 22/1,152 patients (1.9 %) the dose was given earlier than recommended. The second booster dose, i.e., the fifth dose of the vaccine was administered in the 6th year of life in 347/528 children (65.7 %). The mean age at which this dose was given was 5.2 ± 0.6 years (range: 4.3–11.1 years) and the average delay was 0.8 ± 1.1 years, ranging: 0.03–5.1 years, median: 0.4 years. In 145/528 children (27.5 %) the fifth dose was given earlier than recommended. In general, all three doses of the primary vaccination were given according to recommendations in 779/1,310 children (59.5 %).

The average age at which the whole-cellular or acellular vaccine was given did not differ significantly: the first dose of DTP was administered at the age of 64 *vs*. 64 days; the second dose at the age of 118 *vs*. 117 days, the third dose at the age of 178 *vs*. 174 days and the fourth dose at the age of 609 *vs*. 598 days, respectively, all $P>0.05$.

The majority of the patients having received all three doses of the vaccination (748/1,310; 55.2 %) were given all the doses of acellular vaccine, which is still not subsidized in Poland, with the exception of some risk groups (preterm infants, infants with a low birth weight, and children with chronic neurological disorders). The complete whole-cellular vaccine course was administered in 495/1,310 patients (36.5 %) and in 67/1,310 (4.9 %) patients both vaccines were used – some whole-cellular doses and some acellular vaccine doses.

3.2 Pneumococcal Vaccination

Pneumococcal vaccine was administered to 499 children (36.8 %). In most of the patients (230/499; 46.1 %) immunization started in the first 6 months of life as recommended, in 63/499 patients (12.6 %) at age 7–11 months, in 63/499 children (12.6 %) at age 12–23 months, and in 143 patients (28.7 %) at age 24 months or over. The mean age at which the first dose was given was 15.4 ± 18.5 months, the second dose – 8.0 ± 6.5 months, the third dose – 9.0 ± 6.0 months, and the fourth dose – 18.8 ± 4.1 months.

In the group of patients, who started the vaccination course in the first 6 months of life (between the 6th week and the 6th month of life), only 122/230 children (53.0 %) were given all the recommended four doses. Three doses of pneumococcal vaccine were given in the first year of life in 206/230 (89.6 %) children. The 4th dose, which should have been given between the 11–15th month of life, was administered in a timely manner in only 16/122 (13.1 %) children, who were given all four doses. Thus, only 16/230 children (7.0 %) in this group were vaccinated according to the correct vaccination dosage regimen based on the producer's recommendations.

In the group of children who started their pneumococcal immunization between the 7–11th month of life, 46/63 (73.0 %) received all three recommended doses. Two doses in the first year of life were administered in 54/63 patients (85.7 %). The third dose was given in a timely manner (i.e., in the second year of life) in 34/46 patients (73.9 %) who were given all three doses. The vaccination schedule was correct in only 4/63 patients (6.4 %).

In the group of children starting their vaccination between the 12–23rd month of life, the majority (58/63, 92.1 %) received two recommended doses and the vast majority,

Table 1 Influenza immunizations in children from different age groups in the period 1996–2012

Year of vaccination	Number of vaccinated patients	6 to <12 mo (%)	≥12 to <24 mo (%)	≥2 to <5 yr (%)	≥5 yr (%)
1996	1	1 (100)	–	–	–
1997	2	1 (50.0)	–	1 (50.0)	–
1998	2	–	1 (50.0)	1 (50.0)	–
1999	3	–	–	3 (100)	–
2000	3	–	–	1 (33.3)	2 (66.7)
2001	6	–	–	3 (50.0)	3 (50.0)
2002	6	–	–	1 (16.7)	5 (83.3)
2003	16	1 (6.3)	–	4 (25.0)	11 (68.8)
2004	7	–	–	1 (14.3)	6 (85.7)
2005	20	1 (5.0)	1 (5.0)	3 (15.0)	15 (75.0)
2006	14	2 (14.3)	4 (28.6)	2 (14.3)	6 (42.9)
2007	22	1 (4.6)	6 (27.3)	3 (13.6)	12 (54.6)
2008	4	–	2 (50.0)	1 (25.0)	1 (25.0)
2009	31	1 (3.2)	2 (6.5)	16 (51.6)	12 (38.7)
2010	24	2 (8.3)	1 (4.2)	12 (50.0)	9 (37.5)
2011	16	1 (6.3)	3 (18.8)	3 (18.8)	9 (56.3)
2012	1	1 (100)	–	–	–
Total	178	12 (6.7)	20 (11.2)	55 (30.9)	91 (51.1)

mo months, *yr* years

54/58 children (93.1 %), took the both doses separated by an interval of at least 2 months as recommended. The schedule was correct in the vast majority of children, 54/63 children (85.7 %), in this group.

To conclude, the pneumococcal vaccination schedule was correct in only 16 children (7.0 %), who started their immunization in the first 6 months of life, in only 4 children (6.4 %), who started their immunization at aged 7–11 months, and in 54 children (85.7 %) who started their pneumococcal immunizations at aged 12–23 months.

3.3 Influenza Vaccination

Finally, we focused on immunizations against influenza, which should be administered annually starting at the age of 6 months. The number of patients who received at least one dose of the influenza vaccine was 109/1,356 (8.0 %) including 48/109 (36.7 %) children vaccinated once in their lifetime. The proper dosage regimen, meaning the administration of two doses separated by a 4-week interval in children under 9 years of age, was used in 30/61 patients (49.2 %) vaccinated more than once. Only 3.5 % of all the patients (47/1,356) took both recommended vaccinations against respiratory infections, influenza, and pneumococcal vaccine.

Patients immunized against influenza in the following years were assigned to the following age groups: 0 to <12 months, ≥12 to <24 months, ≥2 to <5 years, and ≥5 years. The distribution of patients within particular group in each vaccination year is presented in Table 1. The majority of children were immunized aged over 5 years. Children aged 2–5 years were immunized less frequently with the exception of 2009 and 2010. However, it should be taken into consideration that children immunized more than once, i.e., in subsequent years, were assessed each time as a new patient, because each vaccination was in a different age range. Younger children (infants and children under 24 months) forming the highest risk group were immunized very rarely.

4 Discussion

4.1 Pertussis Vaccination

Pertussis immunization has been mandatory and has been offered free of charge in Poland as the combined diphtheria, tetanus and whole cell pertussis vaccine (DTPw) since the 1950s. Official recommendations for the routine annual immunization of children in Poland are made annually by the Chief Sanitary Inspector in collaboration with the advisory committee. The recommendations specify the type of vaccines, the number of doses that should be given, and the age ranges for their administration. With the addition of new vaccines, the Polish immunization program is increasingly complex (Chief Sanitary Inspector 2012). Nevertheless, the uptake of mandatory vaccinations in Poland is generally high. Three doses of the combined diphtheria, tetanus, and pertussis vaccine (DTP3) were received by around 99 % of Polish children in 2011, the figure being very close to the results of our study (96.6 %) (WHO 2011). Pertussis immunization coverage in our study was similar to that in other developed countries (WHO 2011). The timely administration of the vaccine doses remains a major problem.

The most important finding of our study was that a significant proportion of children fell behind with the schedule set out by the Polish National Immunization Program (Chief Sanitary Inspector 2012). In our study only 2/3 of children received the first dose of the vaccine on time, with delays ranging from 0.1 to 24.3 months, median: 0.8 months. The second and the third doses were administered in about ¾ of children (72.9 % and 72.6 %), with delays ranging from 0.03 to 45.7 months (median: 0.8 months) and from 0.7 to 46.7 months (median: 1.1 months), respectively. The percentage of children who fell behind with vaccinations is also high in other developed countries. Our data correspond well with the results of the National Immunization Survey in the U.S. children, which revealed that about 28 % of children did not comply with official vaccination recommendations and 19 % of children had missed one or more doses (Luman et al. 2008).

There are many possible reasons for vaccinations delays. The reasons can be divided into missed vaccination visits and missed opportunities for simultaneous multiple immunizations during one visit. To start with, parents and doctors have concerns about false contraindications to immunization, e.g., common colds, low grade fever, history of neonatal jaundice, or contact with a patient suffering from an infectious disease (ACIP 2011). Secondly, the frequency of visits required during the first 2 years of life could be difficult to adhere to for busy parents. As a result, some of the vaccinations, if not most of them, may be missed unintentionally, e.g., forgetting appointments, lack of time after the mother returns to work, or another child in the family who needs care (Tickner et al. 2006). The migration of young people to large cities and the reorganization of public healthcare in Poland may also contribute negatively. From time to time children are exempt due to real medical contraindications, e.g., acute disease or serious allergy, but actual contraindications to vaccinations are either rare, e.g., severe allergic reaction after a prior dose, or more commonly are short-term acute febrile illness. Sometimes not vaccinating children or delaying the scheduled vaccination visit is a conscious decision. Lack of faith in the vaccines may be a significant barrier (Ozawa and Stack 2013). Lack of the perceived threat of childhood diseases is also significant – parents may not be familiar with infectious diseases and do not appreciate the benefit of immunization (Ozawa and Stack 2013). Parents who see fewer benefits are less likely to vaccinate their children (Meszaros et al. 1996). Additionally, there is a growing anti-vaccine movement and sometimes the media may spread disinformation that induces a negative attitude to immunizations in Polish society.

The results of a study of Luman and Chu (2009) were that while missed vaccinations visits are responsible for children falling behind in the first 6 months of life, missed opportunities for simultaneous immunizations accounted for more than 90 % of children aged 7–16 months. The vaccination schedule set out by the Polish National Program on Immunization (Chief Sanitary Inspector 2012) in addition to

immunization given after birth at the hospital, includes 6 immunization visits in the first 2 years of life. Four visits consist of three injections and two of a single injection. Even parents who understand the long-term benefits of immunizations acknowledge a trade-off with short-term disadvantages such as pain related to injections (Bennett and Smith 1992). According to a study by Bedford and Lansley (2007), the majority of parents agree to no more than one or two injections during one visit as this is less painful for the child. However, a previous study of Melman et al. (1999) showed that despite potential resistance to multiple injections, parents overwhelmingly complied with a physician's recommendations of five injections during one visit. The study indicated that parental resistance may be less of a barrier than previously feared. Although we did not examine the reasons for delays in vaccinations directly, our results indicate that simultaneous administration of vaccinations may matter in Poland. While only 3.5 % of our study group were immunized with both influenza and pneumococcal vaccines, the indications for their use are very similar (i.e., age and risk factors) and as many as 55.2 % of children were given the self-paid acellular vaccine as a 'five in one' or 'six in one' combined vaccine, which reduces the number of injections.

4.2 Pneumococcal and Influenza Vaccinations

While pertussis immunization has been mandatory and offered free of charge in Poland for many years, the influenza and pneumococcal vaccines are relatively new immunizations. They are recommended by the Polish National Immunization Program (Chief Sanitary Inspector 2012), but not subsidized with the exception of the pneumococcal vaccine for infants born prematurely and with a low birth weight (since 2013). Patients or their guardians have to pay for the vaccines. The immunization rates for this self-paid vaccines were significantly lower in our study. Price was important but not the only factor contributing to the unfavorable situation from a health perspective. While pneumococcal conjugated vaccines are expensive (regular retail price about 60 Euro), but perceived as very important, influenza vaccines are relatively low-cost (regular retail price about 7 Euro), but need annual administration and suffer from societal perception as being less effective. In our study, more than 1/3 of children received pneumococcal vaccines (36.8 %) while only 8.0 % were ever immunized against influenza. On the other hand, in less than half (46.1 %) of the children immunized with the pneumococcal vaccine, the vaccination program started in the first 6 months of life, when children benefit most from immunization but more doses (four) are necessary. We think this could be explained by economic factors because the dosage regimen between 12 and 23 months of life includes only 2 doses of vaccine, thus it is definitely less expensive.

The results of a recent study of Ganczak et al. (2013) performed in 3 randomly selected general practices in Poland were very similar. The authors showed that combination vaccines are the most commonly used (62.3 %) followed by pneumococcal (36.4 %) and influenza vaccines (14.7 %). The high cost of the vaccines is associated with immunization rates being more than five times lower.

Influenza vaccine coverage in our study group was relatively low (8.0 %), although much higher than that among all children in Poland (2.6 %). The city of Wroclaw is large, where access to medical services is easy, and parents are usually well educated. Influenza vaccination coverage was similar or higher than that in other developed countries. In the U.S., the estimated national coverage among children aged 6 months to 17 years ($\geq$1 or more doses for children $<$9 years) was 51.0 % ranging from 37.3 % in Montana to 79.3 % in Rhode Island (CDC 2011). In Spain, the influenza vaccine coverage rate among children older than 6 months was similar (6.8 %) (Jiménez-García et al. 2008). Considering the fact that indications for immunization against *Streptococcus pneumoniae* and influenza are similar and that coverage of the conjugated pneumococcal vaccine was relatively high (36.8 %), the very low immunization coverage

(3.5 %) with both recommended vaccinations indicates that Polish parents do not accept the influenza vaccination for children. We assume that neither doctors nor parents are conscious of the importance of this immunization in prophylaxis of this serious respiratory disease in children. Compliance with the dosage regimen is very important in inactivated vaccines against respiratory diseases. The recommended schedules are based on the results of controlled clinical trials assessing the effectiveness and immunogenicity of vaccines. While a single dose of a live vaccine is usually sufficient to produce immunity, inactivated vaccines require multiple doses to protect children.

Our study is subject to at least two limitations. Firstly, we analyzed data from children from a single healthcare establishment where the personal preferences of a few doctors could significantly influence the results. To minimize the aforementioned negative impact we selected a large primary healthcare practice characterized by high staff turnover (i.e., many doctors working for a short period of time). Secondly, we did not collect the information on medical contraindications to vaccinations. However, true contraindications to vaccinations are rare or temporary and should not have had much impact on our results. Our study, although relatively limited, clearly identifies problems complying with the Polish National Immunization Program (Chief Sanitary Inspector 2012).

Our study showed that although immunization rates of mandatory vaccinations remain high, a substantial proportion of children in Poland fall behind the schedule. The high rate of delays in our study (32.9 % of children had postponed the first dose of the combined pertussis vaccine) indicates an urgent need for action. Polish parents care about the number of injections, cost of vaccines, and opinions about their effectiveness. Immunizations against vaccine-preventable respiratory diseases should be used more effectively, e.g., immunization against one disease should be regarded as an opportunity for immunization against other respiratory diseases.

5 Conclusions

Compliance with the Polish National Immunization Program should be increased by reducing the number of injections and the cost of vaccines, and introducing combination vaccines. Furthermore, education about immunizations is essential to facilitate acceptance of simultaneous administration of several vaccines during one visit and to prepare parents for judicious decision-making when it comes to immunization.

Conflicts of Interest The authors have no financial or otherwise relations that might lead to a conflict of interest.

References

ACIP – Advisory Committee on Immunization Practices of the National Center for Immunization and Respiratory Diseases (2011) General recommendations on immunization. Morb Mortal Wkly Rep Recomm Rep 60:1–64

Bedford H, Lansley M (2007) More vaccines for children? Parents' views. Vaccine 25:7818–7823

Bennett P, Smith C (1992) Parents attitudinal and social influences on childhood vaccination. Health Educ Res 7:341–348

CDC – Centers for Disease Control and Prevention (2011) Final state-level influenza vaccination coverage estimates for the 2010–11 season–United States, National Immunization Survey and Behavioral Risk Factor Surveillance System, August 2010 through May 2011. Available online from: http://www.cdc.gov/flu/fluvaxview/coverage_1011estimates.htm. Accessed 30 Sept 2013

Chief Sanitary Inspector (2012) National Program on Immunization. Available online from: http://www.wsse.krakow.pl/Files/Attachments/phpvb5fSg_program%20szczepien.pdf. Accessed 30 Sept 2013

Cohen SA, Chui KK, Naumova EN (2011) Influenza vaccination in young children reduces influenza-associated hospitalizations in older adults, 2002–2006. J Am Geriatr Soc 59:327–332

Feikin DR, Lezotte DC, Hamman RF, Salmon DA, Chen RT, Hoffman RE (2000) Individual and community risks of measles and pertussis associated with personal exemptions to immunization. JAMA 284:3145–3150

Ganczak M, Dmytrzyk-Daniłów G, Karakiewicz B, Korzeń M, Szych Z (2013) Determinants influencing self-paid vaccination coverage in 0–5 years old Polish children. Vaccine 31:5687–5692

Grant CC, Roberts M, Scragg R, Stewart J, Lennon D, Kivell D, Ford R, Menzies R (2003) Delayed immunisation and risk of pertussis in infants: unmatched case-control study. Br Med J 326:852–853

Grant GB, Campbell H, Dowell SF, Graham SM, Klugman KP, Mulholland EK, Steinhoff M, Weber MW, Qazi S, World Health Organization Department of Child and Adolescent Health and Development (2009) Recommendations for treatment of childhood non-severe pneumonia. Lancet Infect Dis 9:185–196

Hurwitz ES, Haber M, Chang A, Shope T, Teo S, Ginsberg M, Waecker N, Cox NJ (2000) Effectiveness of influenza vaccination of day care children in reducing influenza-related morbidity among household contacts. JAMA 284:1677–1682

Jefferson T, Rivetti A, Di Pietrantonj C, Demicheli V, Ferroni E (2012) Vaccines for preventing influenza in healthy children. Cochrane Database Syst Rev 8: CD004879. doi:10.1002/14651858.CD004879.pub4

Jiménez-García R, Hernández-Barrera V, Carrasco-Garrido-P, López de Andrés A, Pérez N, de Miguel AG (2008) Influenza vaccination coverages among children, adults, health care workers and immigrants in Spain: related factors and trends, 2003–2006. J Infect 57:472–480

Luman ET, Chu SY (2009) When and why children fall behind with vaccinations missed visits and missed opportunities at milestone ages. Am J Prev Med 36:105–111

Luman ET, Shaw KM, Stokley SK (2008) Compliance with vaccination recommendations for U.S. children. Am J Prev Med 34:463–470

Mahon BE, Ehrenstein V, Nørgaard M, Pedersen L, Rothman KJ, Sørensen HT (2007) Perinatal risk factors for hospitalization for pneumococcal disease in childhood: a population-based cohort study. Pediatrics 119:e804–812

Melman ST, Nguyen TT, Ehrlich E, Schorr M, Anbar RD (1999) Parental compliance with multiple immunization injections. Arch Pediatr Adolesc Med 153:1289–1291

Meszaros JR, Asch DA, Baron J, Hershey JC, Kunreuther H, Schwartz-Buzaglo J (1996) Cognitive processes and the decisions of some parents to forego pertussis vaccination for their children. J Clin Epidemiol 49:697–703

Neuzil KM, Wright PF, Mitchel EF Jr, Griffin MR (2000) The burden of influenza illness in children with asthma and other chronic medical conditions. J Pediatr 137:856–864

Neuzil KM, Hohlbein C, Zhu Y (2002) Illness among schoolchildren during influenza season: effect on school absenteeism, parental absenteeism from work, and secondary illness in families. Arch Pediatr Adolesc Med 156:986–991

Ozawa S, Stack ML (2013) Public trust and vaccine acceptance – international perspectives. Hum Vaccin Immunother 9:1774–1778

Poehling KA, Edwards KM, Weinberg GA, Szilagyi P, Staat MA, Iwane MK, Bridges CB, Grijalva CG, Zhu Y, Bernstein DI, Herrera G, Erdman D, Hall CB, Seither R, Griffin MR, New Vaccine Surveillance Network (2006) The underrecognized burden of influenza in young children. New Engl J Med 355:31–40

Tickner S, Leman PJ, Woodcock A (2006) Factors underlying suboptimal childhood immunization. Vaccine 24:7030–7036

Verma R, Khanna P (2012) Pneumococcal conjugate vaccine: a newer vaccine available in India. Hum Vaccin Immunother 8:1317–1320

Wendelboe AM, Njamkepo E, Bourillon A, Floret DD, Gaudelus J, Gerber M, Grimprel E, Greenberg D, Halperin S, Liese J, Muñoz-Rivas F, Teyssou R, Guiso N, Van Rie A, Infant Pertussis Study Group (2007) Transmission of Bordetella pertussis to young infants. Pediatr Infect Dis J 26:293–299

WHO. Poland: WHO and UNICEF estimates of immunization coverage: 2011 revision (2011) Available online from: http://www.who.int/immunizationmonitoring/data/pol.pdf. Accessed 30 Sept 2013

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 5: 19–28
DOI 10.1007/5584_2014_22

Published online: 14 October 2014

Effectiveness of Immunoprophylaxis in Suppressing Carriage of *Neisseria Meningitidis* in the Military Environment

K. Korzeniewski, A. Skoczyńska, A. Guzek, M. Konior, A. Chciałowski, I. Waśko, M. Markowska, and E. Zwolińska

Abstract

Neisseria meningitidis, etiological factor of invasive meningococcal disease, is a human commensal that colonizes the nasopharynx. Colonization is usually asymptomatic, but it is a prerequisite for disease. Asymptomatic carriers are the major source of infection. In the present study, a survey of *N. meningitidis* carriage was conducted between January and March 2013 in a military unit in Poland. Single-time throat culture samples were collected from professional 559 soldiers (302 unvaccinated vs. 257 vaccinated individuals with the quadrivalent conjugate vaccine AC YW-135). Bacterial identification was performed with classic microbiological methods (culture, incubation, identification). Non-culture method (PCR) was used for confirmation of detected strains of *N. meningitidis* and determination of serogroups. We found 29 carriers in the group of unvaccinated soldiers (9.6 % of examined individuals) whereas among vaccinated soldiers only 3 persons were carriers of *N. meningitidis* (1.2 %). The most frequently identified serogroups among the carriers serving in the same military facility were serogroup B (28 %), followed by Y (25 %), and C (22 %). In conclusion, the initiation of mass vaccination with the quadrivalent conjugate vaccine ACYW-135 in the military environment seems an effective method of suppressing *N. meningitidis* carriage.

Keywords

Carriage • *Neisseria meningitidis* • Professional soldiers • Vaccination

K. Korzeniewski (✉) and M. Konior
Department of Epidemiology and Tropical Medicine, Military Institute of Medicine, 4 Grudzińskiego St., Gdynia, 81-103, Poland
e-mail: kktropmed@wp.pl

A. Skoczyńska, I. Waśko, and M. Markowska
National Reference Centre for Bacterial Meningitis, National Medicines Institute, Warsaw, Poland

A. Guzek
Department of Medical Diagnostics, Military Institute of Medicine, Warsaw, Poland

A. Chciałowski
Department of Science, Military Institute of Medicine, Warsaw, Poland

E. Zwolińska
Department of Obstetrics, St Family Maternity Hospital, Warsaw, Poland

1 Introduction

Neisseria meningitidis are gram-negative, aerobic diplococci which cause severe infections in the form of bacterial meningitis and sepsis, defined as an invasive meningococcal disease (IMD). Bacteria colonize the nasopharynx and spread through inhalation of droplets of respiratory secretions or through a direct contact. The sources of infection are carriers and infected individuals. Humans are the only natural reservoir of *N. meningitidis* (Rosenstein et al. 2001). In most cases, the IMD is transmitted from asymptomatic nasopharyngeal carriers of *N. meningitidis* strains, who account for 5–10 % of the general population (Yazdankhah and Caugant 2004). In closed environments such as prisons, boarding institutions, orphanages, and military camps, meningococcal carriage may reach 40–80 % (Cartwright et al. 1991). Some studies have shown that meningococcal carriage prevalence differs across age groups, increasing during childhood and peaking in 15–24-year-olds (Soriano-Gabarro et al. 2011; Rosenstein et al. 1999). Another surveys assessed carriage across all ages, with increasing prevalence through childhood: from 4 to 5 % in infants to the peak of 23–27 % in 19-years-old persons and subsequently decreasing prevalence in adulthood to 7–8 % in 50-year-old persons (Christensen et al. 2010). Most of *N. meningitidis* strains in the population of asymptomatic carriers are not pathogenic. Only a minority of the nasopharyngeal isolates penetrate the human mucosa and gain access to the bloodstream, causing invasive disease (Rosenstein et al. 2001). Despite the opportunity to implement antibiotic therapy at an early stage of the disease and the development of intensive care facilities, the IMD remains one of the most severe contagious diseases in the world, with mortality reaching 10–13 %, and in case of a septic shock – as much as 50 % (Caugant et al. 1994). Invasive infections caused by *N. meningitidis* are a serious public health problem worldwide and have a heavy economic impact, not only in epidemic areas but also in regions where it occurs sporadically. It is estimated that approximately 500,000 cases and 50,000 deaths due to IMD occur worldwide every year (Wilder-Smith 2007). The incidence of invasive disease due to *Neisseria meningitidis* is highly variable according to geographical area and serogroup distribution (Panatto et al. 2011). The biochemical composition of the polysaccharide capsule determines the serogroups of meningococcal strains. There are usually 13 serogroups described, but the WHO reports 12 serogroups (Harrison et al. 2009). Of the 12 different polysaccharide capsular types, only six (A, B, C, W135, Y, and X) frequently cause disease globally. The major disease burden is in developing countries (e.g., 'the meningococcal belt' in Africa); in industrialized communities the IMD occurs sporadically. The European Surveillance System has revealed a considerable variability from one country to another in the incidence of meningococcal disease. The serogroups mostly associated with invasive cases are B and C, but serogroups W-135 and Y are also present, while serogroup A is only responsible for sporadic cases. The IMD due to serogroups Y and W-135, uncommon in most European countries, contributes to 10–23 % of all cases in Scandinavia and Slovenia (Trotter et al. 2007). In the US, serogroup Y is a major cause of meningococcal disease, accounting for more than one third of all cases (Bona and Guidi 2012). Serogroup X determines substantially invasive disease in sub-Saharan Africa, rarely in other parts of the world (Harrison et al. 2009). In recent years, especially in Europe, the incidence of invasive disease caused by serogroup C has declined owing to the introduction of vaccination programs with conjugated vaccine C in children and adolescents in some countries (Cohn et al. 2010). Immunoprophylaxis seems one of the most effective methods of inducing herd protection by preventing nasopharyngeal meningococcal acquisition. Mass vaccinations are particularly important in closed communities, where the risk of increasing meningococcal carriage and invasive disease is exceptionally high. An example of such a community are soldiers staying in military barracks, who living in crowded conditions, together with people from different

geographic areas, have contact with diverse strains of *N. meningitidis* (Brundage et al. 2001). The international medical literature often reports on prevalence of meningococcal carriage and the use of immunoprophylaxis among new military recruits. However, there have only been a few reports on professional soldiers who, unlike recruits, are not living in military barracks, and rarely eat their meals in military canteens, which causes that the risk of meningococcal carriage, just like in the general population, is much lower among professionals than among recruits.

The aim of the study was to assess the prevalence of meningococcal carriage among professional soldiers, the effectiveness of immunoprophylaxis in suppressing the carriage of *N. meningitidis* in the military environment, and the serogroups of *N. meningitidis* on the basis of classic and non-culture microbiological methods.

2 Methods

2.1 Study Population

The study was approved by the Ethics Committee of the Military Institute of Medicine in Warsaw, Poland. The study of *Neisseria meningitidis* carriage was conducted in the military unit in Poland (25th Air Cavalry Brigade in Tomaszów Mazowiecki) between January and March 2013. The single-time throat culture samples were collected from 559 professional soldiers. They were on 8-h duties 5 days a week on the premises of the military unit, while the rest of time they stayed at homes, outside the military environment with the exception of 24-h duties assigned, on average, once a month. Among the 559 soldiers, 302 had never been vaccinated against meningococcal disease, while 257 had been vaccinated over the past 1–3 years with the quadrivalent conjugate vaccine ACYW-135. Apart from the data relating to vaccinations, socio-demographic and behavioral variables of soldiers, such as age, sex, place of residence, smoking of cigarettes were also assessed.

2.2 The Carrier Investigation

2.2.1 Classic Microbiological Laboratory Methods

Biological material was collected single-time from professional soldiers in a military unit (always by the same health care worker throughout the whole study period), on swabs from the back of the nasopharynx. Next, the samples were transported to a microbiological laboratory in the Military Institute of Medicine in Warsaw, Poland, where they were plated onto appropriate medium (Columbia agar with 5 % sheep blood, chocolate agar + PolyVitex VCAT3) in a laminar flow chamber. The plates were incubated in increased atmospheric CO_2 concentration at 37 °C for 48 h. After incubation, the colonies grown were macroscopically evaluated. ATCC (MicroBioLogics, St. Cloud, Minnesota, USA) living reference strains including *N. meningitidis* ATCC 13077 (serogroup A), ATCC 13090 (serogroup B), ATCC 13102 (serogroup C) were used for quality control. Colonies morphologically similar to the reference strains were isolated onto chocolate agar + PolyVitex (PVX) plates, and then incubated for 24–48 h (depending on the growth of microorganisms), in CO_2 atmosphere at 37 °C. The incubated pure colonies of bacteria (transparent, round, and slightly convex) were used to make gram-stained preparations, which were then observed under a light microscope (Gram-negative granuloma, most often in the shape of diplococci). Subsequently, catalase and cytochrome oxidase tests were performed (indicative tests ID Color Catalase, Oxidase Reagent; bioMerieux Polska, Warsaw, Poland). The identification was carried out by means of API NH biochemical sets and an automated system for identification of microorganisms using NH cards in compliance with the manufacturer's instructions (bioMerieux Polska, Warsaw, Poland). The strains identified as *N. meningitidis* were stored frozen in a temperature of −20 °C in Viabank, and then transported to the National Reference Center for Bacterial Meningitis in the National Medicines Institute in Warsaw, Poland, for re-identification and subsequent

characterization of isolates by means of molecular methods.

2.2.2 Non-culture Laboratory Methods

DNA isolated from detected strains was used for polymerase chain reactions (PCR) to identify *N. meningitidis* to the species level by presence of *crg*A and *ctr*A genes and to capsular group level with primers specific for the genogroups A, B, C, W-135, and Y. The relatedness of isolates was determined by the restriction fragment length polymorphism (RFLP) analysis of genomic DNA in pulsed-field gel electrophoresis (PFGE), using *Spe*I restriction enzyme for DNA digestion. RFLP-PFGE patterns were analyzed using Bionumerics Software Package (Applied Maths NV, Sint-Martens-Latem, Belgium) and a dendrogram was constructed using the Dice coefficient of similarity and cluster analysis with the unweighted-pair group method with arithmetic averages (UPGMA). Both the position tolerance and the optimization were set up at 1 %.

2.3 Statistical Elaboration

The quantitative variables were characterized by arithmetic means ±SD or median, min/max (range) and 95 % confidence interval. The qualitative variables were presented in the absolute or percentage terms. Normality of distribution was checked with the Shapiro-Wilk test and homogeneity of variances with the Leven (Brown-Forsythe) test. Significance of differences between two unmatched groups was checked with an unpaired *t*-test (or Welch test in case of lack of homogeneity) or Mann-Withney *U* test. A paired *t*-test or Wilcoxon signed-rank test was used for matching groups, as required. For more than three unmatched groups, one-way ANOVA or Kruskal-Wallis tests were used, followed by *post hoc* Tukey's or Dunn's test, respectively. For matched groups, one-way ANOVA for repeated measurements or Friedman test was used, as required. Chi-squared tests for independence were used for qualitative variables, with Yates' correction for cell counts below 10 and a check of Cochrane's conditions for Fisher's exact test. To determine dependence, strength, and direction between variables, Pearson or Spearman's correlation coefficients were determined. Statistical significance was set at $p \leq 0.05$. The analysis was performed using a Statistica vr. 10.0 commercial package.

3 Results

The results revealed 29 carriers of *N. meningitidis* among the 302 unvaccinated (9.6 %) and 3 carriers (1.2 %) among the 257 vaccinated soldiers. There were no significant differences relating to age, military rank, or place of residence between carriers and non-carriers of *N. meningitidis*. Regression analysis indicated that females were 2.6-fold times more likely to become carriers of *N. meningitidis,* smoking cigarettes increased the risk of carriage, and vaccination reduced it. Among the carriers, there were significantly more females than males, more smokers than non-smokers, and more soldiers vaccinated against meningococcal infections than unvaccinated ones (Table 1).

In a group of 529 soldiers who were identified as non-carriers of *N. meningitidis*, the vaccinated soldiers were significantly older. There were notably more non-commissioned officers among the vaccinated. There were no other significant differences in the socio-demographic and behavioral variables between non-vaccinated and vaccinated individuals (Table 2), nor were there any such differences among 32 soldiers identified as carriers of *N. meningitides* (Table 3).

Testing at the National Reference Center for Bacterial Meningitis showed that all isolates belonged to *N. meningitidis* species and possessed *crg*A gene, but in two cases PCR product for *ctr*A gene was not detected. Genogrouping revealed that among the 32 carriage isolates, 9 belonged to genogroup B (28.1 %), 8 to genogroup Y (25.0 %), and 7 to genogroup C (21.9 %). Isolates belonged to rare genogroups were nongroupable in 8 cases (25.0 %), including 2 isolates with the lack of PCR product for *ctr*A gene. Among three vaccinated soldiers being

Table 1 Sociodemographic and behavioral variables in non-carriers and carriers of *Neisseria meningitidis*

Sociodemographic and behavioral variables	Non-carriers of *N. meningitidis* (*n* = 527)	Carriers of *N. meningitidis* (*n* = 32)	p
Age			
Mean ± SD	30.2 ± 4.8	29.3 ± 4.4	0.407
Range	21.0–52.0	22.0–43.0	
Median	29.0	29.0	
95 % CI	29.8–30.6	27.7–30.9	
Military rank			
Officer	30 (5.7 %)	3 (9.4 %)	0.093
Noncommissioned officer	137 (26.1 %)	3 (9.4 %)	
Private	360 (68.3 %)	26 (81.2 %)	
Sex			
Female	7 (1.3 %)	2 (6.3 %)	0.032
Male	520 (98.7 %)	30 (93.7 %)	
Place of residence			
City	341 (64.7 %)	21 (65.6 %)	0.916
Country	186 (35.3 %)	11 (34.4 %)	
Smoking of cigarettes			
Yes	174 (33.0 %)	22 (68.7 %)	0.001
No	353 (67.0 %)	9 (31.3 %)	
Vaccination			
Vaccinated	250 (47.4 %)	3 (9.4 %)	0.001
Non-vaccinated	277 (52.6 %)	29 (90.6 %)	

p values for the differences between non-carriers and carriers of *Neisseria meningitidis*

Table 2 Sociodemographic and behavioral variables between non-vaccinated and vaccinated with conjugate meningococcal vaccine A, C, Y, and W-135 non-carriers of *N. meningitidis*

Sociodemographic and behavioral variables	Non-vaccinated non-carriers (*n* = 277)	Vaccinated non-carriers (*n* = 250)	p
Age			
Mean ± SD	29.1 ± 4.4	31.4 ± 5.0	0.001
Range	21.0–46.0	22.0–52.0	
Median	28.0	30.0	
95 % CI	28.6–29.6	30.8–32.0	
Military rank			
Officer	14 (5.1 %)	16 (6.4 %)	0.001
Noncommissioned officer	54 (19.5 %)	83 (33.2 %)	
Private	209 (75.4 %)	151 (60.4 %)	
Sex			
Female	5 (1.8 %)	2 (0.8 %)	0.314
Male	272 (98.2 %)	248 (99.2 %)	
Place of residence			
City	174 (62.8 %)	167 (66.8 %)	0.339
Country	103 (37.2 %)	83 (33.2 %)	
Smoking of cigarettes			
Yes	89 (32.1 %)	85 (34.0 %)	0.649
No	188 (67.9 %)	165 (66.0 %)	

p values for the differences between non-vaccinated vs. vaccinated individuals

Table 3 Sociodemographic and behavioral variables between non-vaccinated and vaccinated carriers of *N. meningitidis*

Sociodemographic and behavioral variables	Non-vaccinated carriers of *N. meningitidis* ($n = 29$)	Vaccinated carriers of *N. meningitidis* ($n = 3$)	p
Age			
Mean ± SD	29.2 ± 4.6	30.0 ± 1.0	0.516
Range	22.0–43.0	29.0–31.0	
Median	29.0	30.0	
95 % CI	27.5–31.0	27.5–32.5	
Military rank			
Officer	3 (10.3 %)	0	0.683
Noncommissioned officer	3 (10.3 %)	0	
Private	23 (79.4 %)	3 (100 %)	
Sex			
Female	2 (6.9 %)	0	0.639
Male	27 (93.1 %)	3 (100 %)	
Place of residence			
City	20 (69.0 %)	1 (33.3 %)	0.216
Country	9 (31.0 %)	2 (66.7 %)	
Smoking of cigarettes			
Yes	19 (65.5 %)	3 (100 %)	0.220
No	10 (34.5 %)	0	
Serogroup of *N. meningitidis*			
B	7 (24.1 %)	2 (66.7 %)	0.555
Y	8 (27.6 %)	0	
C	7 (24.1 %)	0	
A, B, C, W, Y (−)[a]	7 (24.1 %)	1 (33.3 %)	

p values for the differences between non-vaccinated vs. vaccinated individuals
[a]Serogroups A, B, C, W-135 or Y have not been identified

meningococcal carriers, two carried isolates of genogroup B and one meningococci not belonging to any of the above outlined genogroups.

Patterns generated by PFGE of *Spe*I-digested DNA of 32 meningococcal isolates were classified into 25 PFGE types. Among them, two types were subdivided into two PFGE subtypes. Isolates of genogroups B and C were heterogenous because except for one pair of meningococci in each genogroup, they possessed dissimilar PFGE patterns. Eight isolates of genogroup Y were divided into five PFGE types, including three patterns (1, 2, and 3) showing more than 80 % similarity. Three Y meningococci had the same PFGE pattern (type 4A) and were also closely related to one isolate not belonging to the genogroups A, B, C, W-135, and Y, with PFGE type 4B (Fig. 1).

4 Discussion

Studies into the prevalence of meningococcal carriage in the military environment are widely available in international medical literature. However, they are limited to one type of community only, i.e., young recruits who had just been drafted into the military. Compulsory military service was abolished in some European countries, including Poland in 2009, which led to professionalization of the national armed forces. The changes caused that the age of soldiers has risen (19–20 year old recruits have been replaced by 25–30 year and older professional privates). Recruits used to serve in a given military facility 24 h a day, 7 days a week, and they were provided with full board in military canteens. Professional soldiers,

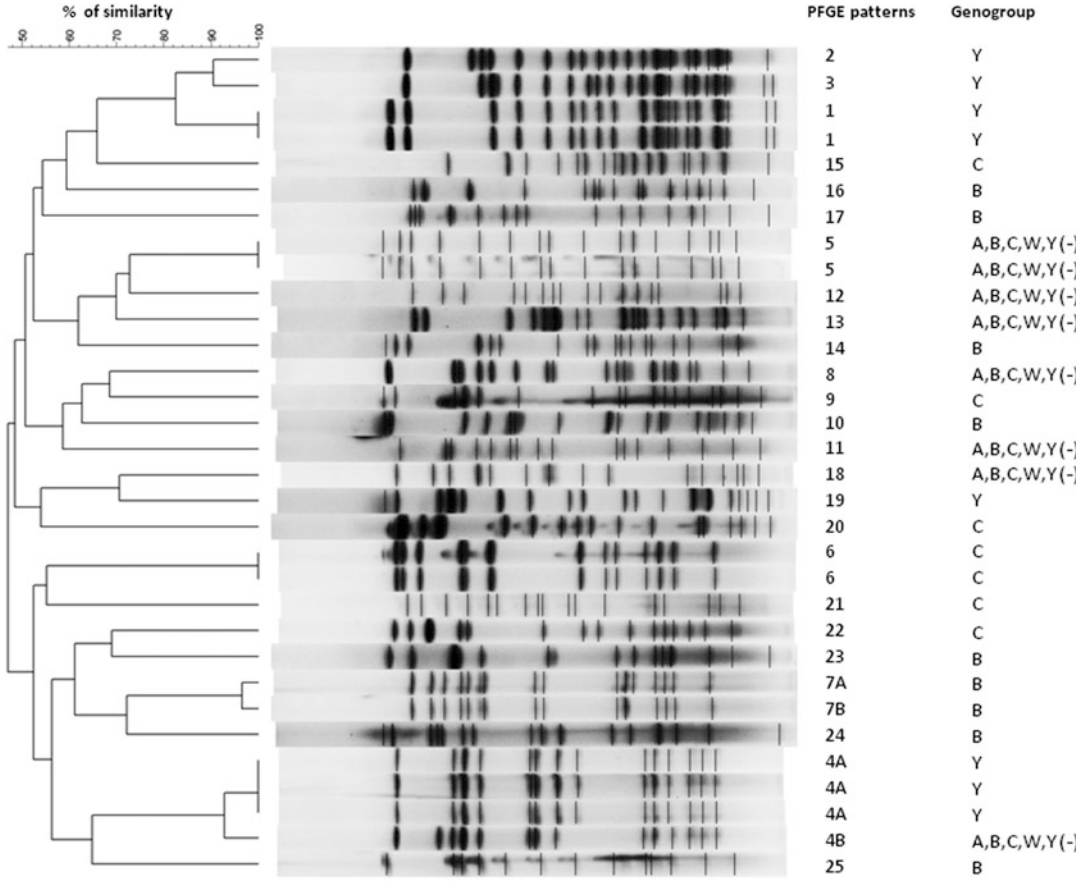


Fig. 1 Dendrogram of similarity obtained with Dice coefficients and arithmetic averages (UPGMA) clustering method, using BioNumerics software, showing the relatedness of meningococcal isolates by PFGE (n = 32)

on the other hand, work for 8 h a day and they are not provided with board and accommodation on the premises of a military unit, with the exception of infrequent 24-h duties and military exercise or military operation. In fact, professional military service has changed from 24 h/7 days service in crowded and close contact conditions to a typical regular job.

Data on the rate of *Neisseria meningitidis* carriage among recruits serving in European armed forces show a high carriage prevalence, regardless of the size of a given study group or country of origin. Recruits constitute a high risk group for meningococcal carriage and invasive disease, with a reported incidence of four to ten times greater than that of the general population (Biselli et al. 1993). Two longitudinal surveys of *N. meningitidis* carriage were conducted in Polish military recruits in non-outbreak settings in 1998 (n = 151 and n = 168). Overall, carriage prevalence in these studies was 36 and 61 %, respectively (Tyski et al. 2001). Andersen et al. (1998) have described that the carriage rate among 3 cohorts of 1,069 Danish recruits was constant over time and season at the level of 39–47 %. The meningococcal carriage surveillance study was conducted between November 1999 and March 2000 among 1,179 German recruits in 6 military camps in Bavaria. Three hundred and eighty-four soldiers (32.6 %) were carriers of *N. meningitidis* (Claus et al. 2005). In Norway, meningococcal carriage study in 126 military recruits was performed between February and July 2003. A total of 78 carriers

(61.9 %) was identified (Caugant et al. 2007). In the present study, carriage of *N. meningitidis* among 559 Polish professional soldiers amounted to 32 individuals, which was 5.7 % of the study population; the rate comparable with that in the general population. Due to the progressive professionalization of the Polish Armed Forces, it is highly probable that meningococcal carriage in the military environment will considerably decrease. The examination of carriers revealed a substantial disproportion between non-vaccinated (9.6 %) and soldiers vaccinated with the quadrivalent conjugate vaccine ACYW-135 (1.2 % persons), which may confirm the effectiveness of immunoprophylaxis in suppressing carriage of *Neisseria meningitidis* and invasive meningococcal disease. Mass vaccinations carried out in some European armed forces also confirm this hypothesis. In the 1980s, high rates of meningococcal disease, mainly caused by serogroup C, were observed among Italian recruits. A mass immunization campaign against meningococcal infections has been carried out in the Italian army since 1987. Thanks to the program the disease prevalence has been largely reduced (Stroffolini 1990). In 1985, 52 cases of meningococcal disease were diagnosed in the Italian military (48 cases with serogroup C) and in 1991 only 1 case (serogroup B). The authors demonstrated that vaccination is highly effective as to seroconversion (18 days after immunization 84 and 91 % had seroconversion against serogroup A and C, respectively). The protective efficacy of the vaccine A + C was 91.2 % (12 cases of serogroup C and A from 150,000 unvaccinated and 1 case of serogroup C from 150,000 vaccinated Italian soldiers in 1987) (Biselli et al. 1993). In connection with a sharp increase in meningococcal disease prevalence among Israeli soldiers, the Department of Epidemiology of the Army Health Branch in Israel adopted an immunization policy with the quadrivalent vaccine for all recruits. As a result of the mass vaccination program, there has been a dramatic drop in the incidence of vaccine-preventable meningococcal disease (from 1.3 cases per 100,000 person-years in 1983–1994 in the period preceding the start of immunization to 0 cases in 1995–2007). From 1983 to 2007, 42 cases of laboratory-confirmed meningococcal disease were reported, all caused by serogroup B after the onset of the vaccination program (Mimouni et al. 2010). According to Panatto et al. (2011), since the introduction of vaccination programs with conjugated vaccine C in children and adolescents, most cases of invasive meningococcal disease in reported countries have been caused by meningococcus B. It is important to underline that invasive meningococcal disease will not be controlled, until safe and effective vaccines for meningococcal B are available and widely used. Scientific publications issued in 2012 confirm the immunogenicity and safety of the 4CMenB (Bexsero, Novartis) vaccine against meningococcal disease caused by serogroup B. The European Medicine Agency registered this vaccine in the territory of European Union in January 2013. The Polish Armed Forces are considering the purchase of the vaccine for their troops. Serogroup B is still a leading cause of meningococcal carriage in the military environment. It was the most frequently identified serogroup (46 %) among 151 carriers of *N. meningitidis* diagnosed in French soldiers serving in the same military facility in 1991 (Chapalain et al. 1992). In 1998, 156 carriers were identified in the population of Polish recruits, 54 % of isolates were nongroupable, among the remaining strains serogroup B was predominant (32 % of all carrier strains) (Tyski et al. 2001). The meningococcal carriage surveillance studies which were conducted between 1999 and 2000 among German soldiers in military facilities revealed that serogroup B was also the most common (42 %) (Claus et al. 2005). In our present study, serogroup B was the most frequently identified as well (28 %). According to most surveys, roughly 50 % of the strains isolated from meningococcal carriers are not serogroupable (Yazdankhah and Caugant 2004; Yazdankhah et al. 2004). In this study, we were able to establish the genogroup for 75 % of isolates tested. Interestingly, we found a high percentage of genogroup Y vs. C carriage in comparison with the general population tested in the Czech Republic, Greece, and Norway:

25.0 % vs. 10.2 % and 21.9 % vs. 4.8 %, respectively (Yazdankhah et al. 2004). An increase in carriage frequency of serogroup Y was recently indirectly reported in young adult population in the UK. The authors concluded that the carriage rise may help explain the recent growth in the incidence of serogroup Y disease in the country, as it was the case during the late 1990s in the US, where a similar increase in serogroup Y carriage was linked to a concomitant increase in serogroup Y disease (Ala'aldeen et al. 2011). Increasing prevalence of meningococcal carriage of serogroup Y demonstrated in this study among non-vaccinated soldiers as well as in other surveys seems to confirm the validity of implementing the quadrivalent conjugate vaccine ACYW-135, which may induce immunological response in humans and facilitate the flow of antibodies to the nasopharyngeal mucosa and eventually affect the colonization of *Neisseria meningitidis*. Additional carriage studies, including extensive molecular strain characterization, should be performed before and after vaccination in countries where mass vaccination programs have been implemented (Caugant et al. 2007). Apart from the analysis of serogroups, it is also important to assess the risk factors affecting the rise in *N. meningitidis* carriage. Smoking, active as well as passive, is one of the strongest risk factors for becoming a meningococcal carrier (Stuart et al. 1989). This has been confirmed by our studies carried out in Polish soldiers. Some studies have also demonstrated a coincidence between meningococcal carriage and symptoms of upper respiratory tract infections. There are also slightly more carriers in males than females and a low socioeconomic status appears to increase the risk factors for carriage (Caugant et al. 2007). Our research revealed that respiratory tract infections occurred sporadically among carriers of *N. meningitidis*, while analyzing gender of soldiers we demonstrated that females exhibit a higher risk for meningococcal carriage than males. Given that the meningococcal carrier state may be chronic, intermittent, or transient (Broome 1986), it is necessary to continue the research into the prevalence of *Neisseria meningitidis* carriage in the military environment taking into consideration its dynamics, risk factors, and serogroup characteristics.

5 Conclusions

The initiation of mass vaccination with the quadrivalent conjugate vaccine ACYW-135 in the military seems to be an effective tool in suppressing *N. meningitidis* carriage. Vaccination contributes to the elimination of carriage, but does eliminate serogroup B. Therefore, it is recommended that the 4CMenB vaccine should be introduced into the vaccination schedule. Surprisingly, a high prevalence of carried serogroup Y observed in the soldiers of this study can indicate the presence of this serogroup also in the general Polish population.

Acknowledgments We are grateful to the Commander of 25th Air Cavalry Brigade from Tomaszów Mazowiecki, Poland for permission to carry out this study and to the soldiers of this military unit for their participation in examination. The paper was supported by the Polish Ministry of Science and Higher Education (Subject No 296/213, Military Institute of Medicine in Warsaw, Poland).

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

References

Ala'aldeen DA, Oldfield NJ, Bidmos FA, Abouseada NM, Ahmed NW, Turner DP, Neal KR, Bayliss CD (2011) Carriage of meningococci by university students, United Kingdom. Emerg Infect Dis 17:1762–1763

Andersen J, Barthelsen L, Bech Jensen B, Lind I (1998) Dynamics of the meningococcal carrier state and characteristics of the carrier strains: a longitudinal study within three cohorts of military recruits. Epidemiol Infect 121:85–94

Biselli R, Fattorossi A, Matricardi PM, Nisini R, Stroffolini T, Amelio RD (1993) Dramatic reduction of meningococcal meningitis among military recruits in Italy after introduction of specific vaccination. Vaccine 11(5):578–581

Bona G, Guidi C (2012) Meningococcal vaccine evolution. J Prev Med Hyg 53:131–135

Broome CV (1986) The carrier state: Neisseria meningitidis. J Antimicrob Chemother 18(Suppl A):25–34

Brundage JF, Ryan MAK, Feighner BH, Erdtmann FJ (2001) Meningococcal disease among United States military service members in relation to routine uses of vaccines with different serogroup-specific components, 1964–1998. Clin Infect Dis 35:1376–1381

Cartwright KA, Stuart JM, Robinson PM (1991) Meningococcal carriage in close contact of cases. Epidemiol Infect 106:133–141

Caugant DA, Hoiby EA, Magnus P, Scheel O, Hoel T, Bjune G, Wedege E, Eng J, Froholm LO (1994) Asymptomatic carriage of *Neisseria meningitidis* in a randomly sampled population. J Clin Microbiol 32:323–330

Caugant DA, Tzanakaki G, Kriz P (2007) Lessons from meningococcal carriage studies. FEMS Microbiol Rev 31:52–63

Chapalain JC, Guibourdenche M, Perrier-Gros-Claude JD, Bartoli M, Riou JY (1992) The chemoprophylaxis of cerebrospinal meningitis using rifampin in a military population. Pathol Biol 40:230–233

Christensen H, May M, Bowen L, Hickman M, Trotter C (2010) Meningococcal carriage by age: a systematic review and meta-analysis. Lancet Infect Dis 10:853–861

Claus H, Maiden MC, Wilson DJ, McCarthy ND, Jolley KA, Urwin R, Hessler F, Frosch M, Vogel U (2005) Genetic analysis of meningococci carried by children and young adults. J Infect Dis 191:1263–1271

Cohn AC, MacNeil JR, Harrison LH, Hatcher C, Theodore J, Schmidt M, Pondo T, Arnold KE, Baubach J, Bennett N, Craig AS, Farley M, Gershman K, Petit S, Lynfield R, Reingold A, Schaffner W, Shutt KA, Zell ER, Mayer LW, Clark T, Stephens D, Messonnier NE (2010) Changes in *Neisseria meningitidis* disease epidemiology in the United States, 1998–2007: implications for prevention of meningococcal disease. Clin Infect Dis 50(2):184–191

European Medicines Agency. Bexsero. Available at: http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/002333/human_med_001614.jsp&mid=WC0b01ac058001d124. Accessed 28 Jan 2013

Harrison LH, Trotter CL, Ramsay ME (2009) Global epidemiology of meningococcal disease. Vaccine 27 (Suppl 2):B51–B63

Mimouni D, Bar-Zeev Y, Huerta M, Balicer RD, Grotto I, Ankol O (2010) Preventive effect of meningococcal vaccination in Israeli military recruits. Am J Infect Control 38:56–58

Panatto D, Amicizia D, Lai PL, Gasparini R (2011) Neisseria meningitidis B vaccines. Expert Rev Vaccines 10:1337–1351

Rosenstein NE, Perkins BA, Stephens DS, Lefkowitz L, Cartter ML, Danila R, Cieslak P, Shutt KA, Popovic T, Schuchat A, Harrison LH, Reingold AL (1999) The changing epidemiology of meningococcal disease in the United States, 1992–1996. J Infect Dis 180:1894–1901

Rosenstein NE, Perkins BA, Stephens DS, Popovic T, Hughes JM (2001) Meningococcal disease. N Engl J Med 344:1378–1388

Soriano-Gabarro M, Wolter J, Hogea C, Vyse A (2011) Carriage of *Neisseria meningitis* in Europe: a review of studies undertaken in the region. Expert Rev Anti Infect Ther 9:761–774

Stroffolini T (1990) Vaccination campaign against meningococcal disease in army recruits in Italy. Epidemiol Infect 105:579–583

Stuart JM, Cartwright KA, Robinson PM, Noah ND (1989) Effect of smoking on meningococcal carriage. Lancet 2(8665):723–725

Trotter CL, Chandra M, Cano R, Larrauri A, Ramsay ME, Brehony C, Jolley KA, Maiden MC, Heuberger S, Frosch M (2007) A surveillance network for meningococcal disease in Europe. FEMS Microbiol Rev 31:27–36

Tyski S, Grzybowska W, Dulny G, Berthelen L, Lind I (2001) Phenotypical and genotypical characterization of Neisseria meningitidis carrier strains isolated from Polish recruits in 1998. Eur J Clin Microbiol Infect Dis 20:350–353

Wilder-Smith A (2007) Meningococcal vaccine in travelers. Curr Opin Infect Dis 20(5):454–460

Yazdankhah SP, Caugant DA (2004) *Neisseria meningitidis*: an overview of the carriage state. J Med Microbiol 53:821–832

Yazdankhah SP, Kriz P, Tzanakaki G, Kremastinou J, Kalmusova J, Musilek M, Alvestad T, Jolley KA, Wilson DJ, McCarthy ND, Caugant DA, Maiden MC (2004) Distribution of serogroups and genotypes among disease-associated and carried isolates of *Neisseria meningitidis* from the Czech Republic, Greece, and Norway. J Clin Microbiol 42:5146–5153

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 5: 29–33
DOI 10.1007/5584_2014_31

Published online: 25 September 2014

Detection of Influenza and Other Respiratory Viruses Carried Out in the Influenza Project – Monitoring Vaccine Effectiveness (I-MOVE)

Agnieszka Woźniak-Kosek

Abstract

The project Influenza Vaccine Effectiveness-Monitoring (I-MOVE) is part of the European research carried out by the ECDC (European Center for Disease Prevention and Control), aimed at monitoring the effectiveness of vaccination in Europe during the growing incidence of flu and influenza-like illnesses in the coming epidemic seasons. Laboratory studies using molecular RT-PCR biology methods for detection of genetic material of influenza virus and other respiratory viruses were performed by Voivodeship Sanitary-Epidemiological Stations in Poland. The validation of the results of swabs taken from the nose and throat were carried out in the Department of Influenza Research, National Influenza Center in Warsaw. The study involved 210 samples from patients across Poland. Positive results were recorded for 72.4 % of the samples; influenza virus type A was detected in 43 and type B in 38 cases, whereas in 71 cases other respiratory viruses were detected, which included Human parainfluenza virus type 1–4; Human respiratory syncytial virus type A and B; Human coronavirus 229E/NL63, OC43; Human rhinovirus type A, B, and C; Human enterovirus; and Human adenovirus. The results show that although influenza viruses predominated in the 2010/2011 season in Poland, other flu-like viruses also abounded.

Keywords

I-MOVE project • Influenza virus • Molecular biology • Respiratory viruses

1 Introduction

A great number of influenza cases are recorded in each epidemic season, although with varying intensity (Van-Tam and Sellwood 2013; Kissling et al. 2011; Brydak 2008). Influenza occurs in

A. Woźniak-Kosek (✉)
Department of Influenza Research, National Influenza Center, National Institute of Public Health-National Institute of Hygiene, 24 Chocimska St., 00-791 Warsaw, Poland
e-mail: kaj12@poczta.fm

every climate and latitude. In Central Europe, the incidence of influenza and influenza-like illnesses usually starts increasing from January to March (Globinska and Kowalski 2012; Brydak 2008). In Poland, the number of cases of influenza and influenza-like illnesses in a single season varies from a few thousands to several million (Globinska and Kowalski 2012; Brydak 2008; Romanowska and Brydak 2007). The recorded cases are tallied from the reports of family doctors who are on the frontline of contact with patients. Depending on the number of cases they report, the epidemiological and virological interpretation is conducted on a national scale as well as a comparative evaluation on a global scale.

The project Influenza Vaccine Effectiveness-Monitoring (I-MOVE) is part of the European research coordinated by the ECDC, the primary purpose of which is to monitor the effectiveness of influenza vaccination in Europe during the growth of the disease (Kissling et al. 2011). The project has been implemented in 12 countries in the European Union. Poland joined this program for the first time in the epidemic season of 2010/2011.

2 Methods

The preparation of this manuscript was approved by an Institutional Board for Research. The material consisted of 210 swabs from the nose and throat taken by primary care physicians who classified patients of all ages with symptoms of upper respiratory tract infection for the examination according to the European criteria for influenza-like illness (ECDC 2013). Detection of the genetic material of influenza virus was performed by an RT-PCR method in the Voivodeship Sanitary-Epidemiological Stations. The collection and validation process of virological data was coordinated by the Influenza Virus Research Institute of the National Center for Influenza in Warsaw, Poland. The tests were designed to investigate the genetic presence of RNA of influenza viruses A and B, and also that of another 15 respiratory viruses, including Human parainfluenza virus type 1–4, Human respiratory syncytial virus type A and B, Human coronavirus 229E/NL63 and OC43, Human rhinovirus type A, B, and C, Human enterovirus, and Human adenovirus (RV 15 OneStep ACE Detection; Seegene, Seoul, South Korea). The methodology consisted of the determination in the reaction mixture of specific amplification product corresponding by its multiplicity to the fragment of a searched virus genome, limited at both sides with the sequences of primers used in the PCR. In order to estimate the size of the resulting PCR product, it was necessary to conduct a comparative analysis with the pattern of the size of DNA which contained fragments of various known sizes of DNA. The reading was performed by applying Amplicon to the agarose gel and carrying out an electrophoretic separation of the swabs, followed by inspecting and documenting the results with a GelDoc device and Quantity One software (Bio-Rad; Hercules, CA).

2.1 Collection of Clinical Material for Testing

Obtaining a reliable laboratory result of a virological test depends on a number of factors, most of which depend on the physician's collecting the material. They include the following:

- type of material and how it is collected
- time of sample collection after the onset of disease
- storage conditions of material until it is transported to the laboratory
- conditions and time of transporting a sample to the laboratory

Failure to meet the specified requirements may result in a false negative result, which may have an impact on patients' treatment (Wozniak-Kosek and Brydak 2013; Romanowska and Brydak 2007). The optimal time for the sample collection is within 4–5 days, when symptoms are the strongest. The material could be a swab from the nose or throat, nasopharyngeal washes, aspirates from the nasopharynx, or the cerebrospinal fluid (Nitsch-Osuch et al. 2013;

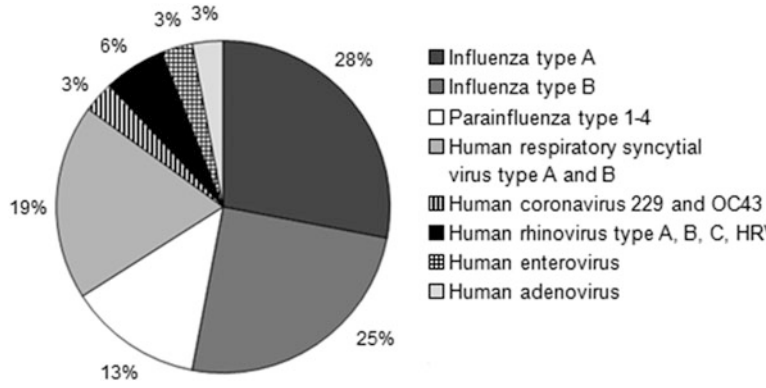


Fig. 1 Infections caused by influenza and influenza-like viruses causing respiratory disease in Poland in 2010–2011 epidemic season; investigated within the I-MOVE project

Wozniak-Kosek and Brydak 2013). Each sample taken from a patient and sent to the laboratory included information about the patient's age, gender, influenza vaccination, the anti-viral medicines taken, the onset of symptoms, and the date of material collection.

3 Results and Discussion

Of the 210 samples obtained from physicians participating in the I-MOVE project, detection of respiratory viral genetic material was successful in 152 cases (72.4 %). Other pathogens responsible for respiratory tract infections, in addition to bacteria and viruses, were fungi and parasites. Figure 1 presents the percentage of viruses detected in clinical specimens.

The results show that influenza and other respiratory viruses are a major clinical, virological, and epidemiological problem. In the samples from the epidemic season of 2010–2011 in the I-MOVE project, the presence of the genetic material of influenza virus type A and B was detected in 81 cases, which represented 38.6 % of all samples investigated; the share held by the influenza virus type A/H1N1/pdm09 is shown in Fig. 2. In the remaining 71 positive samples, i.e., 33.8 %, the following respiratory viruses were detected: Human parainfluenza virus type 1–4, Human respiratory syncytia virus type A and B, Human coronavirus 229E/NL63 and OC43, Human rhinovirus type A, B, and C, Human enterovirus, and Human adenovirus.

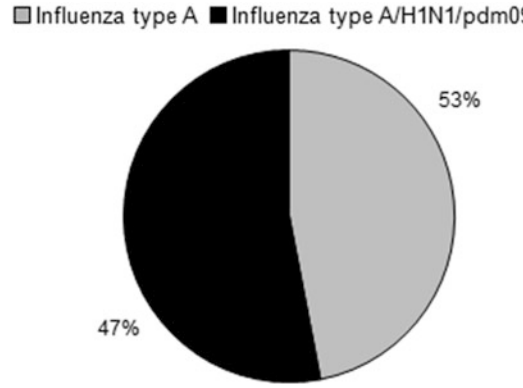


Fig. 2 Infections caused by influenza A non-subtype and influenza A/H1N1/pdm09 in Poland in 2010–2011 epidemic season; investigated within the I-MOVE project

In 23 (11 %) cases, a co-infection with two respiratory viruses was reported, which constitutes a serious clinical problem because of the lack of suitable, effective antiviral drugs. The neuraminidase inhibitors, such as oseltamivir and zanamivir, are suitable only influenza treatment. These drugs are not effective in preventing infections caused by other viruses in the respiratory system. The arrangement of co-infecting viral pairs detected is presented in Table 1.

Outbreaks of the influenza and influenza-like viruses occur every epidemic season, covering 5–25 % of the world's population (Brydak 2008). For many years, medicine has been using molecular biology methods of a rapid viral diagnosis that is beneficial not only for the individual patient but also results in the economic benefits for the country (Van-Tam and

Table 1 Respiratory virus co-infections

Type of co-infection	Positive samples
Human parainfluenza virus 2	1
Human respiratory syncytial virus B, Influenza B	
Human coronavirus 229E/NL63	1
Influenza A	
Human rhinovirus	4
Human respiratory syncytial virus A	
Human respiratory syncytial virus A	1
Influenza B	
Human parainfluenza virus 3	1
Human coronavirus OC43	
Human parainfluenza virus 3	1
Human rhinovirus	
Influenza A	1
Human parainfluenza virus 4	
Human adenovirus	1
Influenza A	
Human parainfluenza virus 1	1
Human respiratory syncytial virus B	
Human adenovirus	1
Human parainfluenza virus 3	
Human rhinovirus	
Human respiratory syncytial virus B	2
Human parainfluenza virus 4	
Influenza B	2
Human parainfluenza virus 4	
Human respiratory syncytial virus B	2
Influenza B	
Human respiratory syncytial virus B	1
Human parainfluenza virus 1	
Influenza A	2
Human enterovirus	
Human parainfluenza virus 2	1
Influenza A	
Total	23

Sellwood 2013). The most cost-effective way to combat influenza is prevention and vaccination. Viruses infecting epithelial cells of the respiratory tract cause a diverse defense response of the organism and try in different ways to avoid the immune mechanisms aimed at eliminating infectious agents (Globinska and Kowalski 2012). An understanding of ways by which viruses avoid body's antiviral response may serve as a tool for developing new methods for treatment of respiratory diseases. The integrated epidemiological and virological influenza surveillance system conducted in Poland (Brydak et al 2013; Wozniak-Kosek and Brydak 2013), as well as international projects such as I-MOVE, significantly contribute to the evaluation of the spread of the influenza and other respiratory viruses in the population investigated. Such programs also allow assessing and comparing the epidemiological and virological situation in different countries, which results in a greater awareness of vaccinations, such as against influenza.

Acknowledgements The author would like to thank Dr. Iwona Paradowska-Stankiewicz, the I-MOVE project coordinator in Poland for enabling the implementation of virological tests in this regard and Prof. L.B. Brydak for supervising the virological work associated with the project.

Conflicts of Interest The author declares no conflict of interests in relation to this article.

References

Brydak LB (2008) Diagnostics, prophylaxis and treatment of influenza in the practice of a family physician. Med Stand/Pediatr 5:261–269 (in Polish)

Brydak LB, Wozniak-Kosek A, Nitsch-Osuch A (2013) Influenza diagnosis and vaccination in Poland. Respir Physiol Neurobiol 187:88–93

ECDC – European Centre for Disease Prevention and Control (2013) http://ecdc.europa.eu/en/activities/surveillance/eisn/surveillance/pages/influenza_case_definitions.aspx. Accessed on 03 Feb 2014

Globinska A, Kowalski ML (2012) Natural immunological response to respiratory viruses – intracellular signalling pathways. Allergy Ashtma Immunol 17 (2):66–75 (in Polish)

Kissling E, Valenciano M, Cohen MJ, Oroszi B, Barret AS, Rizzo C, Stefanoff P, Nunes B, Pitigoi D, Larrauri A, Daviaud I, Horvath JK, O'Donnell J, Seyler T, Paradowska-Stankiewicz IA, Pechirra P, Ivanciuc AE, Jiménez-Jorge S, Savulescu C, Ciancio BC, Moren A (2011) I-MOVE multicentre case control study 2010–11: overall and stratified estimates of influenza vaccine effectiveness in Europe. PLoS One 6(11):e27622. doi:10.1371/journal.pone.0027622

Nitsch-Osuch A, Wozniak-Kosek A, Korzeniewski K, Zycinska K, Wardyn K, Brydak LB (2013) Accuracy of the rapid influenza detection test in the diagnosis of

influenza A and B viruses in children less than 59 months old. Adv Exp Med Biol 788:71–76

Romanowska M, Brydak LB (2007) Family physicians as the basis for the functioning of the integrated epidemiological and virological SENTINEL surveillance of influenza. New Clin 14:1166–1175 (in Polish)

Van-Tam J, Sellwood C (eds) (2013) Pandemic influenza, 2nd edn. CAB International, Wallingford

Wozniak-Kosek A, Brydak LB (2013) Virological monitoring of influenza activity and influenza-like illnesses in the epidemic season of 2011/2012 in Poland. Adv Exp Med Biol 788:77–82

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 5: 35–40
DOI 10.1007/5584_2014_25

Published online: 24 September 2014

Cytokines and Toll-Like Receptors in the Immune Response to Influenza Vaccination

A. Mastalerz-Migas, M. Pokorski, K. Kiliś-Pstrusińska, K. Doskocz, B.J. Sapilak, and L.B. Brydak

Abstract

Toll-like receptors (TLRs) are involved in immunogenicity. However, little information is available on the role of TLRs in the immune response to vaccination against influenza virus. The aim of the study was to analyze the relationship between the immunogenic response to influenza vaccine and the presence of soluble forms of TLRs and selected cytokines in the serum. There were two groups of subjects participating in the main protocol of the study: 55 chronically hemodialyzed patients (Group A) and 55 healthy volunteers (Group B) participated in the study. Both groups were vaccinated against influenza using a subunit Agrippal vaccine. The concentrations of human TNF-α, IL-1β/IL-1F2, IL-6, and IL-10 were measured by a high sensitivity enzyme-linked immunosorbent assay. The soluble forms of TLR-2, TLR-4, and TLR-7 were determined in serum samples by ELISA as well. The findings were that vaccination did not appreciably influence the level soluble TRL-2, TRL-4, and TRL-7 or the cytokines investigated either in patients on hemodialysis or in healthy volunteers. Nor were there any relevant correlations between Toll-like receptors or pro-inflammatory cytokines and the immune response to influenza vaccination. On the other hand, the study showed that Toll-like receptors are increased in hemodialyzed patients, which may enhance the anti-inflammatory IL-10 and counter the downgrade of the immune response to influenza vaccine.

A. Mastalerz-Migas (✉) and B.J. Sapilak
Department of Family Medicine, Medical University of Wrocław, 1 Syrokomli St., Wrocław 51-141, Poland
e-mail: agnieszka.mastalerz-migas@umed.wroc.pl

M. Pokorski
Public Higher Medical Professional School in Opole, 68 Katowicka St., 45-060 Opole, Poland

Institute of Psychology, Opole University, Plac Staszica 1, 45-052 Opole, Poland

K. Kiliś-Pstrusińska
Department of Pediatric Nephrology, Medical University of Wrocław, Wrocław, Poland

K. Doskocz
Dialysis Center 'Diaverum', Nysa, Poland

L.B. Brydak
Department of Influenza Research, National Influenza Center, National Institute of Public Health-National Institute of Hygiene, Warsaw, Poland

Department of Immunology, Division of Biology, University of Szczecin, Szczecin, Poland

Keywords
Antihemagglutinin antibodies • Cytokines • Hemodialysis • Influenza vaccine • Toll like receptors

1 Introduction

Influenza virus infection induces the immune response which includes the synthesis of cytokines and chemokines by macrophages and endothelial cells of the respiratory system. Viral antigens activate the antigen presenting cells and subsequently induce adaptive immune responses and the elimination of viruses from the body. Toll-like receptors (TLR) that react with viruses, bacteria, and numerous factors from the organism, such as, e.g., heat shock proteins (HSP), play a significant role in the mechanisms of immune responses.

So far 13 TLR were described, including 10 of them in humans. The most important TLR are TRL-2 and TLR6 – specific mostly for bacteria, TLR3 – specific for viral RNA, TRL-2 – specific for bacteria, HSP, taxol and RNA viruses, TLR5 – specific for flagellin, and TRL-7, TLR8, and TLR9 – specific for viral DNA and RNA (TRL-7 – for type A influenza virus). It has been shown that TLR, after binding with a ligand (pathogen associated molecular patterns – PAMP), activate immunity after the induction of synthesis of various pro-inflammatory cytokines and the upregulation of major histocompatibility complex (MHC) and co-stimulatory molecules present on immune cells (Boehme and Compton 2004; Beutler 2004; Janssens and Beyaert 2003; Beutler and Wagner 2002).

Apart from Toll-like receptors present on different cells, their soluble forms can be found in peripheral blood. There is little information on the involvement of TLR in the immune response to vaccination against influenza virus. It is suggested that they may react with viral antigens and block their ability to bind the cells and may also facilitate antigens' removal by phagocytosis (Liew et al. 2005; Lebouder et al. 2003). TRL-2 is considered the major TLR receptor. In humans it is activated by lipopolysaccharide of Gram-negative bacteria and, together with TRL-4, it increases the synthesis of TNF-alpha, IL-10, IL-12, and chemokines (Orme 2004; Martin et al. 2003).

TRL-7 and TLR9 are the most important Toll-like receptors involved in the immune response to influenza infection. It has been shown that ssRNA is recognized on plasmacytoid cells by TRL-7 and the adaptor molecule MyD88, while on myeloid cells and fibroblasts by retinoic acid with the PS-1 and interferon-beta promoter stimulator-1 adaptor molecules. To become functionally active, TRL-7 and TLR9 responsible for the viral antigen recognition require enzymatic degradation in endosomes. TRL-7 are activated after proteolysis mediated by endopeptide asparaginase while TLR9 after proteolysis to C-terminal fragment mediated by cathepsin (Pang and Iwasaki 2011; Takeda and Akira 2004).

The aim of the present study was to analyze the correlation between the immunogenic response to influenza vaccine and the presence of soluble forms of TRL-2, TRL-4, and TRL-7, as well as selected cytokines in the serum obtained from patients on hemodialysis and healthy volunteers.

2 Methods

The study was performed in conformity with the Declaration of Helsinki for Human Experimentation of the World Medical Association and was approved by the Ethics Committee of the Medical University of Wroclaw, Poland. In addition, patients enrolled into the study gave written informed consent for the study procedure.

2.1 Subjects

Fifty five patients on hemodialysis – Group A (40 men and 15 women; mean age: 65.7 ± 15.4 years) and 55 healthy volunteers – Group B (21 men and 32 women; mean age: 50.0 ± 21.3) were participated in the study. Both groups were vaccinated against influenza with a subunit vaccine (Agrippal; Novartis, Frimley, Camberley, Surrey, UK). The vaccine was injected into the deltoid muscle. The study was performed in the epidemic season 2009/2010.

2.2 Technical Notes

One vaccine dose contained 15 μg of hemagglutinin from the three different influenza strains each: A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2), and B/Brisbane/60/2008 in a volume of 0.5 ml. Ten milliliters of venous blood were collected from each subject for immunologic studies twice, before the vaccination and 1 month after it. Blood was drawn into a sterile 10 mL serum separator tube. Samples were centrifuged at 3,000 rpm for 15 min and stored at −80 °C until use.

The serologic response to vaccination was assessed from the level of antihemagglutinin (anti-HA) antibodies. The anti-HA level was assayed with a hemagglutination inhibition assay (Receptor Destroying Enzyme, Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instruction. After the removal of non-specific hemagglutination inhibitors, the highest serum dilution in which the blood cell agglutination was still inhibited, was assumed to be the anti-HA antibody titer for a given viral strain.

Serum concentrations of human TNF-α, IL-1β/IL-1F2, IL-6, and IL-10 were measured by a high sensitivity enzyme-linked immunosorbent assay (ELISA) using commercially available kits according to the manufacturer's instruction (R&D Systems, Minneapolis, MN). The detection range for TNF-α was 0.5–32 pg/mL with a sensitivity of 0.106 pg/mL, for IL-1β/IL-1 F2 was 0.125–8 pg/mL with a sensitivity of 0.057 pg/mL, for IL-6 was 0.156–10 pg/mL with a sensitivity of 0.039 pg/mL, and for IL-10 was 0.106–50 pg/mL with a sensitivity of 0.09 pg/mL. Concentrations of TLR-2, TLR-4, and TLR-7 were also determined with an ELISA assay (USCN, Wuhan, China). The detection range for TLR-2 was 0.312–20 pg/mL with a sensitivity of 0.125 pg/mL, for both TLR-4 and TLR-7 were 0.156–10 pg/mL with a sensitivity of 0.061 pg/mL and 0.057 pg/mL, respectively. The measured values of optical density of the variables above outlined were interpolated the standard curves.

2.3 Statistical Analysis

Data were given as means ±SD. Differences between groups were assessed with one-way ANOVA, Kruskal-Wallis test, Wilcoxon test, chi-squared, and Fisher's test. Statistical significance was defined as $p < 0.05$. All calculations were performed using a commercial Statistica 7.1 package.

3 Results

To assess the ability to serologically respond to specific influenza antigens, the subjects of both groups (hemodialyzed and healthy controls) were stratified into responders (R) (at least a four-fold increase in anti-HA titer) and non-responders (NR) to the three influenza strains tested. There was one basic difference between the hemodialyzed patients and healthy controls, which was that nearly all controls had an increase in anti-HA antibodies against A/H1N1 as opposed to the patients of who only two thirds responded (Table 1).

Table 1 Percentages of responders (R) and non-responders (NR) to influenza vaccination, as assessed from ≥4-fold increase in anti-HA titer, in hemodialyzed patients (Group A) and control healthy subjects (Group B)

	Hemodialyzed ($n = 55$)		Healthy ($n = 55$)	
	R	NR	R	NR
A/H1N1	65	35	95	5
A/H3N2	89	11	89	11
B	80	20	75	25

Data are % of subjects

Table 2 Serum concentrations of Toll-like receptors (TLRs), tumor necrosis factor alpha (TNF-α), and interleukins (IL) in hemodialyzed patients and control healthy subjects before and after influenza vaccination with regard to the A/H1N1 antigen response

	Hemodialyzed ($n = 55$)				Healthy ($n = 55$)			
	Before vaccination		After vaccination		Before vaccination		After vaccination	
	R	NR	R	NR	R	NR	R	NR
TRL-2	10.9 ± 4.0	11.8 ± 5.0	11.3 ± 3.6	11.5 ± 3.9	0.1 ± 0.3**	0.0 ± 0.0**	1.3 ± 2.3***	5.4 ± 6.2
TRL-4	0.3 ± 0.3	0.4 ± 0.3	0.3 ± 0.2	0.3 ± 0.2	0.0 ± 0.0**	0.0 ± 0.0**	0.0 ± 0.1***	0.1 ± 0.1
TRL-7	0.7 ± 1.7	0.1 ± 0.1	0.5 ± 1.4	0.1 ± 0.1	0.2 ± 0.5**	0.0 ± 0.0**	0.2 ± 1.0***	0.4 ± 0.8
TNF-α	6.9 ± 4.3	5.5 ± 1.9	7.0 ± 4.5	5.6 ± 3.6	2.0 ± 1.1**	2.4 ± 0.6**	2.0 ± 1.2***	2.4 ± 0.7***
IL-10	0.6 ± 0.9	0.4 ± 0.4*	0.6 ± 1.0	0.3 ± 0.2*	0.3 ± 1.0**	0.2 ± 0.3	0.2 ± 0.4***	0.1 ± 0.2
IL-6	2.7 ± 2.0	3.1 ± 2.4	3.4 ± 2.3	2.9 ± 2.1	1.2 ± 1.2**	1.2 ± 0.3	1.1 ± 0.9***	1.2 ± 0.4
IL-1β	0.2 ± 0.3	0.1 ± 0.1	0.2 ± 0.3	0.3 ± 0.9	0.2 ± 0.4	0.1 ± 0.1	0.2 ± 0.2	0.5 ± 0.8

All data are in pg/mL

R responders, *NR* non-responders

*$p < 0.05$ responders *vs.* non-responders in a given group; **$p < 0.05$ healthy subjects *vs.* hemodialyzed patients before vaccination (correspondingly concerning the serologic response); and ***$p < 0.05$ healthy subjects *vs.* hemodialyzed patients after vaccination (correspondingly concerning the serologic response)

Further analysis focused on the A/H1N1 antibody titer, as the response to this antigen is considered the most relevant for immunity. Serum concentrations of TLRs were inappreciably different before and 1 month after influenza vaccination in either hemodialyzed patients or healthy subjects, in both serologic responders and non-responders. Regarding the cytokine levels, IL-10 was appreciably higher before vaccination in the hemodialyzed responders than non-responders and remained so also after vaccination. The levels of other cytokines did not show any relation with the response to the A/H1N1 antigen (Table 2). On the other hand, TLRs and cytokines were appreciably lower in the healthy subjects than in hemodialyzed patients both before and after influenza vaccination, particularly in the serologic responders, with the exception of IL-1β which did not change much (Table 2).

4 Discussion

In the present study we have analyzed if the immune response to influenza vaccine depends on the concentrations of soluble TRL-2, TRL-4, and TRL-7, and if it has any relation to the level of cytokines represented by IL-10, IL-6, IL-1β, and TNF-α. We chose to investigate the relationship between the immune response, Toll-like receptors, and cytokines in the hemodialyzed patients on the premise that immunogenicity and cytokine expression are hampered in chronic kidney disease, which might help bring out the relevant relations to the factors above outlined while comparing with healthy subjects. In addition, there is a previous study pointing to the influence of TLR on the immune response in patients with chronic kidney disease on hemodialysis (Kazimierczak et al. 2007). In contrast to that study, however, the present findings were that there was no correlation between vaccination and concentration of soluble forms of Toll-like receptors type 2, 4, and 7. Vaccination did not influence the concentrations of TLRs or cytokines in either hemodialyzed patients with compromised immunogenicity or healthy subjects. Moreover, both groups of subjects failed to show any immunogenic-responsive differences in TLRs and cytokines, despite evidently higher levels of these biomarkers in hemodialyzed patients. Understandably, increased pro-inflammatory cytokines in patients on hemodialysis point to enhanced inflammatory state in these patients.

Increased concentration of TLRs in hemodialyzed patients is less readily explainable. TLRs exert a role in innate immune responses being members of pattern recognition receptors. The expression of TLRs has been observed in immune system cells, aside from other cell types. Various types of TLRs exert different functions. Of the TRLs investigated in

the present study, TRL-2 and TRL4 are present in membranes and TRL-7 in the cytoplasm (Werkling and Jungi 2003; Beutler and Wagner 2002). After binding with pathogen-associated molecular patterns (PAMPs) from many pathogens, TRL-4 increases immunity through induction of TNF-α, IL-1, and IL-12 synthesis. Decreased synthesis of cytokines hampers the immune response against pathogens in mammals without TRL-4 (Janssens and Beyaert 2003; Martin et al. 2003; Sabroe et al. 2003). In the present study we noted that IL-10, known as anti-inflammatory cytokine (cytokine synthesis inhibitory factor), was appreciably enhanced in the hemodialyzed patients, particularly in those who responded to the influenza vaccine. A similar trend also was observable in healthy subjects. That suggests that IL-10 may facilitate the humoral response. TRL-7 activates the immune system as well. They bind small antiviral compounds, but also specific viruses, including influenza, RSV, HIV, and Birnaviridae and Reoviridae families of viruses. The induction of immune responses is mediated through activation of B cells and myeloid dendritic cells. In humans, TRL-7, aside TRL9, are natural receptors for the viruses mentioned above (Diebold et al. 2004; Heil et al. 2004; Beutler et al. 2003; Sabroe et al. 2003; Werkling and Jungi 2003). Therefore, in the context of our present study, a plausible assumption might be that TLRs are enhanced to counter a downgrade in immunogenicity in such patients and to uphold the ongoing immune responses at a reasonable biological level.

The expression of TLR-2 and TLR-4 on granulocytes and peripheral blood monocytes in patients on hemodialysis is similar to that observed in healthy subjects. However, after lipopolysaccharide-induced TLRs expression on cells obtained from patients on hemodialysis, increased synthesis of pro-inflammatory TNF-α and IL-6 has been observed (Wolf et al. 2006). Concerning the influenza virus, TRL-7 is considered most influential as it recognizes viral RNA and starts the immune response; both in case of infection and vaccination. Viral genome – ssRNA (single-stranded RNA) is recognized by plasmacytoid dendritic cells through TRL-7 and its adaptor protein MyD88, which results in induction of interferon type 1 expression. In the mouse model, TRL-7 appears essential for the immune response to influenza type A vaccine, as the lack of these receptors deprived the animals of the ability to produce antibodies after administration of the experimental vaccine M2e-AP205(+RNA) (Schmitz et al. 2012; Koyama et al. 2010). However, apart from TLR activation, the immune system might be activated through retinoic acid-inducible gene I (RIG-I), a cytosolic RNA helicase. Thus, the immune response to influenza type A virus is regulated by several intracellular signaling pathways, rather than by a single receptor (Koyama et al. 2007; Kato et al. 2005).

Jeisy-Scot et al. (2012) showed that TRL-7 plays a role in the induction of immune responses to a monovalent split vaccine against influenza A viruses. Geeraedts et al. (2008) showed that patients vaccinated with the whole inactivated viruses produce higher levels of anti-HA antibodies compared with patients vaccinated with split or subunit vaccines. Possibly, the difference has to do with activation of TRL-7 by viral RNA resent in vaccine containing the whole inactivated viruses. It is presumed that commercially available split and subunit vaccines are based only on the production of specific antibodies against hemagglutinin and viral neuraminidase, with no influence on the TRL-7 receptor system. The development of an influenza vaccine containing an adjuvant stimulating these receptors would be beneficial. Yet, such a vaccine is unavailable at present.

Influenza is a serious viral disease of the respiratory tract. Because of its contagiousness and epidemic spreading, influenza is still an important problem of public health. The most effective form of protection against influenza virus is vaccination, which is recommended in broad groups of patients, including both healthy population and high risk groups such as hemodialyzed patients. These patients are usually immunocompromised and thus there is a need to develop a vaccine that would evoke a stronger immunogenic response (Jeisy-Scot et al. 2012).

In conclusion, vaccination with a subunit influenza vaccine does not change the level of soluble

TRL-2, TRL-4, TRL-7 and pro-inflammatory cytokines either in patients on hemodialysis or in healthy subjects, despite that these biomarkers are increased in immunodeficient patients. The study failed to substantiate the presence of correlations between Toll-like receptors or pro-inflammatory cytokines and the immune response to influenza vaccination. On the other hand, the study showed that TRLs are increased in hemodialyzed patients, which may enhance the anti-inflammatory IL-10 and counter the downgrade of the immune response to influenza vaccine.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

References

Beutler B (2004) Innate immunity: an overview. Mol Immunol 40:845–859

Beutler B, Wagner H (2002) Toll-like receptor family members and their ligands. Curr Top Microbiol Immunol 270:121–143

Beutler B, Hoebe K, Du X, Ulevitch RJ (2003) How to detect microbes and respond to them: the Toll-like receptors and their transducers. J Leukoc Biol 74:479–485

Boehme KW, Compton T (2004) Innate sensing of viruses by Toll-like receptors. J Virol 78:7867–7873

Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C (2004) Innate antiviral responses by means of TRL-7-mediated recognition of single-stranded RNA. Science 303:1529–1531

Geeraedts F, Goutagny N, Hornung V, Severa M, de Haan A, Pool J, Wilschut J, Fitzgerald KA, Huckriede A (2008) Superior immunogenicity of inactivated whole virus H5N1 influenza vaccine is primarily controlled by Toll-like receptor signaling. PLoS Pathog 4, 10.1371/journal.ppat.1000138

Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S (2004) Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303:1526–1529

Janssens S, Beyaert R (2003) Role of Toll-like receptors in pathogen recognition. Clin Microbiol Rev 16:637–646

Jeisy-Scot V, Kim JH, Davis WG, Cao W, Katz JM, Sambhara S (2012) TRL-7 recognition is dispensable for influenza virus A infection but important for the induction of hemagglutinin-specific antibodies in response to the 2009 pandemic split vaccine in mice. J Virol 86:10988–10998

Kato HS, Sato M, Yoneyama M, Yamamoto S, Uematsu K, Matsui T, Tsuimura K, Takeda T, Fujita O, Takeuchi O, Akira S (2005) Cell-type specific involvement of RIG-1 in antiviral response. Immunity 23:19–28

Kazimierczak K, Kopec W, Klinger M (2007) Toll-like receptors (TLR) in the pathogenesis of kidney diseases. Pol Merkur Lekarski 23(137):382–385 (in Polish)

Koyama S, Ishii KJ, Kumar H, Tanimoto T, Coban C, Uematsu S, Kawai T, Akira S (2007) Differential role of TLR- and RLR-signaling in the immune responses to influenza A virus infection and vaccination. J Immunol 179:4711–4720

Koyama S, Aoshi T, Tanimoto T, Kumagai Y, Kobiyama K, Tougan T, Sakurai K, Coban C, Horii T, Akira S, Ishii KJ (2010) Plasmacytoid dendritic cells delineate immunogenicity of influenza vaccine subtypes. Sci Transl Med 2:25ra24. doi:10.1126/scitranslmed.3000759

Lebouder E, Rey-Nores JE, Rushmere NK, Grigorov M, Lawn SD, Affolter M, Griffin GE, Ferrara P, Schiffrin EJ, Morgan BP, Labéta MO (2003) Soluble forms of Toll-like receptor (TLR)2 capable of modulating TRL2 signaling are present in human plasma and breast milk. J Immunol 171:6680–6689

Liew FY, Xu D, Brint EK, O'Neill LA (2005) Negative regulation of Toll-like receptor-mediated immune responses. Nat Rev Immunol 5:446–458

Martin M, Michalek SM, Katz J (2003) Role of innate immune factors in the adjuvant activity of monophosphoryl lipid A. Infect Immun 71:2498–2500

Orme I (2004) Adaptive immunity to mycobacteria. Curr Opin Immunol 7:58–61

Pang IK, Iwasaki A (2011) Inflammasomes as mediators of immunity against influenza virus. Trends Immunol 32:34–41

Sabroe I, Read RC, Whyte MK, Dockrell DH, Vogel SN, Dower SK (2003) Toll-like receptors in health and disease: complex questions remain. J Immunol 171:1630–1635

Schmitz N, Beerli RR, Bauer M, Jegerlehner A, Dietmeier K, Maudrich M, Pumpens P, Saudan P, Bachmann MF (2012) Universal vaccine against influenza virus: linking TLR signaling to anti-viral protection. Eur J Immunol 42:863–869

Takeda K, Akira S (2004) TLR signaling pathways. Semin Immunol 16:3–9

Werkling D, Jungi TW (2003) TOLL-like receptors linking innate and adaptive immune response. Vet Immunol Immunopathol 91:1–12

Wolf G, Bohlender J, Bondeva T, Roger T, Thaiss F, Wenzel UO (2006) Angiotensin II upregulates toll-like receptor 4 on mesangial cells. J Am Soc Nephrol 17:1585–1593

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 5: 41–46
DOI 10.1007/5584_2014_33

Published online: 14 October 2014

Vaccination Status and Perception of Influenza Vaccination in the Polish Population

A. Wozniak-Kosek, M. Mendrycka, A. Saracen, J. Kosek, E. Hallmann-Szelińska, B. Zielnik-Jurkiewicz, and B. Kempińska-Mirosławska

Abstract

Influenza is still considered to be the most dangerous infectious disease of the twenty-first century. Outbreaks of influenza occur worldwide and affect all ages. The disease is severe, often with threatening complications and can lead to death, albeit many people have it in disregard. One of the main ways of preventing the disease is vaccination. The most effective method of prevention against influenza illness and its complications are annual vaccinations. Vaccinations, although recommended by the Ministry of Health in Poland, are not subject to refund. This paper presents the results of research conducted with the use of an anonymous questionnaire containing 18 questions to be completed by parents of school children, students of technical and medical universities, patients, medical staff, and people over 65 years of age. The study was conducted in the season of 2012/2013 in Poland. The survey involved 1,203 people in various age groups with different educational background. The analysis of the study shows that respondents very rarely use this form of prevention. Even if the vaccination were refunded, the percentage of people vaccinated against

A. Wozniak-Kosek (✉) and E. Hallmann-Szelińska
Department of Influenza Research, National Influenza Center, National Institute of Public Health-National Institute of Hygiene, 24 Chocimska St., 00-791 Warsaw, Poland
e-mail: kaj12@poczta.fm

M. Mendrycka
Department of Organic Materials Technology, Faculty of Material Science, Technology and Design, Kazimierz Pulaski University of Technology and Humanities in Radom, Radom, Poland

A. Saracen
Faculty of Health Sciences and Physical Culture, Kazimierz Pułaski University of Technology and Humanities in Radom, Radom, Poland

J. Kosek
Otolaryngology Clinic, Military Institute of Medicine, Warsaw, Poland

B. Zielnik-Jurkiewicz
Ear, Nose and Throat Department, Children's Hospital, Warsaw, Poland

B. Kempińska-Mirosławska
Department History of Medicine and Pharmacy, Medical University of Łódź, Łódź, Poland

influenza would not increase significantly. Among the respondents, those who are in favor of influenza vaccination are in the minority.

Keywords
Influenza virus types A and B • Influenza like virus • Epidemiological surveillance

1 Introduction

Influenza remains a serious medical problem. An effective method of preventing infections caused by influenza viruses is annual vaccination. For patients at risk of complications from influenza, vaccination of this type is recommended by the World Health Organization and the Advisory Committee on Immunization Practices of the Centers for Disease Control and Prevention (CDC 2000). In Poland in the years 1969–1999, among the majority of infectious diseases (70 %) was caused by respiratory viruses, including the influenza virus, about 23 % was due to other viral factors, and only 7 % of the cases were of non-viral aetiology (Kruszewski and Brydak 2000).

Reports of outbreaks of influenza that date back to ancient times indicate that the disease had proceeded with very high morbidity and mortality. A characteristic feature of influenza is a tendency for the annual incidence of epidemics and occasional pandemics that occur with a frequency of every 10–40 years, spreading then to the whole continent. The incidents occur in all ages, races, and regions of the world. In the twentieth century, three pandemics occurred across the world. The most severe pandemic, known as the 'Spanish flu', caused 50–100 million deaths due to severe course of the disease or complications. Health costs associated with influenza can reach billions of dollars (Lew 1996). The above mentioned factors cause influenza to be one of the most extensively researched diseases around the world, both in terms of the laboratory research and public health.

Outbreaks of influenza occur seasonally in temperate climates of both hemispheres mainly in autumn and winter months (September–April). The disease can occur at any age, but most commonly affects children and adolescents. The factor conducive to getting sick is smoking. However, the greatest mortality occurs among the elderly, probably due to other chronic co-morbid conditions (Kark et al. 1982; Kark and Lebiush 1981). The reliability of this data is incomplete, because the majority of cases are not confirmed virologically, and the causes of death are supposedly post-infectious complications (Douglas 1976). The situation in modern times seems not much different.

2 Methods

This study has been accepted by an institutional review Board for Human Research. The investigation was based on a questionnaire addressed to various social and professional groups in such a way as to get the widest possible knowledge of the Polish public awareness about the influenza vaccination. In total, the survey included a group of 1,335 people. There were 1,012 (75.8 %) women and 323 (24.2 %) men; 67 % of respondents were urban dwellers. The education status of respondents was as follows: 455 (34.1 %), had university education, 850 (63.7 %) had high school education, and vocational and primary education declared 23 and 7 persons, representing 1.7 % and 0.5 %, respectively. The participants belonged to the following groups:

- students of different specialties of the University of Science and Humanities in the city of Radom;
- students of the Faculty of Medicine and Pharmacy of the Medical University of Lodz;

- postgraduate students majoring in medical analytics at the Medical University of Gdansk;
- students of the University of the Third Age in Czestochowa, at the Czestochowa University of Technology;
- parents of children attending kindergartens and schools in a town near Radom;
- parents of children attending kindergarten and a primary school in Warsaw;
- young people studying at the School of Fine Arts in a town near Radom.

The questionnaire, containing 20 items, was developed at the Department of Influenza Virus Research Institute, National Center for Influenza of the National Institute of Public Health-National Institute of Hygiene in Warsaw, Poland. The results were entered into Excel spreadsheet assigning to each response a value of 0 or 1. This made it possible to determine the percentage of individual responses and to perform statistical calculations. Detailed results obtained by applying the respondents' answers to the questions were presented in tabular and graphical forms.

3 Results

One of the major factors influencing the reduction of morbidity is to promote influenza vaccination. The investigation conducted identified differences in the methods of acquiring the knowledge about vaccination by the respondents. More than 30 % of respondents were the people who made a decision about vaccination due to the opinion of their physicians. An important role was also played by 'buzz marketing', i.e., the opinions of members of the immediate family, which amounted to 13.1 %. The results show that advertising in the mass media and leaflets to encourage vaccinations factored in undertaking the decision in 33.6 % of respondents. However, a group of people over 64 years of age had a distrust of ads presented on the television. They also rarely or never use modern media such as the internet (13 % of respondents). This group of respondents preferred direct contact with the staff of health institutions and submitted to their recommendations and suggestions concerning the vaccination procedure.

There was a low level of positive answers of respondents declaring their intention to be vaccinated if the vaccination were refunded, amounting to just 24.9 %; 33.2 % of persons were negative and 41.9 % were undecided in this regard, out of the 1,058 persons who responded to this question. The results show that the best time for vaccination against influenza, according to the respondents, was the moment the vaccine becomes available on the market for the epidemic season – 217 (21.2 %) persons or if a person is at higher risk (26.7 %). The largest group were the undecided – 443 (41.9 %), while the remaining 277 persons did not provide any answer to this item. Figure 1

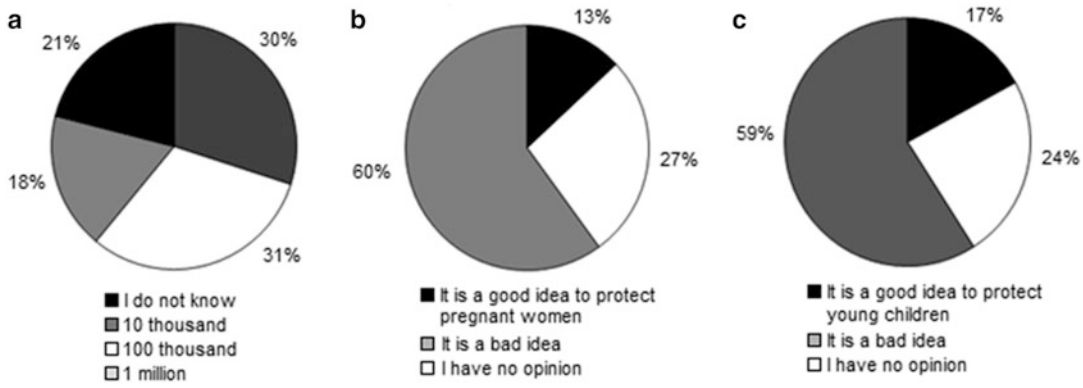


Fig. 1 Data from respondents concerning the presumed number of annual deaths from influenza and its complications worldwide (*Panel A*), the vaccine-related protection of pregnant women (*Panel B*), and young children (*Panel C*)

shows low awareness of the mortality caused by influenza, and of the protection of pregnant women and young children through seasonal influenza vaccinations.

4 Discussion

Influenza in children is a significant clinical, epidemiological, and economical problem, but the present investigation shows that public awareness in this regard was not significant. The respondents regard the protection of children over 6 months of age as beneficial in 59 %, while only 13 % of them believed that vaccination of pregnant women is a good way to protect the mother and the child. The low percentage above outlined is rather surprising in view of the data of others showing that 24 % of all outpatient visits concerning sick children are due to influenza (Nitsch-Osuch et al. 2012). Thus, influenza in the pediatric population seems an underrated and under-evaluated issue. The reason for that may be non-specific symptoms and a relative difficult conclusive diagnosis.

Influenza virus infection is particularly dangerous for pregnant women and for new-borns and infants. The risk of birth defects increases if one catches influenza during pregnancy. On the other hand, influenza in neonates, although rare, is associated with a high risk of complications. Following the recommendations of the Advisory Committee on Immunization Practices of the Centers for Disease Control and Prevention (CDC 2000), all women who are pregnant or are planning to be pregnant during the influenza season should be vaccinated. Observational research shows that antibodies produced against influenza by a woman during pregnancy also protect the infant during the first months of life.

Infections caused by the influenza virus cause a significant increase in mortality in high-risk groups, the increase in medical costs, and substantial economic losses. Influenza remains a serious public health problem that requires reaching for all available methods of control and prevention in order to minimize the annual effects. Currently, the recommendations for influenza vaccination are issued not only by the Advisory Committee on Immunization Practices of the Centers for Disease Control and Prevention (CDC 2000), but also by many Scientific Medical Societies. Recommended vaccines are safe and do not cause serious adverse reactions, and their effectiveness in preventing influenza illness and post-influenza complications is high. Vaccination of 80 % of the worldwide population would give savings of $2.1 billion per year. International studies have shown that the direct costs of influenza are four times higher than the cost of vaccination. This indicates the need of prophylaxis by vaccination (Lew 1996; Nichol et al. 1990).

Meanwhile, despite the best efforts of the World Health Organization to determine the actual composition of the vaccine and to increase its safety and efficiency, the influenza vaccines have not yet been widely used. The technology of production of influenza vaccines (chick embryo) does not allow obtaining the number of doses needed to vaccinate the entire populations. Thus, in the first place vaccination should be recommended for persons at increased risk of complications and mortality after influenza infection, and for epidemiological indications (major population centres, medical staff, large workplaces, etc.). The level of use of influenza vaccine in a given country is defined by providing a number of doses per 1,000 persons. For example, at the beginning of the twenty-first century the use of vaccine was 140 doses per 1,000 inhabitants in Iceland and France, 90–100 in Canada, USA, Australia, and the U.K., whereas in Sweden the indicator was 60–70 doses. In Poland, the use of vaccine in the epidemic season 2000/01 increased 178 times compared with the season 1992/93, from 0.52 doses to 92.4 doses per thousand inhabitants (Brydak 2001).

The obligation to report the cases of influenza was introduced in Poland in 1936. In the years 1946–2000, there were 65 million cases of influenza, ending up with 17,000 deaths, recorded in Poland. Since 1994, influenza vaccinations in Poland can be found in the calendar of recommended vaccinations. In recent years,

several millions people became voluntarily vaccinated, which constitutes a substantial progress from the 1980s when influenza vaccinations had been almost never applied. The rarity of vaccination during that time caused the cessation of vaccine production and closure of a vaccine plant in Cracow. Vaccinations on a larger scale were resumed in the epidemic seasons 1990/91 and 1991/92 with the use of a subunit vaccine within the American Foundation 'Project Hope' (a total of 50,000 doses and 100,000 doses, respectively). Vaccinations were carried out in all voivodeships of Poland in proportion to the number of inhabitants. People from 6 months to 85 years of age were vaccinated. The results indicated the effectiveness of vaccine in terms of seroconversion and hence, the desirability of using this type of prophylaxis (Brydak 1998). In Poland, however, there is no tradition to vaccinate, although in recent years medical staff and the public at large are becoming interested in this form of prophylaxis (Brydak 2001). Scarce knowledge in the society about post-influenza complications is a major cause of the relatively low interest in such prevention. Research carried out among the staff of nursing care at Neonatal Departments and Intensive Care Units in Germany showed that only 15–17 % were vaccinated at least once during a lifetime, 9 % – twice, and 2 % thrice (Glathe and Langer 1995).

Annual vaccination of people with chronic diseases, posing a higher risk of complications and death after influenza infection was recommended by the World Health Organization and the Advisory Committee on Immunization Practices of the Centers for Disease Control and Prevention (CDC 2000) for the first time in 1964. Since then, the groups recommended for annual influenza vaccination gradually extended to residents of nursing homes, medical staff, employees of services essential for the economy, and the general public. The effectiveness of inactivated influenza vaccine in preventing infection in healthy subjects was assessed at the turn of the twentieth and twenty-first centuries to be 70–90 % (Palache 1992; Powers 1992; Powers et al. 1984). A key to effective influenza vaccinations is that all patients who need to be immunized are proposed vaccination (CDC 2000; Brydak 1998). The fact that the Advisory Committee on Immunization Practices of the Centers for Disease Control and Prevention (CDC 2000) lowered the age of adults who are recommended to be vaccinated from 65 to 50 years of age shows how dangerous an infection caused by the influenza virus is considered (CDC 2000).

5 Conclusions

1. Knowledge about vaccinations against influenza is insufficient.
2. Vaccinations against influenza are poorly accepted by medical personnel. Promoting prophylaxis against influenza, particularly among medical professionals should be priority.
3. Data on influenza vaccination in children show that parents do not vaccinate their children because they are afraid of vaccination in general, and in case of influenza they do not have enough knowledge about the benefits from vaccination.
4. The results of a survey conducted in the present study lead to the generalized conclusions on influenza vaccination and the perception of vaccination in various groups of society. In the season 2012/2013, out of the 1,335 respondents, 89 persons, i.e., 8.2 % were vaccinated, the vast majority of 999 persons (91.8 %) were not vaccinated, and 247 (18.5 %) persons did not respond to the question. The percentage of vaccinated people still seems to remain below any reasonable and rationally justified level.

Conflicts of Interest Authors declare no conflict of interests in relation to this article.

References

Brydak LB (1998) Flu and its prevention. Springer PWN, Warsaw, pp 1–216 (in Polish)

Brydak LB (2001) Efficacy and safety of vaccination against influenza in children. Pediatr Pol 9:631–638 (Article in Polish)

CDC – Centers for Disease Control and Prevention (2000) Delayed supply of influenza vaccine and adjunct CDC

influenza vaccine recommendations for the 2000–01 influenza season. Advisory Committee on Immunization Practices. MMWR Morb Mortal Wkly Rep 49:619–622

Douglas RG (1976) Influenza: the disease and its complications. Hosp Pract 11:43–50

Glathe H, Langer W (1995) Influenza vaccination in older patients. Immunogenicity, epidemiology and available agents. Drug Ther 6:368–387

Kark JD, Lebiush M (1981) Smoking and epidemic influenza-like illness in female military recruits: a brief survey. Am J Public Health 71:530–532

Kark JD, Lebiush M, Rannon L (1982) Cigarette smoking as a risk factor for epidemic A/H1N1/influenza in young men. N Engl J Med 307:1042–1046

Kruszewski K, Brydak LB (2000) The epidemiology and history of influenza. Biomed Pharmacother 54:188–195

Lew E (1996) French economic evaluations of influenza and influenza vaccination. Pharmacoeconomics 9:62–66 (Article in Polish)

Nichol KL, Kom JE, Margolis KL, Poland GA, Petrel R (1990) Achieving the national heath objective for influenza immunization: success of an institution – wide vaccination program. Am J Med 89:156–160

Nitsch-Osuch A, Wozniak-Kosek A, Brydak LB (2012) Seasonal influenza in children – the underestimated problem. Med Overv 69:1029–1214

Palache AM (1992) Influenza subunit vaccine – ten years experience. Eur J Clin Res 3:117–138

Powers DC (1992) Immunological principles and emerging strategies of vaccination for the elderly. J Am Geriatr Soc 40:81–94

Powers DC, Hayden FG, Samuelson I, Gwaltney JM Jr (1984) Immune response of adults to sequential influenza vaccination. J Med Virol 14:169–175

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 5: 47–54
DOI 10.1007/5584_2014_36

Published online: 24 September 2014

Immune Efficacy of First and Repeat Trivalent Influenza Vaccine in Healthy Subjects and Hemodialysis Patients

Agnieszka Mastalerz-Migas, Maria Bujnowska-Fedak, and Lidia B. Brydak

Abstract

Influenza vaccination is recommended to patients from groups at risk and to healthy persons alike. It is not completely clear whether persons vaccinated every year benefit more from the vaccination in any given season in comparison with those who are vaccinated for the first time. The aim of the study was to analyze whether influenza vaccination in previous seasons influences the response to ongoing vaccination in the healthy population and in hemodialyzed patients. The outcome measure was the production of anti-hemagglutinin antibodies in 71 hemodialyzed patients (Group A) and 63 patients of primary healthcare clinic, without chronic renal failure (Group B). The patients of these two groups were subdivided into never vaccinated before and previously vaccinated against influenza. After the current vaccination, significantly lower levels of anti-A/H1N1/ antibodies were found in the hemodialyzed compared with non-hemodialyzed previously vaccinated, but not unvaccinated, patients. The hemodialyzed patients, previously unvaccinated, had at baseline significantly lower levels of anti-A/H3N2/ and anti-B antibodies than those who were previously vaccinated; the differences were no longer significant after the current vaccination. We conclude that although antiflu immunization in previous seasons leads to higher baseline antibody titers in hemodialysis compromised patients, which is less evident in non-hemodialyzed patients, it is of little influence on the immunoresponse to current influenza vaccination, in both hemodialyzed and non-hemodialyzed patients.

A. Mastalerz-Migas (✉)
Department of Family Medicine, Medical University of Wroclaw, 1 Syrokomli St., Wroclaw 51-141, Poland

Public Higher Professional Medical School in Opole, Opole, Poland
e-mail: agnieszka.migas@gmail.com

M. Bujnowska-Fedak
Department of Family Medicine, Medical University of Wroclaw, 1 Syrokomli St., Wroclaw 51-141, Poland

L.B. Brydak
Department of Influenza Research, National Influenza Center, National Institute of Public Health-National Institute of Hygiene, Warsaw, Poland

Department of Immunology, Biology Division, University of Szczecin, Szczecin, Poland

Keywords
Hemagglutinin • Hemodialysis • Influenza • Vaccination

1 Introduction

Influenza vaccination is recommended to more and more groups of patients because vaccination indications in risk groups have been broadened. The Advisory Committee on Immunization Practice (ACIP) and a number of scientific societies have recommend influenza vaccination to chronic immunosuppressed patients, including hemodialyzed patients (ACIP 2011). Infections of different etiology are the second most common cause of death among patients undergoing chronic hemodialysis therapy. It is worth realizing that the immune system dysfunction can worsen the course of any severe disease, lead to the occurrence of post-influenza complications, and decrease the influenza vaccine efficiency (Kunisaki and Janoff 2009).

Doubts arise about the efficiency of influenza vaccine in hemodialysis patients due to the immune system insufficiency and repeat hemodialysis procedures (Ott et al. 2012). Most studies conducted in hemodialyzed patients have indicated that the serological response to vaccination is weaker than that in healthy persons, although the patients do produce the protective levels of antibodies (Mastalerz-Migas et al. 2013; Eiselt et al. 2010; Vogtländer et al. 2004; Cavdar et al. 2003; Antonen et al. 2000). Immune deficiency in hemodialyzed patients is multifactorial and includes incorrect activation of the complement or disorders of neutrophil and T and B lymphocyte function (Cohen and Hörl 2012; Vaziri et al. 2012).

Immune insufficiency in hemodialyzed patients prompts medical recommendations to introduce changes in vaccination scheme or to increase the dose in comparison with healthy persons in case of some other vaccinations (e.g., vaccinations against hepatitis B). With regard to influenza, there are no such recommendations and administration of a single vaccine dose in a given epidemic season is recommended. Seasonal influenza vaccination is recommended, because a new composition of vaccine is developed every epidemic season due to a significant antigen variation of the virus. However, one may surmise that 'immune memory' related to each seasonal shot improves the current immune response, although the issue is unsettled. The aim of the present study was, therefore, to assess whether previously applied influenza vaccination would enhance the immune response to the current vaccination in the healthy population and in chronically hemodialyzed patients, as compared with the first time vaccinees.

2 Methods

The study was performed in conformity with the Declaration of Helsinki for Human Experimentation of the World Medical Association and was approved by the Ethics Committee of the Medical University of Wroclaw, Poland. In addition, patients enrolled into the study gave written informed consent.

There were two groups of the study patients: 71 hemodialyzed patients (F/M – 23/48), mean age 65 $\pm$ 15 years; mean time of dialysis therapy 39 $\pm$ 32 months (Group A) and 63 patients of a primary health care clinic (F/M – 41/22), without chronic renal failure, mean age 44 $\pm$ 21 years (Group B). The patients of each group were stratified into two subgroups:

- A1 – hemodialyzed who were never previously vaccinated against influenza (n = 24/71; 33.8 %)
- A2 – hemodialyzed who were vaccinated against influenza in previous seasons (n = 47/71; 64.2 %)
- B1 – non-hemodialyzed who were never previously vaccinated against influenza (n = 27/63; 42.8 %)
- B2 – non-hemodialyzed who were vaccinated against influenza in previous seasons (n = 36; 57.2 %)

There was an additional sham group of 39 - age-matched hemodialyzed patients, never vaccinated before and not going to be currently vaccinated whose humoral status was assessed before and after a month's time-lapse. The rationale behind having this group was to check if severely sick patients do not have inherent immunogenic fluctuations which could get overlaid, and thus mask the true responses to vaccination. We found that hemodialyzed patients, have a stable, although it might be deficient, immunogenic pattern, with no appreciable fluctuations as based on the level of anti-hemagglutinin (anti-HA) antibodies (data not shown). All the patients filled out a self-reported questionnaire on past vaccinations, post-vaccination reactions, past events of influenza, causes of renal failure, and the length of hemodialysis therapy.

Vaccinated patients got a single dose of the influenza vaccine Agrippal (Novartis; Frimley, Camberley, Surrey, UK), which is a deactivated subunit vaccine, i.e., a vaccine that contains only the hemagglutinin and neuraminidase surface glycoproteins of the virus. One vaccine dose contained 15 μg of hemagglutinin from three different influenza strains each: A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2), and B/Brisbane/60/2008 in a volume of 0.5 ml. The vaccine was injected into the deltoid muscle. Vaccination was performed in September–-October 2009 and the patients were instructed to report symptoms of upper respiratory tract infections, which would enable to perform diagnostics for influenza in the epidemic season September 2009/March 2010.

Venipuncture was performed twice in each patient, before and 1 month after vaccination, to draw 10 ml of blood on clot to obtain the serum for the assessment of the serologic response to the vaccination, consisting of the level of anti-HA antibodies. Samples were stored at −70 °C until further collective use.

Anti-HA antibodies were assayed using a hemagglutination inhibition assay (Receptor Destroying Enzyme, Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instruction. After the removal of non-specific hemagglutination inhibitors, the highest serum dilution in which the blood cell agglutination was still inhibited, was assumed to be the anti-HA antibody titer for a given viral strain. The humoral response was assessed based on the following parameters:

- geometric mean titer (GMT) of antibodies before and after influenza vaccination;
- mean of by how many times antibody index (MFI) increases after influenza vaccination;
- protection rate, the percentage of patients with antibody titer $\geq$1:40 before and after influenza vaccination;
- response rate, the percentage of patients with at least a fourfold increase in antibody titer after vaccination

Statistical comparisons of inter-group differences were performed with a *t*-test for normally distributed data or Mann Whitney U and Wilcoxon tests for not normally distributed data. Statistical significance was defined as $p < 0.05$. Statistical calculations were performed using a commercial Statistica 7.1 package.

3 Results

A statistically lower GMT of anti-A/H1N1/ antibodies a month after the current vaccination was found in the hemodialyzed compared with non-hemodialyzed patients vaccinated in previous seasons (11.6 *vs*. 38.5, respectively; $p < 0.001$). The above difference did not assume significance in patients who were previously unvaccinated. The hemodialyzed patients unvaccinated in previous seasons (A2) had lower titers of anti-A/H3N2/ and anti-B antibodies before the current vaccination compared with the hemodialyzed vaccinated ones (1.8 *vs*. 6.2; $p = 0.003$, and 1.7 *vs*. 3.7; $p = 0.03$, respectively); the significance of these differences abated after the current vaccination (Table 1).

The MFI parameter indicates by how many times the antibody titer increases after vaccination. It is obvious that the increase in a person who has never been vaccinated before should be greater than in a person who has been vaccinated in previous season or has had contact with a

Table 1 The immune response to influenza vaccination depending on vaccination in previous infection seasons – geometric mean titer (GMT) of anti-hemagglutinin antibodies before and a month after the current vaccination

			GMT		
Antigen	Group	Current vaccination	Unvaccinated previously (A1, B1)	Vaccinated in previous seasons (A2, B2)	p
A/Brisbane/59/2007 (H1N1)	A	Before	1.3 A1	2.0 A2	0.27
		After	19.1 A1	**11.6 A2**	0.25
	B	Before	1.5	2.3	0.22
		After	37.4	**38.5**	0.64
	A *vs.* B	Before	p = 0.71	p = 0.59	
	A *vs.* B	After	p = 0.10	**p < 0.001**	
A/Brisbane/10/2007 (H3N2)	A	Before	**1.8**	**6.2**	**0.003**
		After	42.8	63.5	0.37
	B	Before	3.2	6.3	0.15
		After	55.4	67.3	0.71
	A *vs.* B	Before	p = 0.16	p = 0.61	
	A *vs.* B	After	p = 0.60	p = 0.96	
B/Brisbane/60/2008	A	Before	**1.7**	**3.7**	**0.03**
		After	26.5	**36.7**	0.32
	B	Before	**3.5**	2.5	0.33
		After	37.0	**25.7**	0.09
	A vs. B	Before	**p = 0.04**	p = 0.26	
	A *vs.* B	After	p = 0.99	**p = 0.01**	

Group A – hemodialyzed patients; Group B – non-hemodialyzed patients. GMT was assessed 1 month after vaccination

Table 2 Immune response to influenza vaccination depending on vaccination in previous infection seasons – mean fold increase (MFI) of antihemagglutinin antibodies a month after vaccination

		MFI		
Antigen	Group	Unvaccinated previously (A1, B1)	Vaccinated in previous seasons (A2, B2)	p
A/Brisbane/59/2007 (H1N1)	**A**	**14.3**	**5.8**	**p = 0.04**
	B	**24.4**	**16.4**	p = 0.18
	A *vs.* B	p = 0.26	**p = 0.002**	
A/Brisbane/10/2007 (H3N2)	**A**	**23.6**	**10.3**	**p = 0.004**
	B	**17.1**	**10.7**	p = 0.13
	A *vs.* B	p = 0.37	p = 0.71	
B/Brisbane/60/2008	**A**	**15.5**	**10.0**	p = 0.24
	B	**10.7**	**10.3**	p = 0.98
	A *vs.* B	p = 0.42	p = 0.49	

given virus, which blunts the response (13). Hence, in our study, the patients from subgroups never vaccinated before (A1, B1) had a higher MFI for A/H1N1/ and A/H3N2/ than those vaccinated in previous seasons (A2, B2), but the difference reached statistical significance only in the subgroup of hemodialyzed patients (A1-MFI = 14.3 *vs.* A2-MFI = 5.8; p = 0.04). Simultaneously, a statistically higher MFI for A/H1N1/ was found in healthy patients vaccinated before (B2) as compared with the group of hemodialyzed patients vaccinated before (A2) – MFI 16.4 *vs.* 5.8 (p = 0.002) (Table 2).

Table 3 Immune response to influenza vaccination depending on vaccination in previous infection seasons – protection rate before and a month after the current vaccination

Antigen	Group	Current vaccination	Protection rate (%)		p
			Unvaccinated previously (A1, B1)	Vaccinated in previous seasons (A2, B2)	
A/Brisbane/59/2007 (H1N1)	A	Before	0.0	4.3	p = 0.79
		After	**41.7**	**36.2**	p = 0.85
	B	Before	0.0	0.0	p = 0.31
		After	**74.1**	**72.2**	p = 0.90
	A *vs.* B	Before	p = 0.78	p = 0.6	
	A *vs.* B	After	**p = 0.039**	**p = 0.002**	
A/Brisbane/10/2007 (H3N2)	A	Before	12.5	**17.0**	p = 0.88
		After	58.3	70.2	p = 0.46
	B	Before	0.0	**0.0**	p = 0.31
		After	85.2	83.3	p = 0.88
	A *vs.* B	Before	p = 0.19	**p = 0.026**	
	A *vs.* B	After	p = 0.07	p = 0.26	
B/Brisbane/60/2008	A	Before	0.0	10.6	p = 0.24
		After	58.3	**70.2**	p = 0.46
	B	Before	0.0	0.0	p = 0.31
		After	55.6	33.3	p = 0.13
	A *vs.* B	Before	p = 0.78	**p = 0.002**	
	A *vs.* B	After	p = 0.93	p = 0.12	

Table 4 Immune response to influenza vaccination depending on vaccination in previous infection seasons – response rate a month after vaccination

Antigen	Group	Response rate (%)		p
		Unvaccinated previously (A1, B1)	Vaccinated in previous seasons (A2, B2)	
A/Brisbane/59/2007 (H1N1)	A	**41.7**	**34.0**	p = 0.53
	B	**74.1**	**72.2**	p = 0.87
	A *vs.* B	**p = 0.039**	**p = 0.001**	
A/Brisbane/10/2007 (H3N2)	A	58.3	68.1	p = 0.42
	B	81.5	77.8	p = 0.72
	A *vs.* B	p = 0.13	p = 0.46	
B/Brisbane/0/2008	A	58.3	**70.2**	p = 0.32
	B	55.6	**33.3**	p = 0.08
	A *vs.* B	p = 0.93	**p = 0.002**	

The protection rate for A/H1N1/ was higher 1 month after the vaccination in the group of healthy persons, regardless of the earlier vaccination. Hemodialyzed patients vaccinated in previous seasons (A2) had a higher protection rate for A/H3N2/ before the vaccination than the healthy patients (B2) (Tables 3 and 4).

The response rate in terms of anti-A/H1N1/ antibodies was higher in the group of healthy patients (B1, B2), independently of vaccination in previous seasons. In terms of response to B antigen, a statistically higher response rate was found in the hemodialyzed patients vaccinated in previous seasons (A2), as

compared with healthy patients vaccinated in previous seasons (B2).

4 Discussion

According to the criteria developed by the Committee for Proprietary Medicinal Products of the European Medicines Agency (EMA), an EU regulatory agency for the evaluation of medicinal products (EMA 1997), the following parameters must be taken into account when the serological response to influenza vaccination is assessed: MFI (mean fold antibody index) of anti-hemagglutinin antibodies, protection rate, and response rate, measured about 3 weeks after the vaccination.

It is assumed that anti-hemagglutinin antibody titers above 1:40 constitutes a sufficient level of protection against infection with influenza virus (Beyer et al. 2004). According to the guidelines of the Committee for Proprietary Medicinal Products of the European Medicines Agency (EMA 1997) on influenza vaccinations, the conversion rate (conversion rate = MFI, mean fold increase) for persons aged 18–60 is $\geq$2.5, and for persons older than 60 is $\geq$2.0. The norms of protection rate are $\geq$70 % for persons aged 18–60 and $\geq$60 % for persons older than 60. The norms of response rate are $\geq$40 % for persons aged 18–60 lat and $\geq$30 % for persons older than 60. Such values are indicative of an efficient response to vaccination (Brydak 2008).

The main protective effect of a vaccine is induced by the response to the A/H1N1/ hemagglutinin. Antibodies against A/H1N1/ subunit are capable of neutralizing the influenza viruses. The A/H3N2/ subtype of the virus is connected with a higher risk of hospitalization and death of the infected person than the A/H1N1/ subtype (Antonen et al. 2000). Production of antibodies depends on the immune history of the vaccinated person (Brydak 2008).

In the present study we showed that healthy patients gain a higher response and protection rates than hemodialyzed patients regardless of the influenza vaccination in previous seasons. Undoubtedly, this results from insufficiency of the immune system in the latter group of patients, which is also reported by others (Mastalerz-Migas et al. 2013; Vaziri et al. 2012; Cavdar et al. 2003; Antonen et al. 2000; Beyer et al. 1987).

It is worth noting that hemodialyzed patients who were vaccinated against influenza in previous seasons had appreciably higher GMT and protection rate, before the current vaccination in terms of anti-A/H3N2/ and anti-B antibodies than hemodialyzed patients vaccinated for the first time. This difference between the two groups of hemodialyzed patients was particularly expressed for anti-A/H1N1/ antibodies. This shows that despite the immune system deficiency, memory cells produce antibodies against antigens that the organism had contact with during earlier vaccination.

Künzel et al. (1996) found higher GMT and protection rate for all antibodies before the ongoing vaccination in healthy patients who were vaccinated before, compared with those awaiting the first vaccination. However, GMT values measured after a longer time (280 days) after vaccination were similar in both groups, which is indicative of a minor influence of previous vaccination on the immune response. Sasaki et al. (2008) and Ohmit et al. (2006) demonstrate that the A/H3N2/ antigen is probably more immunogenic that A/H1N1/, which may result in higher titers of anti-H3N2 antibodies maintained over longer periods.

Considering the immune response to influenza vaccination, earlier contact with the antigen can bear on the interpretation of results. Theoretically, the presence of persons who were seropositive for a given vaccine strain before vaccination in the study group could result in higher antibody GMT and protection rate values, which would indicate higher immunogenicity of a vaccine than it is in reality. Consequently, MFI and response rate values should be lower in this case, which, in turn, could lead to an underestimation of vaccine immunogenicity (Beyer et al. 2004; Künzel et al. 1996). In the present study we show that hemodialyzed patients who had never been never

vaccinated before had a higher MFI that those vaccinated in terms of anti-A/H1N1/ and anti-A/H3N2/ antibodies. This is an expected result, taken into account the considerations above outlined. Similar results have also been obtained by Sasaki et al. (2007).

Matsushita et al. (2012) found that influenza vaccination in persons aged 61 and older in a previous season results in higher GMT and protection rates before and after the current vaccination. These parameters decrease over a longer time (22 weeks), but their enhancement is sustained longer than it takes in seronegative persons.

We conclude that influenza immunization in previous seasons has a moderate or little influence on the response to current vaccination in both healthy and chronically hemodialyzed patients. Accordingly, influenza vaccination should be recommended to hemodialyzed patients every season in order to obtain immunologic protection against the influenza viruses that are currently present in the population. Physicians should have no misgivings concerning the value of influenza vaccinations in this group of chronically ill patients.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

References

ACIP – Advisory Committee on Immunization Practices (2011) Recommendations of the prevention and control of influenza with vaccines. MMWR Morb Mortal Wkly Rep 60:11–28

Antonen JA, Hannula PM, Pyhälä R, Saha HH, Ala-Houhala IO, Pasternack AI (2000) Adequate seroresponse to influenza vaccination in dialysis patients. Nephron 86(1):1402–1412

Beyer WE, Versluis DJ, Kramer P, Diderich PP, Weimar W, Masurel N (1987) Trivalent influenza vaccine in patients on hemodialysis: impaired seroresponse with differences for A-H3N2 and A-H1N1 vaccine components. Vaccine 5(1):43–48

Beyer WE, Palache AM, Lüchters G, Nauta J, Osterhaus AD (2004) Seroprotection rate, mean fold increase, seroconversion rate: which parameter adequately expresses seroresponse to influenza vaccination? Virus Res 103(1–2):125–132

Brydak LB (2008) Influenza, pandemic flu myth or a real threat? Rhythm, 1st edn (in Polish), Warsaw

Cavdar C, Sayan M, Sifil A, Artuk C, Yilmaz N, Bahar H, Camsari T (2003) The comparison of antibody response to influenza vaccination in continuous ambulatory peritoneal dialysis, hemodialysis and renal transplantation patients. Scand J Urol Nephrol 37(1):71–76

Cohen G, Hörl WH (2012) Immune dysfunction in uremia – an update. Toxins 4(11):962–999

Eiselt J, Kielberger L, Sedláčková T, Racek J, Pazdiora P (2010) High ferritin, but not hepcidin, is associated with a poor immune response to an influenza vaccine in hemodialysis patients. Nephron Clin Pract 115(2):147–153

EMA – European Medicines Agency, Committee for Proprietary Medicinal Products (1997) Note for guidance on harmonisation of requirements for influenza vaccines. CPMP/BWP/214/96. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003945.pdfl. Accessed 30 Dec 2013

Kunisaki KM, Janoff EN (2009) Influenza in immunosuppressed populations: a review of infection frequency, morbidity, mortality and vaccine responses. Lancet Infect Dis 9(8):493–504

Künzel W, Glathe H, Engelmann H, Van Hoecka C (1996) Kinetics of humoral antibody response to trivalent inactivated split influenza vaccine in subjects previously vaccinated or vaccinated for the first time. Vaccine 14(12):1108–1110

Mastalerz-Migas A, Steciwko A, Brydak LB (2013) Immune response to influenza vaccine in hemodialysis patients with chronic renal failure. Adv Exp Med Biol 756:285–290

Matsushita M, Takeuchi S, Kumagai N, Uehara Y, Matsushita C, Arise K, Seo H, Awatani T (2012) Prevaccination antibody titers can estimate the immune response to influenza vaccine in a rural community-dwelling elderly population. Vaccine 30(6):1101–1107

Ohmit SE, Victor JC, Rotthoff JR, Teich ER, Truscon RK, Baum LL, Rangarajan B, Newton DW, Boulton ML, Monto AS (2006) Prevention of antigenically drifted influenza by inactivated and live attenuated vaccines. New Engl J Med 355(24):2513–2522

Ott U, Sauerbrei A, Lange J, Schäfler A, Walther M, Wolf G, Wutzler P, Zell R, Krumbholz A (2012) Serological response to influenza A H1N1 vaccine (Pandemrix®) and seasonal influenza vaccine 2009/2010 in renal transplant recipients and in hemodialysis patients. Med Microbiol Immunol 201(3):297–302

Sasaki S, He XS, Holmes TH, Dekker CL, Mahmood K, Kemble GW, Arvin AM, Greenberg HB (2007) Comparison of the influenza virus-specific effector and memory B-cell responses to immunization of children and adults with live attenuated or inactivated influenza virus vaccines. J Virol 81(1):215–228

Sasaki S, He XS, Holmes TH, Dekker CL, Kemble GW, Arvin AM, Greenberg HB (2008) Influence of prior influenza vaccination on antibody and B-cell responses. PLoS One 3(8):e2975

Vaziri ND, Pahl MV, Crum A, Norris K (2012) Effect of uremia on structure and function of immune system. J Ren Nutr 22(1):149–156

Vogtländer NPJ, Brown A, Valentijn RM, Rimmelzwaan GF, Osterhaus AD (2004) Impaired response rates, but satisfying protection rates to influenza vaccination in dialysis patients. Vaccine 22(17–18): 2199–2201

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 5: 55–62
DOI 10.1007/5584_2014_38

Published online: 14 October 2014

First-Line Immunosuppressive Treatment in Children with Aplastic Anemia: Rabbit Antithymocyte Globulin

K. Pawelec, M. Salamonowicz, A. Panasiuk, U. Demkow, J. Kowalczyk, W. Balwierz, E. Zaleska-Czepko, A. Chybicka, K. Szmyd, T. Szczepanski, H. Bubala, M. Wysocki, A. Kurylak, J. Wachowiak, D. Szpecht, W. Młynarski, M. Bulas, M. Krawczuk-Rybak, E. Leszczynska, T. Urasinski, J. Peregud-Pogorzelski, A. Balcerska, B. Kaczorowska-Hac, and M. Matysiak

Abstract

Immunosuppressive therapy is the treatment of choice in children with acquired severe aplastic anemia (AA) and no HLA-matched family donor. The paper presents results of a multicenter study of 63 children with AA treated with rabbit antithymocyte globulin (r-ATG) and cyclosporine A as

K. Pawelec (✉), M. Salamonowicz, and M. Matysiak
Department of Pediatric, Hematology and Oncology, Medical University of Warsaw, 24 Marszalkowska St., Warsaw 00-576, Poland
e-mail: katarzyna.pawelec@litewska.edu.pl

A. Panasiuk, M. Krawczuk-Rybak, and E. Leszczynska
Department of Pediatrics, Oncology and Hematology, Medical University of Bialystok, Bialystok, Poland

U. Demkow
Department of Laboratory Diagnostics and Clinical Immunology of Developmental Age, Medical University of Warsaw, Warsaw, Poland

J. Kowalczyk
Department of Pediatric Hematology, Oncology and Transplantology, Medical University of Lublin, Lublin, Poland

W. Balwierz and E. Zaleska-Czepko
Department of Pediatric Oncology and Hematology, Jagiellonian University Medical College, Krakow, Poland

A. Chybicka and K. Szmyd
Department and Clinic of Pediatric Oncology, Hematology and Bone Marrow Transplantation, Medical University of Wroclaw, Wroclaw, Poland

T. Szczepanski and H. Bubala
Department of Pediatric Hematology and Oncology, Medical University of Silesia, Zabrze, Poland

M. Wysocki and A. Kurylak
Department of Pediatric Hematology, Oncology and Transplantology, Collegium Medicum in Bydgoszcz, Mikolaj Kopernik University, Bydgoszcz, Poland

J. Wachowiak and D. Szpecht
Department of Pediatric Hematology, Oncology and Transplantology, University of Medical Sciences, Poznan, Poland

W. Młynarski and M. Bulas
Department of Pediatric Oncology, Hematology and Diabetology, Medical University of Lodz, Lodz, Poland

T. Urasinski and J. Peregud-Pogorzelski
Department of Pediatrics, Hematology and Oncology, Pomeranian Medical University, Szczecin, Poland

A. Balcerska and B. Kaczorowska-Hac
Department of Pediatric, Hematology, Oncology and Endocrinology, Medical University of Gdansk, Gdansk, Poland

the first line treatment in the years 1996–2012. Therapeutic effects were evaluated at Days 112, 180, and 360. At Day 112, remission was achieved in 28 out of the 63 patients (44.4 %), complete remission in 10 patients (15.9 %), and partial remission in 18 (28.5 %). At Day 180, 31 patients (49.2 %) were in remission including 15 cases in complete (23.8 %), and 16 cases in partial remission (25.4 %). One year after therapy onset, 34 patients (64.9 %) were in remission including 24 patients (38.0 %) in complete and 10 (15.9 %) in partial remission. Relapse occurred in 4 patients, from 8 months up to 2 years and 2 months after remission. One child, 5 years after remission, was diagnosed with paroxysmal nocturnal hemoglobinuria. The estimated 10-year overall survival rate and 10-year event-free survival rate were 67 % and 57 %, respectively.

Keywords
Antithymocyte globulin • Aplastic anemia • Children • Cyclosporine • Relapse • Remission

1 Introduction

Acquired aplastic anemia (AA) is a rare pediatric disease characterized by peripheral pancytopenia and bone marrow hypoplasia (Passweg and Marsh 2010; Bagby et al. 2004). The disease etiology in about half of the cases still remains undefined. Immunological mechanisms play a key role in AA pathophysiology. The apoptosis of stem cells is likely mediated by activated cytotoxic T lymphocytes (CD8) that produce gamma-interferon, tumor necrosis factor, and interleukin-2 (Bagby et al. 2004).

Immunosuppressive treatment in patients with severe AA gives very good results, acting through blockade of immune mechanisms and subsequent restoration of bone marrow. Immunosuppression is considered the treatment of choice for children with severe AA and no HLA-matched family donor (Passweg and Marsh 2010; Pulsipher et al. 2011). Until recently, horse-derived ATG (h-ATG) was considered as the first-line therapy of severe AA, however rabbit-derived antithymocyte globulin (r-ATG) came into use due to unavailability of h-ATG in Poland. The aim of the study was to analyze the results of the first-line immunosuppressive therapy with r-ATG in children with severe AA and very severe AA in the years 1996–2012.

2 Methods

2.1 Patients

Retrospective analysis of medical histories of 63 patients treated in 11 centers of the Polish Pediatric Hematology Group in the years 1996–2012 was carried out. The patient characteristics were presented in Table 1. All children fulfilled the diagnostic criteria of severe aplastic anemia, and 9 children were diagnosed with a very severe form of the disease. These criteria were as follows: bone marrow hypocellularity <25 % of the age-reference

Table 1 Patients' characteristics

Number of patients	63
Age at treatment onset; mean ± SD, range (years)	10.5 ± 4.3, 0.5–17.5
Gender; males/females	36/27
Etiology, number of patients	
Idiopathic factors	45 (71.4 %)
Toxic exposure	6 (9.5 %)
Seronegative hepatitis	4 (6.4 %)
Hepatitis B Virus (HBV)	2 (3.2 %)
Hepatitis C Virus (HCV)	3 (4.7 %)
Cytomegalovirus (CMV)	1 (1.5 %)
Parvovirus B19	2 (3.2 %)
Staging of aplastic anemia	
Severe aplastic anemia	54 (85.7)
Very severe aplastic anemia	9 (14.3)

value or 25–50 % of hematopoietic cells representing <30 % of residual cells, plus the presence of two criteria related to the peripheral blood cell count – absolute neutrophil count $<0.5 \times 10^9$/L, reticulocytes $<20.0 \times 10^9$/L, or platelets $<20 \times 10^9$/L. A very severe form of AA was diagnosed when the neutrophil count was lower than 0.2×10^9/L (Camitta 2000). All patients were evaluated to exclude inherited bone marrow failure and paroxysmal nocturnal hemoglobinuria, and had no HLA-matched family donor. Screening for paroxysmal nocturnal hemoglobinuria was carried out using flow cytometry with analysis of CD55 and CD59 expression on neutrophils and red blood cells. The diagnostic procedure towards Fanconi anemia (chromosome instability) was undertaken as well.

2.2 Protocol

All enrolled patients received r-ATG (Lymphoglobulin, Genzyme; Cambridge, USA) intravenously in a dose of 3.75 mg/kg body weight on Days 1–5 and cyclosporine-A (Sandimmun Neoral Novartis; Pharma GmbH, Nurnberg; Germany) orally in a dose of 5 mg/kg body weight over Days 1–180. The dose of cyclosporine was modified according to its serum concentration to keep it in a range of 100–200 ng/ml. Granulocyte colony-stimulating factor (Fligrastim-Neupogen, Amgen; Thousand Oaks; USA) was given subcutaneously or intravenously only to patients with severe infections non-responding to antibiotics and antifungal medications. The presence of remission was evaluated at Days 112, 180, and 360 from the onset of treatment. Complete remission was defined as the absolute neutrophil count $>1.5 \times 10^9$/L, platelets $>100 \times 10^9$/L, and hemoglobin concentration >11.0 g/L. Partial remission was diagnosed when the neutrophil count was $>0.5 \times 10^9$/L, platelets $>20 \times 10^9$/L, and hemoglobin concentration >8.0/L.

2.3 Statistical Analysis

The Kaplan-Meier estimator was used to draw survival curves and curves showing the time of disease progression and relapse. Overall survival, expressed in years, was defined as the interval between the diagnosis and death of a patient. The event free survival, expressed in years, was defined as the time from the diagnosis to death, disease relapse, or the last follow-up. The time to disease relapse was considered as the number of years between the diagnosis of AA and its relapse. If no demise or relapse occurred, the last follow-up with the evaluation of treatment response, was considered as right-censoring. The longest follow-up period equaled 10 years. All statistical tests carried out during the analysis considered a p-value of 0.05 and confidence intervals that did not include 1 as defining significance. Statistical analysis was carried out with a commercial package Stata ver. 11.0.

3 Results

3.1 Response to Immunosuppressive Treatment

At Day 112, complete remission occurred in only 10 patients (15.9 %) and partial by 18 (28.5 %); in total in 28 out of the 63 treated patients (44.4 %) (Table 2). At Day 180, complete remission occurred in 15 patients (23.8 %) and partial in 16 (25.4 %); in total in 31 patients (49.2 %). One year after the onset of therapy, complete remission occurred in 24 patients (38.0 %) and partial in 10 (15.9 %); in total in 34 patients (64.9 %).

The response to treatment in relation to the disease severity at Day 360 is as follows: in the group of nine children with very severe AA, complete remission occurred in one child (11.1 %), partial in three (3.3 %), and five

Table 2 Response to rabbit antithymocyte globulin (r-ATG)

	Day 112	Day 180	Day 360
CR	10	15	24
PR	18	16	10
NR	28	23	14
Death	10	12	15

CR complete remission, *PR* partial remission, *NR* non-responders

children (55.5 %) did not presented any response. In total, therapy was effective to some extend in 4 patients (44.4 %). On the other hand, 30 patients out of the 54 children with severe AA responded to treatment; complete remission was observed in 23 (42.5 %) and partial PR in 7 (13.0 %). The group of patients with severe AA had better treatment results in comparison with the children with very severe AA ($p = 0.003$). Remission was not achieved in 14 out of the 63 children (22.2 %) after 1 year from the onset of therapy.

The treatment course with r-ATG was repeated in four cases and three (75.0 %) of them have remained in complete remission until today. Hematopoietic stem cell transplantation from unrelated donors was carried out in 11 patients out of the 15 non-responders.

Table 3 Causes of deaths over the treatment course

	Before Day 90	After Day 90
Infectious causes:	2	5
E. coli,	1	2
S. aureus		
E. cloacae,	1	
E. faecium,		1
P. aeruginosa,		1
K. pneumoniae		1
Cerebral hemorrhage	1	1
Others:		
Pancreatitis		1
Pneumonia & pleural empyema	1	
Cerebral mycosis		1
Serum sickness	1	
Unknown		3
All	5	10

3.2 Relapses

Severe aplastic anemia relapsed in four patients (6.3 %) out of the 63 children treated with r-ATG. In two of them the course of r-ATG was repeated and one underwent hematopoietic stem cell transplantation from unrelated donors. The estimated probability of no relapse was 98 % after the second, third, fourth, and fifth year of follow-up (95%CI 0.86; 0.99). The estimated probability of no relapse during the next 6–10 years was 93 % (95%CI 0.72; 0.98).

3.3 Clonal Transformation

One child, 5 years after achieving remission, was diagnosed with paroxysmal nocturnal hemoglobinuria. Secondary myelodysplastic syndrome or acute myeloblastic leukemia were not observed in the study group.

3.4 Survival Analysis

Fifteen deaths (23.8 %) out of the 63 patients, including 5 early demises (up to Day 90 from treatment onset) and 10 late (after Day 90) were observed in the study group. The major causes of death were severe infections (7 cases) and cerebral hemorrhage (2 cases) (Table 3).

The estimated 10-year overall survival rate and 10-year event-free survival rate were 67 % and 57 %, respectively. The effectiveness of the first-line treatment with r-ATG was inferior to horse antithymocyte globulin. While analyzing the overall survival rate in the whole group of severe AA patients ($n = 63$) treated with r-ATG, it can be concluded that the estimated survival probability was 80 % (95%CI 0.68; 0.88) 2 years after the onset. However, the overall survival rate was 67 % (95%CI 0.51; 0.80) in the ninth and tenth year of follow-up (Fig. 1a).

Non-responders at Day 112 had the significantly shorter overall survival in comparison with the patients achieving complete or partial remissions ($p = 0.003$). Similar results were observed for the non-responders at Day 360 ($p = 0.003$). While comparing groups of children with severe ($n = 54$) and very severe ($n = 9$) AA, we showed that the 10-year overall survival was significantly shorter ($p = 0.001$ log rang) in the latter group (83 *vs.* 55 %).

Since we observed that some children achieved only partial remission, we also determined the event-free survival rate. During the first year of treatment, 16 % of patients relapsed or died (84 %, 95%CI 0.72; 0.91) Five years after

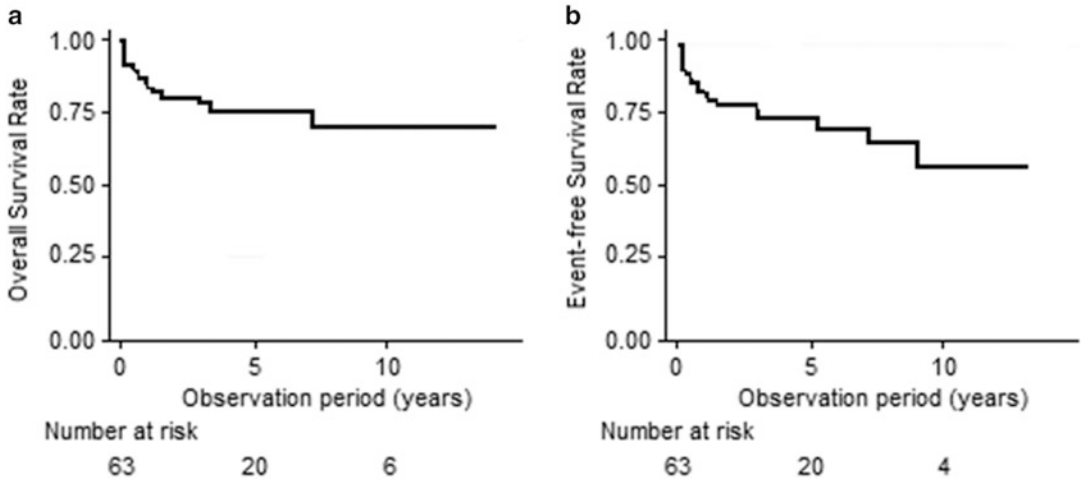


Fig. 1 Overall survival rate (*Panel A*) and event-free survival rate (*Panel B*) in children treated with rabbit antithymocyte globulin (r-ATG)

the onset of treatment, the event-free survival rate was 73 % (95%CI 0.59; 0.83) and after 10 years of follow-up, it was 57 % (95%CI 0.35; 0.74) (Fig. 1b).

4 Discussion

4.1 Survival After Immunosuppressive Therapy

The hematopoietic stem cell transplantation from HLA-matched family donor is a treatment of choice for children with severe aplastic anemia; however, it is possible only in 25 % of cases (Pawelec et al. 2008; Bagby et al. 2004; Ochocka et al. 1995). The majority of patients are treated with combined immunosuppressive therapy, which carries a risk of disease relapse or clonal transformation to paroxysmal nocturnal hemoglobinuria, myelodysplastic syndrome, or leukemia (Pawelec et al. 2008; Bagby et al. 2004; Kojima et al. 2000; Di Bona et al. 1999). Results of therapy of children with severe aplastic anemia have recently improved due to combined treatment with h-ATG and cyclosporine A (Pulsipher et al. 2011; Passweg and Marsh 2010). The 5-year survival rate in patients younger than 16 years was 50 % before 1990 and have risen to about 80 % afterward. The 10-year survival rate after 1997 has been 81 % (Saracco et al. 2008). Data published on the basis of the Polish experience showed the 10-year overall survival rate of 78 % in children treated mainly with h-ATG (Pawelec et al. 2008). Until recently, the first-line treatment was composed of h-ATG. However, due to the unavailability of the drug on the European market, it was substituted by r-ATG. According to the long-standing experience, particularly in immunosuppressive treatment lines, rabbit-derived ATG has been considered as a replacement for h-derived medicine. Di Bona et al. (1999) analyzed long-term results of treatment of severe AA with r-ATG in patients who did not respond to h-ATG and showed the 2.5-year overall survival of 93 %. A randomized study of Scheinberg et al. (2011) showed that the 3-year survival of patients receiving r-ATG as a first-line treatment was inferior to h-ATG amounting to 76 *vs.* 96 %, respectively. The present study, in which r-ATG was used, demonstrated the 3-year overall survival of 78 % and 10-year survival of 67 %. These results are comparable to the observations of Scheinberg et al. (2011). Zheng et al. (2006) noted the 5-year overall survival of 66 %, which is considerably better than about 2-year survival of 55 % reported by Atta et al. (2010). Afable

et al. (2011) also obtained rather unsatisfactory overall survival results with r-ATG of 76 ± 5 % and 64 ± 5 % over the 2 and 5-year time spans, respectively. The 10-year event-free survival rate of Polish children with SAA, r-ATG-treated, was presented by Salamonowicz et al. (2011) as 57.7 %. Similar results were described by the Severe Aplastic Anemia Working Party of the European Group for Blood and Marrow Transplantation (EBMT) (Marsh et al. 2012).

Lower effectiveness of r-ATG treatment in severe AA is emphasized by Marsh et al. (2012). According to the EBMT, 2-year overall survival rate for r-ATG-treated patients is 68 % in comparison with 86 % achieved by h-ATG-treated individuals. Saracco et al. (2008) observed the 10-year survival of 83 % in severe AA patients treated with h-ATG. However, some scientists opine that the effectiveness of both horse and rabbit-derived ATG is equal. Chang et al. (2010) did not show any relevant difference between the survival rate of patient treated with h-ATG (n = 29) and r-ATG (n = 33); the 4-year survival was 75 % in both groups.

4.2 Response to Immunosuppressive Therapy

Complete remission is the ultimate goal of severe AA therapy. Usually, however, patients achieve only partial remission, but some of them could progress to complete remission in the course of further therapy (Chen et al. 2012; Yoshida et al. 2011). The protocol based on r-ATG resulted in a positive response (complete or partial remission) in 31 (49.2 %) children at Day 180. Our results are concordant with those published by Zheng et al. (2006) who achieved response in 53 % of children treated with r-ATG in a randomized study. Afable et al. (2011) also gained 45 % of positive response after six months' therapy. An investigation of Salamonowicz et al. (2011) in a group of severe AA children treated with r-ATG showed the overall response of 50.9 % at Day 180. Atta et al. (2010) and Marsh et al. (2012), in turn, observed the overall remission at Day 180 in 34.5 and 40 % of patients, respectively.

While analyzing the severity of AA, the response to therapy at Day 360 in the present study was weaker in patients with very severe AA (complete remission in 1 child −11.1 % and partial remission in 3 children −33.3 %, a total of 44.4 % of positive responses) in comparison with severe AA children (complete remission in 24 patients – 42.5 % and partial remission in 7 patients – 13 %, a total of 55.5 %). These results differ from those of Führer et al. (2005) who showed a better response to treatment (68 % of complete remission – *vs.* 45 % of partial remission) and better survival rates of patients with very severe AA (93 % in very severe AA *vs.* 81 % in severe AA). This discrepancy could be explained by different protocols applied (children were treated with h-ATG for 8 consecutive days). In the present study, the overall survival in relation to the disease severity was better in patients with severe AA than that in very severe AA (83 *vs.* 53 %, respectively; p = 0.001).

4.3 Relapses and Clonal Transformations

In the present study we noted 4 (7 %) relapses in the 10-year long follow-up. Similar data were published by Gluckman et al. (2002) who showed 9 % of relapses in severe AA patients treated with h-ATG, cyclosporine A, prednisolone, and granulocyte colony stimulating factor (G-CSF). According to Schrezenmeier et al. (1993) the relapse probability might reach even 30 %. Although we diagnosed only one case of paroxysmal nocturnal hemoglobinuria (PNH), which makes its prevalence of 1.8 %, Socie et al. (2007) estimated the probability of clonal diseases at 16 %. A long-term follow-up of severe AA patients after immunosuppressive treatment supplemented with G-CSF or granulocyte-macrophage colony-stimulating factor (GM-CSF), became the starting point of a discussion on the relation between severe AA and malignant diseases (Takahashi et al. 2013; Deyell et al. 2011; Socie et al. 2007; Kojima et al. 2000). According to Kojima et al. (2000), the cumulative risk of developing myelodysplastic syndromes (MDS) or acute myeloid leukemia

(AML) in severe AA children after immunosuppressive therapy reaches as high as 13.7 %. The EBMT group estimated the risk of developing MDS at 4.3 %, AML at 4.6 %, and solid tumors at 0.7 % (Socie et al. 2007); the calculated results are lower than those published in Japanese studies (Kojima et al. 2000). Currently, the use of growth factors is limited to selected cases and not widely recommended due to the risk of developing clonal diseases (Samarasinghe and Webb 2012).

5 Conclusions

The analysis of a 10-year follow-up period after rabbit-derived ATG treatment in severe AA children showed that this protocol is somewhat less effective than the treatment modality based on h-ATG. These observations are consistent with other studies. The specialists agree that hematopoietic stem cell transplantation form unrelated donor is the acknowledged first-line treatment (Marsh et al. 2012; Takahashi et al. 2013). Samarasinghe and Webb (2012) algorithm concerning AA treatment, places rabbit-derived ATG as a third-line therapy. Further clinical investigation is warranted to discern other treatment options for this life-threatening disease.

Conflicts of Interest The authors report no conflicts of interest in relation to this article.

References

Afable MG, Shaik M, Sugimoto Y, Elson P, Clemente M, Makishima H, Sekeres MA, Lichtin A, Advani A, Kalaycio M, Tiu RV, O'Keefe CL, Maciejewski JP (2011) Efficacy of rabbit anti-thymocyte globulin in severe aplastic anemia. Haematologica 96(9):1269–1275

Atta EH, Dias DS, Marra VL, de Azevedo AM (2010) Comparison between horse and rabbit antithymocyte globulin as first-line treatment for patients with severe aplastic anemia: single center retrospective study. Ann Hematol 89(9):851–859

Bagby GC, Lipton JM, Sloand EM, Schiffer CA (2004) Marrow failure. In: Hematology: American Society of Hematology education program book, pp 318–336

Camitta BM (2000) What is the definition of cure for aplastic anemia? Acta Haematol 103:16–18

Chang MH, Kim KH, Kim HS, Jun HJ, Kim DH, Jang JH, Kim K, Jung CW (2010) Predictors of response to immunosuppressive therapy with antithymocyte globulin and cyclosporine and prognostic factors for survival in patients with severe aplastic anemia. Eur J Haematol 84(2):154–159

Chen C, Xue HM, Xu HG, Li Y, Huang K, Zhou DH, Guo HX, Fang JP, Huang SL (2012) Rabbit-antithymocyte globulin combined with cyclosporine A as a first-line therapy: improved, effective, and safe for children with acquired severe aplastic anemia. J Cancer Res Clin Oncol 138(7):1105–1111

Deyell RJ, Shereck EB, Milner RA, Schultz KR (2011) Immunosuppressive therapy without hematopoietic growth factor exposure in pediatric acquired aplastic anemia. Pediatr Hematol Oncol 28(6):469–478

Di Bona E, Rodeghiero F, Bruno B, Gabbas A, Foa P, Locasciulli A, Rosanelli C, Camba L, Saracco P, Lippi A, Lori AP, Porta F, De Rossi G, Comotti B, Lacopino P, Dufour C, Bacigalupo A (1999) Rabbit antithymocyte globulin (r-ATG) plus cyclosporine and granulocyte colony stimulating factor is an effective treatment for aplastic anaemia patients unresponsive to a first course of intensive immunosuppressive therapy. Gruppo Italiano Trapianto di Midollo Osseo (GITMO). Br J Haematol 107(2):330–334

Führer M, Rampf U, Baumann I, Faldum A, Niemeyer C, Janka-Schaub G, Friedrich W, Ebell W, Borkhardt A, Bender-Goetze C (2005) Immunosuppressive therapy for aplastic anemia in children: a more severe disease predicts better survival. Blood 106(6):2102–2104

Gluckman E, Rokicka-Milewska R, Hann I, Nikiforakis E, Tavakoli F, Cohen-Scali S, Bacigalupo A, European Group for Blood and Marrow Transplantation Working Party for Severe Aplastic Anemia (2002) Results and follow-up of a phase III randomized study of recombinant human-granulocyte stimulating factor as support for immunosuppressive therapy in patients with severe aplastic anemia. Br J Haematol 119(4):1075–1082

Kojima S, Hibi S, Kosaka Y, Yamamoto M, Tsuchida M, Mugishima H, Sugita K, Yabe H, Ohara A, Tsukimoto I (2000) Immunosuppressive therapy using antithymocyte globulin, cyclosporine, and danazol with or without human granulocyte colony stimulating factor in children with acquired aplastic anemia. Blood 96:2049–2054

Marsh JC, Bacigalupo A, Schrezenmeier H, Tichelli A, Risitano AM, Passweg JR, Killick SB, Warren AJ, Foukaneli T, Aljurf M, Al-Zahrani HA, Höchsmann B, Schafhausen P, Roth A, Franzke A, Brummendorf T, Dufour C, Oneto R, Sedgwick P, Barrois A, Kordasti S, Elebute MO, Mufti GJ, Socie G, European Blood and Marrow Transplant Group Severe Aplastic Anaemia Working Party (2012) Prospective study of rabbit antithymocyte globulin and cyclosporine for aplastic anemia from the EBMT Severe Aplastic Anaemia Working Party. Blood 119(23):5391–5396

Ochocka M, Karwacki M, Matysiak M (1995) Results of acquired aplastic anemia in children. Pediatr Pol 30:205–209

Passweg JR, Marsh JC (2010) Aplastic anemia: first-line treatment by immunosuppression and sibling marrow transplantation. In: Hematology: American Society of Hematology education program book, pp 36–42

Pawelec K, Matysiak M, Niewiadomska E, Rokicka-Milewska R, Kowalczyk J, Stefaniak J, Balwierz W, Załecka-Czerpko E, Chybicka A, Szmyd K, Sońta-Jakimczyk D, Bubała H, Krauze A, Wysocki M, Kurylak A, Wachowiak J, Grund G, Młynarski W, Bulas M, Krawczuk-Rybak M, Leszczyńska E, Urasiński T, Peregud-Pogorzelski J, Balcerska A, Wlazłowski M (2008) Results of immunosupressive therapy in children with severe aplastic anaemia. Report of the Polish Paediatric Haematology Group. Med Wieku Rozwoj 12:1092–1097

Pulsipher MA, Young NS, Tolar J, Risitano AM, Deeg HJ, Anderlini P, Calado R, Kojima S, Eapen M, Harris R, Scheinberg P, Savage S, Maciejewski JP, Tiu RV, Di Fronzo N, Horowitz MM, Antin JH (2011) Optimization of therapy for severe aplastic anemia based on clinical, biologic, and treatment response parameters: conclusions of an international working group on severe aplastic anemia convened by the Blood and Marrow Transplant Clinical Trials Network, March 2010. Biol Blood Marrow Transplant 17(3):291–299

Salamonowicz M, Pawelec K, Matysiak M, Kowalczyk J, Balwierz W, Zaleska-Czepko E, Chybicka A, Szmyd K, Szczepanski T, Bubala H, Wysocki M, Kurylak A, Wachowiak J, Szpecht D, Mlynarski W, Bulas M, Krawczuk-Rybak M, Panasiuk A, Leszczynska E, Urasinski T, Peregud-Pogorzelski J, Balcerska A, Kaczorowska-Hac B (2011) Results of treatment of severe aplastic anaemia in children using rabbit antithymocyte globulin (r-ATG). Blood 118 (21):3435

Samarasinghe S, Webb DK (2012) How I manage aplastic anaemia in children. Br J Haematol 157(1):26–40

Saracco P, Quarello P, Lori AP, Zecca M, Longoni D, Svahn J, Varotto S, Del Vecchio GC, Dufour C, Ramenghi U, Bacigalupo A, Locasciulli A, Bone Marrow Failure Study Group of the AIEOP (Italian Association of Paediatric Haematology Oncology) (2008) Cyklosporin A response and dependence in children with acquired aplastic anaemia: multicentre retrospective study with long –term observation follow-up. Br J Haematol 140:197–205

Scheinberg P, Nunez O, Weinstein B, Scheinberg P, Biancotto A, Wu CO, Young NS (2011) Horse versus rabbit antithymocyte globulin in acquired aplastic anemia. New Engl J Med 365(5):430–438

Schrezenmeier H, Marin P, Raghavachar A, Mc Cann S, Hows J, Gluckman E, Nissen C, van't Veer-Korthof ET, Ljungman P, Hinterberger W (1993) Relapse of aplastic anemia after immunosuppressive treatment: a report from the European Bone Marrow Transplantation Group SAA Working Party. Br J Haematol 85 (2):371–377

Socie G, Mary JY, Schrezenmeier H, Marsh J, Bacigalupo A, Locasciulli A, Führer M, Bekassy A, Tichelli A, Passweg J (2007) Granulocyte-stimulating factor and severe aplastic anemia: a survey by the European Group for Blood and Marrow Transplantation (EBMT). Blood 109(7):2794–2796

Takahashi Y, Muramatsu H, Sakata N, Hyakuna N, Hamamoto K, Kobayashi R, Ito E, Yagasaki H, Ohara A, Kikuchi A, Morimoto A, Yabe H, Kudo K, Watanabe K, Ohga S, Kojima S, Japan Childhood Aplastic Anemia Study Group (2013) Rabbit antithymocyte globulin and cyclosporine as first-line therapy for children with acquired aplastic anemia. Blood 121(5):862–863

Yoshida N, Yagasaki H, Hama A, Takahashi Y, Kosaka Y, Kobayashi R, Yabe H, Kaneko T, Tsuchida M, Ohara A, Nakahata T, Kojima S (2011) Predicting response to immunosuppressive therapy in childhood aplastic anemia. Haematologica 96(5):771–774

Zheng Y, Liu Y, Chu Y (2006) Immunosuppressive therapy for acquired severe aplastic anemia (SAA) a prospective comparison of four different regiments. Exp Hematol 34:826–831

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 5: 63
DOI 10.1007/5584_2014

Index