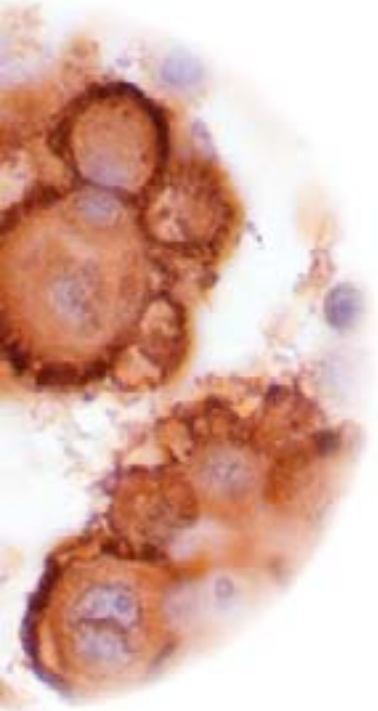
Effusion Cytology

A Practical Guide to Cancer Diagnosis



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A Practical Guide to Cancer Diagnosis

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demosMEDICAL
New York

ISBN 978–1–933864–65–5

Acquisitions Editor: Richard Winters
Cover Design: Joe Tenerelli
Compositor: Techset Composition
Printer: SCI

Visit our website at www.demosmedpub.com

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Library of Congress Cataloging-in-Publication Data

A catalog record for this book is available from the Library of Congress.

Made in the United States of America
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To our families and our patients

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Contents

Preface

The presence of malignant cells in body cavity fluids commonly indicates an advanced stage of cancer and is associated with poor prognosis. The detection of these cells in effusions is extremely important and may present a challenging task for pathologists. Even in the best of hands, conventional cytology fails to detect malignant cells in more than 50% of cases, resulting in missed diagnoses of malignancy. Thus there is an urgent need to reduce the high false negative rates of conventional cytology by using a simple systematic approach to the diagnosis of malignancy, the potential sub classification of the neoplastic process and determine the site of origin whenever possible. The authors of this book have used a practical and simplistic approach for the diagnosis of malignancy in effusions that includes a primer on the most effective use immunocytochemistry with small panels of 1–2 antibodies for select cases.

In 1980 M. Nadji, Professor of Pathology at our institution and a pioneer in immunohistochemistry, first described a variant immunocytochemistry (ICC) technique for the diagnosis and sub classification of malignancies in cytologic material. The methodology involves direct use of alcohol-fixed, papanicolaou-stained slides for immunocytochemistry (Nadji M. The Potential Value of immunoperoxidase Technique in Diagnostic Cytology. Acta Cytologica, 1980;24, 442–447). Since then we have utilized this ICC technique in our daily practice of cytology for the diagnosis, sub classification, and determination of site of origin for malignancies in cytologic specimens. There are several advantages to using the original cytospin slides for ICC, particularly when the effusion material is limited to the smears and no additional material is available for cell block preparation.

This book is the result of over 25 years of experience in the field of diagnostic cytology at the Miller School of Medicine, University of Miami, Jackson Memorial Medical Center, Miami, Florida, one of the largest cytology and immunocytochemistry laboratories in the country. Since 1992 we have shared our experience with serous effusion cytology with pathologists and cytotechnologists in the US and around the world by conducting workshops at the

American Society of Cytopathology (ASC) and the American Society for Clinical Pathology (ASCP) and many other cytology organizations in Canada, Europe, and the Middle East. The idea for this book arose as a result of the feedback we received from those who attended our workshops and invitation by our publisher.

This book is written in an outline format, emphasizing the diagnostic clues, and making them easily accessible to the reader. Chapter 1 offers an introduction and discussion on cellular content of pleural, peritoneal, and pericardial effusions. The second chapter discusses general diagnostic criteria used to differentiate benign from malignant cells. Chapter 3 lists and details the types of malignancies found in effusions. Chapter 4 covers the major differential diagnoses such as adenocarcinoma vs reactive mesothelium and malignant mesothelioma. The focus of Chapter 5 is on the detection of primary tumor site based on morphology, site of sample and patients' gender. Chapter 6 is dedicated to cerebrospinal fluid cytology. In Chapter 7, sample collection, fixation, and preparation of slides are discussed. The immunocytochemistry protocol we use in our laboratory is included in this chapter. We have presented illustrated examples and discussed the basic cytodiagnostic criteria that we have found most useful. We have provided illustrated examples of malignant cells and their markers as well as pointing out the pitfalls in the use of these immunomarkers. Besides describing the various immunomarkers that we find useful for the positive identification of malignant cells, we have presented tables listing the differential expression of specific markers for the diagnosis of various types of tumors.

Chapter 8 briefly discusses laser flow cytometry as a diagnostic tool for detection of malignant cells in body cavity fluids. In this chapter we have shown examples of flow cytometric data used to identify malignant cells on the basis of their DNA aneuploidy and tumor cell associated markers expression.

This book will serve as a practical text for the cytopathologist and cytotechnologist who face daily diagnostic dilemmas in effusion cytology, requiring the use of ICC. Cross-references in the text are extensive and are intended to help the reader who scans the book or selects a specific topic without having to read preceding chapters. A short list of Suggested Readings complements each chapter for further information.

The authors would like to acknowledge the assistance of the cytology and immunohistochemistry faculty and staff of the Departments of Pathology at Jackson Memorial Hospital and the Miller School of Medicine, University

of Miami. We would especially like to thank Fred Cordoves for his technical expertise and Dr. Carmen Gomez-Fernandez for her editorial assistance. We would also like to thank the staff of Demos Medical Publishing who has been very patient with us. And finally, we would like to thank our families who have been a constant source of support during the preparation of this book.

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Effusion Cytology

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1

Body-Cavity Fluid Cytology

Introduction, Source, and Cellular Content

Introduction

- This work represents an illustrated practical handbook that enables quick reference in the diagnosis of malignancy based on the study of body-cavity fluid. Specific differential diagnoses encountered in the daily practice of diagnostic cytology will be discussed using a simplistic approach.
- In this chapter, we will discuss major causes of fluid accumulation in the pleural, pericardial, and peritoneal cavities. The different cell types found in these effusions are usually as a result of similar reactive or neoplastic etiologies. The cell types described and illustrated in these effusions have similar morphology regardless of their site of collection. In addition, in patients with bilateral pleural effusions, the main cellular and biochemical features on the right and left side are shown to be similar; therefore, a diagnostic thoracentesis may not need to be performed on both sides, unless there is a specific clinical indication.

> The major purpose of cytologic examination of serous effusions is to determine whether malignant cells are present. This is an extremely important task since in most cases the presence of malignant cells in effusions indicates an advanced or terminal stage of malignancy and it is associated with poor survival.

- Cytologic diagnosis may be difficult and many diagnostic pitfalls exist.

- False-positive results may prevent a necessary surgery and false-negative results lead to unnecessary surgical treatment and delay otherwise indicated medical treatments.
- In order to avoid diagnostic pitfalls, special attention should be given to proper collection, preparation, and fixation of cytologic material (see Chapter 7).
- Diagnostic criteria should be followed carefully and, in equivocal cases, when diagnostic material is inadequate or poorly preserved, repeat cell study should be recommended.

Knowledge of the complete clinical history, patient's age, gender, occupation, prior history of cancer, and treatment modalities such as radiation and/or chemotherapy should be available for proper interpretation and meaningful clinico-pathological correlation.

- In this book, we will first discuss a simplistic approach in the diagnosis of serous effusions (pleural, pericardial, and peritoneal) with emphasis on the cytomorphologic criteria found in alcohol-fixed Papanicolaou-stained cytospin slides.
- The cytomorphologic diagnosis of cerebrospinal fluid specimens (CSF) will follow our general discussion on the serous fluid specimens in Chapter 6.
- We will also provide cytopreparatory techniques and the modified Papanicolaou staining method used in our laboratory.

Use of immunocytochemistry on previously Pap-stained slides, without destaining is discussed, and selection of markers used in the differential diagnosis of diagnostic entities will be presented in detail.

- Flow cytometric and molecular methods available in diagnostic cytology are described in Chapter 8.

Source and Cellular Content

- Serous effusion is referred to the accumulated fluid within the body cavities, that is, pleural, pericardial, and peritoneal. The pleura encloses the lungs, pericardial the heart, and peritoneum the intra-abdominal organs. Each cavity is lined by an external (parietal) and a visceral layer that directly covers the underlying organs (lungs, heart, and abdominal viscera). All these cavities have a common embryologic origin of the mesenchyme.
  - Structurally, all three cavities are similar and lined by a single layer of small cuboidal or flat mesothelial cells supported by a connective tissue.
  - In normal condition, the parietal and visceral layers of mesothelium are separated by a small amount of fluid that facilitates the movement of the underlying organs. In reality, the cavities are not true cavities unless they contained abnormally accumulated fluid as a result of neoplastic or nonneoplastic conditions, that is, *exudate* and *transudate*.
- The *transudate* is characterized by its low protein (below 3 g/100 ml) and a low specific gravity (below 1.015). Transudate is a result of filtration of the blood serum across the intact capillaries.
- Accumulation of transudates within the body cavities is commonly seen in cases of congestive heart failure, cirrhosis of the liver and other liver and kidney diseases associated with hypoproteinemia.
- The *exudates* on the other hand are characterized by relatively high protein content (usually above 3 g/100 ml) and, therefore a high specific gravity (above 1.015). This type of fluid results from endothelial damage to the capillaries in neoplastic, infectious, or inflammatory conditions such as rheumatoid arthritis, or lupus erythematosus or as a result of trauma and the presence of any long-standing, space-occupying mass (usually benign) within the serosal cavities.

> The presence of malignant cells in serous effusions indicates the involvement of serous membranes by cancerous cells, and is of clinical interest. Thus, it is the main focus of this book.

- In noncancerous conditions due to the chronic irritation of the mesothelial-lined surfaces, a varying degree of mesothelial proliferation occurs.

Exfoliated mesothelial cells often demonstrate changes that may be confused for malignancy. These benign mesothelial cells will be referred to as "*reactive*" mesothelial cells.

- *Reactive mesothelial cells:* Mesothelial cells are present in the majority of effusions, but especially in sterile inflammations. They are absent or scarce when a serous cavity becomes infected by pyogenic organisms.
  - Mesothelial cells that shed into the serous cavities appear in loosely cohesive clusters or in single/isolated form (Figure 1.1).
  - When mesothelial cells are aspirated during a surgical procedure (as in peritoneal washings), they are more commonly found in sheets, presumably as a result of their forceful detachment from the serosal surfaces (Figure 1.2).
  - Reactive mesothelial cells are arranged in loosely cohesive groups, in contrast to those tight molding cellular clusters seen in malignant effusions (Figure 1.3).
  - The cytoplasmic borders are sharp and usually remain intact.
  - In sheet-like arrangements, the cells have a mosaic pattern.

Reactive mesothelial cells exfoliated from the pericardium, on the other hand, have a tendency to roll up as in tight clusters due to the constant cardiac movements. This may present a diagnostic pitfall when dealing with pericardial fluid specimens of patients with benign conditions.

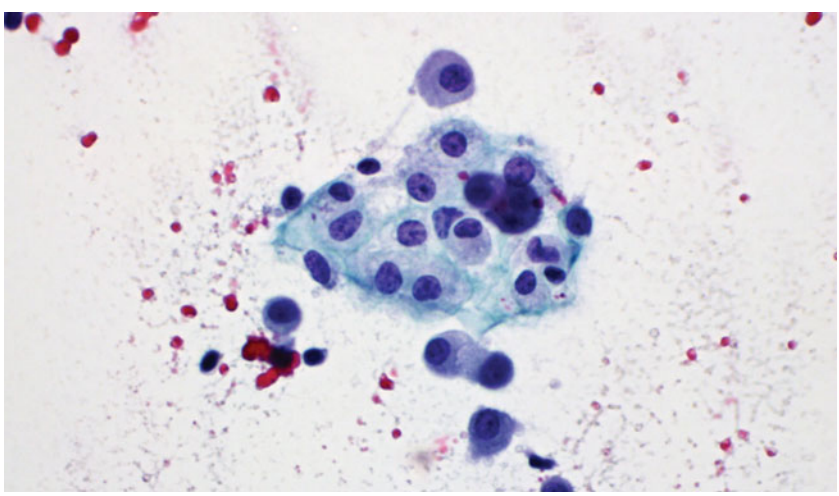

FIGURE 1.1 Ascitic fluid: Negative for malignancy. Cellular evidence of reactive mesothelium. *Comment:* The mesothelial cells are arranged in loosely cohesive clusters. The patient had long standing cirrhosis (PAP, 60×).

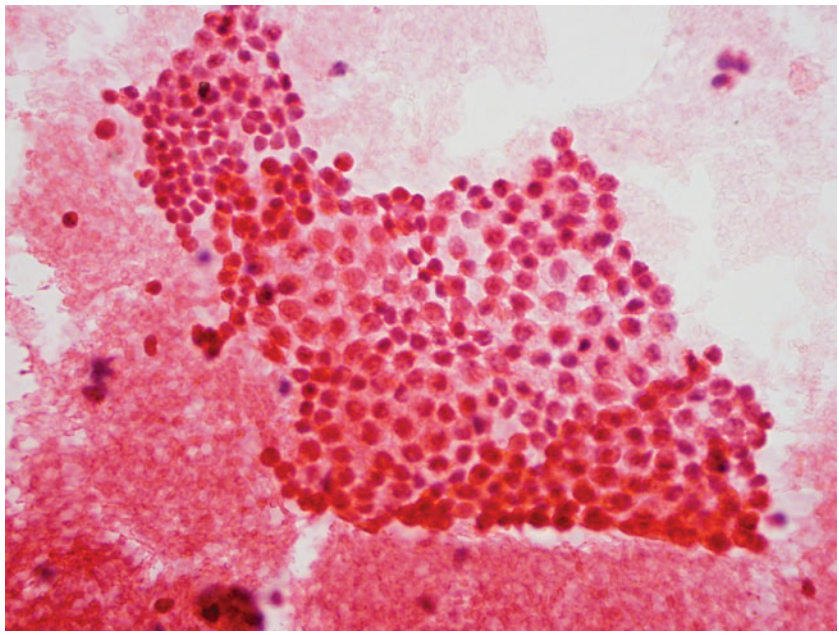

FIGURE 1.2 Peritoneal washings: Negative for malignancy. Cellular evidence of reactive mesothelium. *Comment:* The mesothelial cells are arranged in a flat sheet (PAP, 40×).

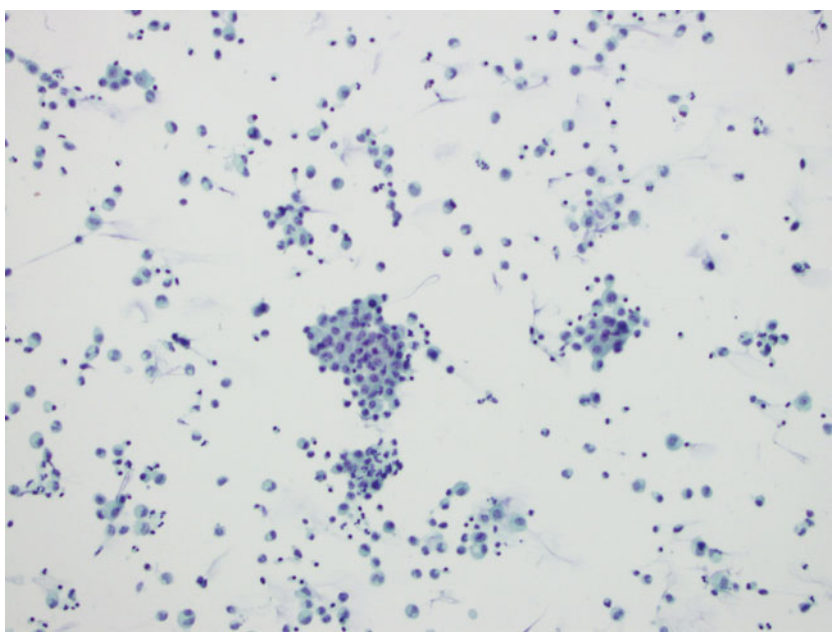

FIGURE 1.3 Pleural fluid: Negative for malignancy. Cellular evidence of reactive mesothelium. *Comment:* The mesothelial cells are arranged in loosely cohesive groups in contrast to tight groups seen in malignancies (PAP, 25×).

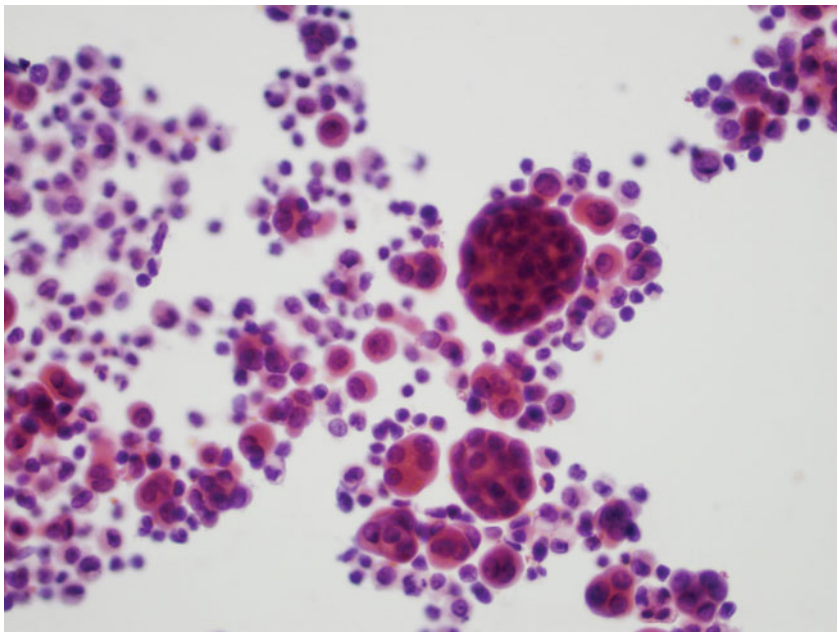

FIGURE 1.4 Pericardial fluid: Negative for malignancy. Cellular evidence of reactive mesothelium. *Comment:* The mesothelial cells are arranged in tight clusters due to constant cardiac movement. This may represent a diagnostic pitfall in pericardial fluids. (PAP, 40×).

- Isolated mesothelial cells may also be present in variable number. They are round or oval measuring 10–12 μm. The cytoplasm is dense and two toned.
- A foamy rim may be present in some cells; however, the perinuclear portion of the cytoplasm is usually denser than the periphery.
- The nuclei are usually centrally located. They are round or oval with a smooth nuclear membrane, and evenly distributed chromatin. Nucleoli may be prominent (Figure 1.5).
- Binucleation or multinucleation is not uncommon in reactive mesothelial cells. In binucleated cells, the nuclei are mirror images of each other (Figure 1.6). Bi- and multinucleated mesothelial cells/macrophages may be seen that may be mistaken for Langhans giant cells seen in granulomatous inflammations (Figure 1.7).

It is important to note that the diagnosis of granulomatous inflammation should not be made based on finding a few multinucleated giant cells in the body-cavity fluid.

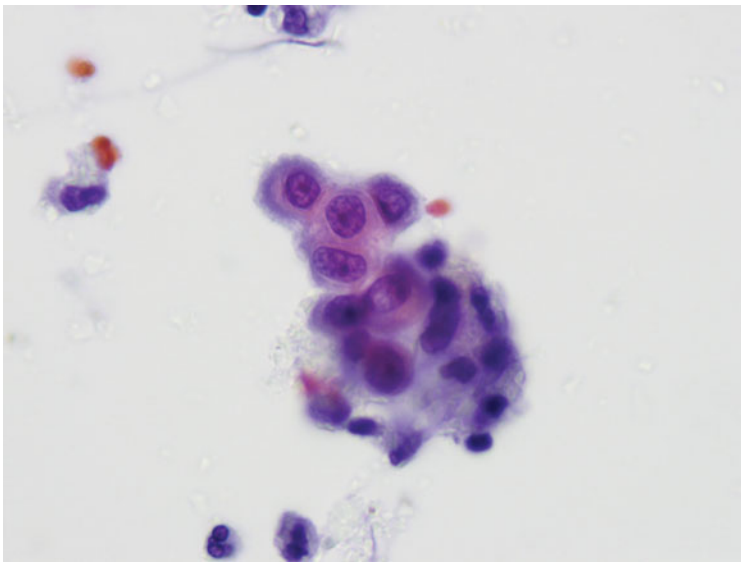

FIGURE 1.5 Ascitic fluid: Negative for malignancy. Cellular evidence of reactive mesothelium. *Comment:* Reactive mesothelial cells with prominent nucleoli and even distributed chromatin (PAP, 100×).

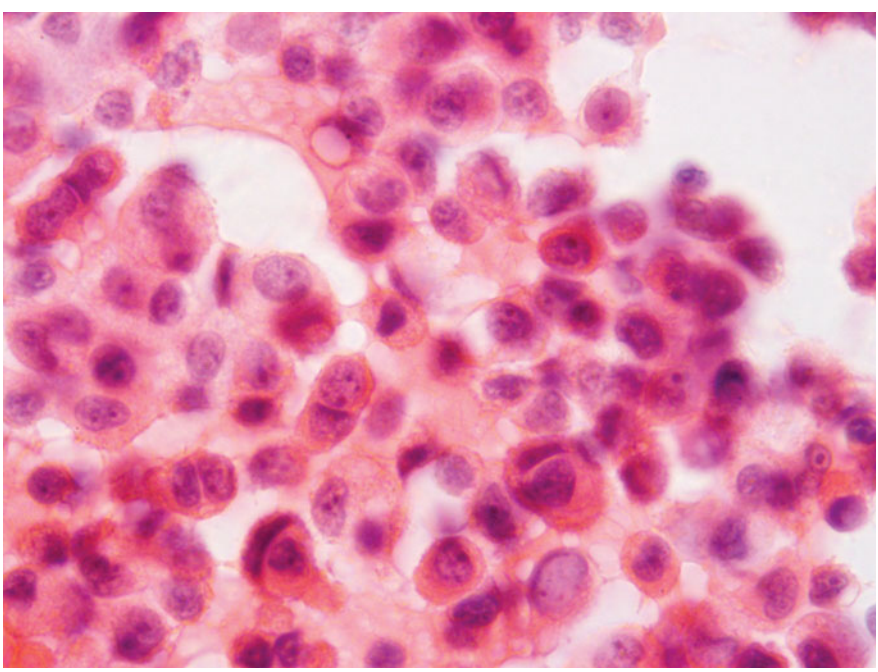

FIGURE 1.6 Pleural fluid: Negative for malignancy. Cellular evidence of reactive mesothelium. *Comment:* Binucleation of mesothelial cells is common (PAP, 40×).

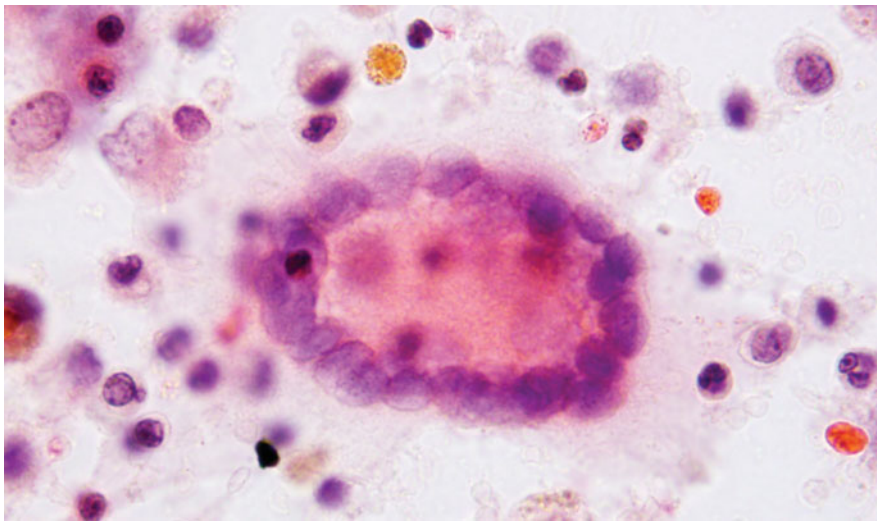

FIGURE 1.7 Peritoneal washings: Negative for malignancy. Cellular evidence of reactive mesothelium. *Comment:* Multinucleated mesothelial cell (PAP, 40×).

- Foreign body-type multinucleated giant cells may be seen in patients with injury of serosal surfaces as a result of trauma or treatment modalities such as radiotherapy. Therefore, the presence of multinucleated giant cells in the serous effusion, in our experience is of no diagnostic significance.
- Reactive mesothelial cells are capable of dividing and multiplying while floating in the effusions and mitotic figures can be found in benign effusions. The mitotic figures, however, are usually of normal configuration and atypical mitoses are rare.

> For all practical purposes, the presence of mitosis in the effusions should be disregarded and not considered a sign of malignancy.

- *Macrophages:* Macrophages are found in almost every serous fluid. They are usually larger than mesothelial cells and contain bean-shaped nuclei (Figure 1.8).
  - The cytoplasm is foamy and may contain phagocytized material or large vacuoles displacing the nucleus to the periphery resembling "*signet ring cells*" (Figure 1.9).

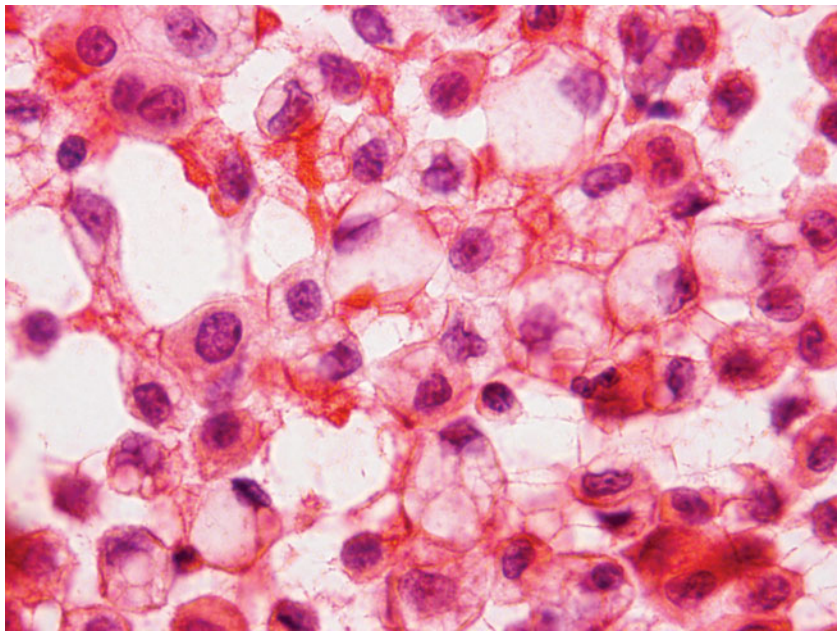

FIGURE 1.8 Pleural fluid: Negative for malignancy. *Comment:* Macrophages are usually larger than mesothelial cells and contain bean-shaped nuclei (PAP, 100×).

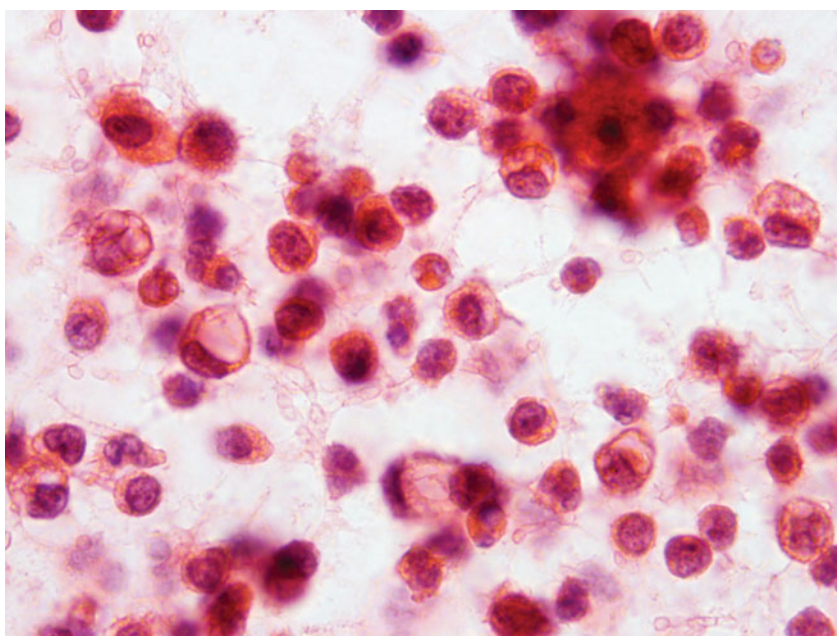

FIGURE 1.9 Ascitic fluid: Negative for malignancy. Note isolated cell pattern and bland chromatin. *Comment:* Large vacuoles displacing the nucleus to the periphery resembling "signet ring cells. (PAP, 100×).

- Degenerating mesothelial cells may contain cytoplasmic vacuoles that are generally confused with macrophages. On the other hand, vacuolated degenerated mesothelial cells may be confused with adenocarcinoma cells. Several histochemical and immunohistochemical stains have been used for this distinction; however, this cytomorphologic distinction is not of any clinical significance.

Normochromatic bland appearing nuclei and isolated cellular arrangement of degenerated mesothelial cells with a *"signet ring cell"* appearance serve as an important diagnostic clue in differentiating these cells from their malignant counterpart, that is, signet ring cell carcinomas.

- *Red blood cells*: A bloody aspirate is rare in nontraumatic fluid collections except in neoplastic involvement of the serosal membrane.
  - Excess number of red blood cells may prevent proper evaluation of the fluid specimen. In such cases, to dissolve red blood cells, preservative media containing acetic acid may be used (see Chapter 7).

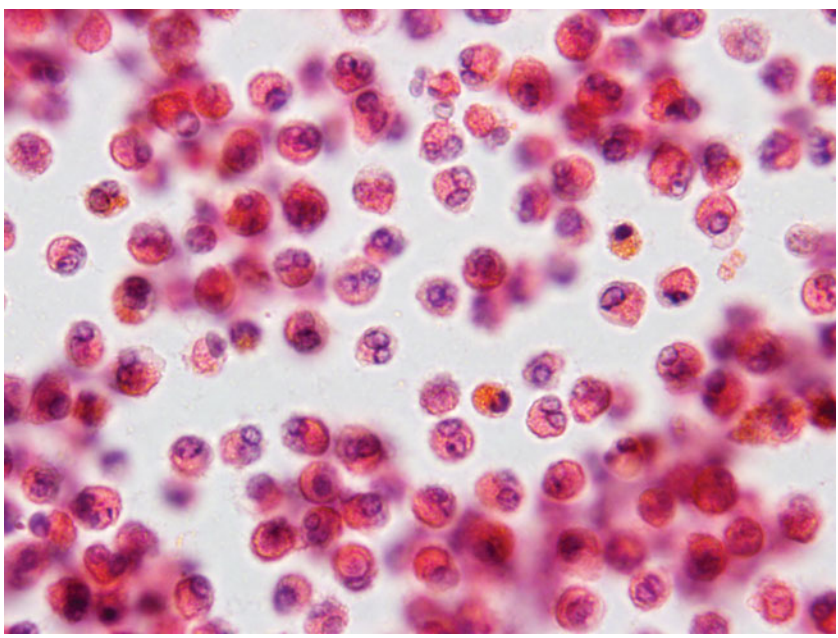

FIGURE 1.10 Pleural fluid: Negative for malignancy. Numerous eosinophils are present. (PAP, 100×).

- *Eosinophils:* Pleural cavities are the most common sites for eosinophilic effusions. Eosinophilic pleural effusion, defined as the presence of 10% or more eosinophils in the pleural fluid. This condition accounts for a small number of exudative pleural effusions (Figure 1.10).
  - Traditionally, blood and air in the pleural space was considered to be the most common condition associated with eosinophilic pleural effusion. However, more recent studies have shown that repeated thoracenteses is not an important factor in the development of eosinophilic infiltrate in the cavity fluids. Further, eosinophilic pleural effusions may be seen in patients with underlying malignant conditions. Therefore, pleural eosinophilia at the initial thoracentesis cannot be considered as a predictor of an underlying benign disorder.
- *Leukocytes*: Leukocytes are extremely common in body-cavity effusions. Polymorphonuclear neutrophils (PMN) invariably indicate an inflammatory process, which may be secondary to malignancy or other disorders (Figure 1.11).

Purulent effusions are composed of abundant neutrophils only. Majority of them arise in the pleural cavity and are associated with pneumonia, lung carcinoma, and tuberculosis.

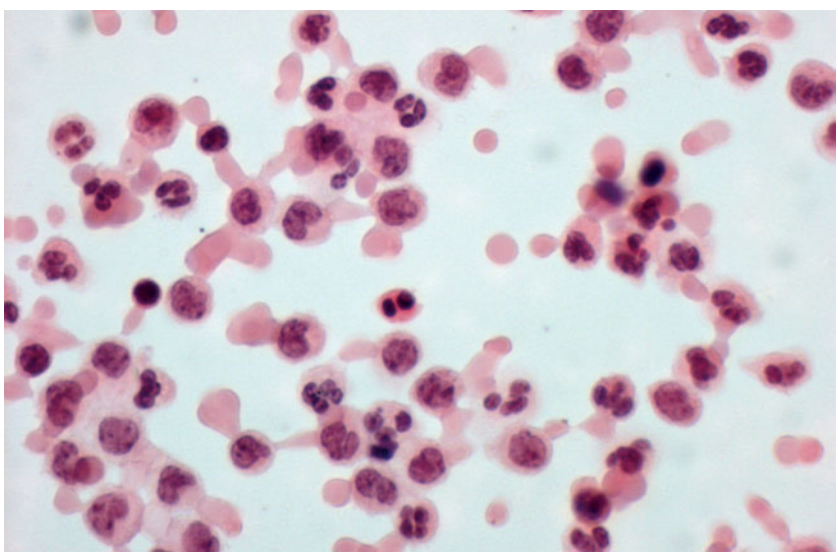

FIGURE 1.11 Pleural fluid: Negative for malignancy. Cellular evidence of acute inflammation (PAP, 100×).

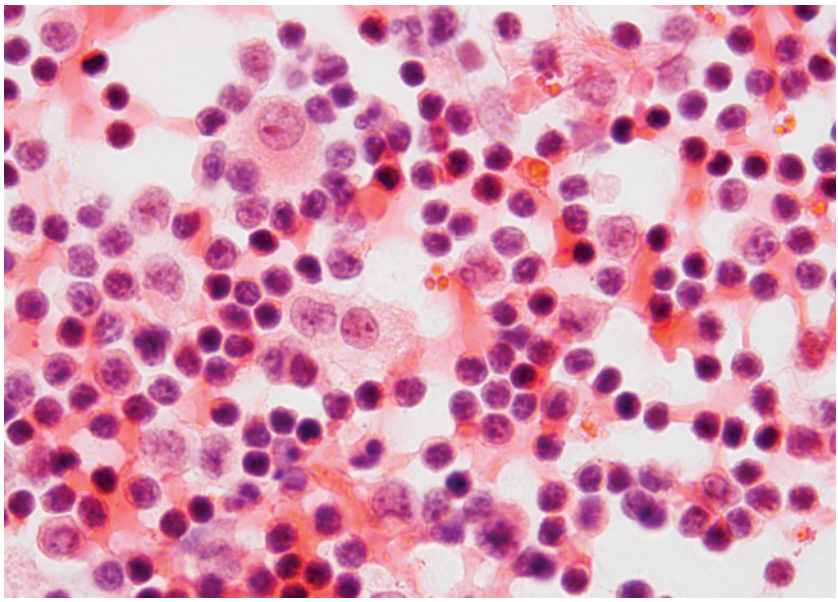

FIGURE 1.12 Pleural fluid: Negative for malignancy. Cellular evidence of lymphocytic infiltrate. *Comment:* Commonly seen in cases of pulmonary tuberculosis (PAP, 100×).

- *Lymphocytes*: In cases of long-standing effusions, lymphocytes may be predominant. When they are numerous—especially if no other type of leukocytes are present (Figure 1.12)—the possibility of tuberculosis and lymphoproliferative disorders should be considered.

Immunophenotyping of lymphocytes into B- and T-cell subgroups may be of diagnostic value. A predominance of small T-cell population usually represents a benign process such as tuberculosis.

  - Proliferation of small B lymphocytes usually indicates a lymphoproliferative process such as small lymphocytic lymphoma/leukemia. Combined morphology and immunophenotyping by flow cytometry (FCM) has been shown to have a high (up to 100%) sensitivity as well as specificity.

In the presence of large lymphocytes in body-cavity fluids, the possibility of a diffuse large-cell lymphoma should be investigated. In the absence of a known primary lymphoma in HIV/AIDS patients, the possibility of a primary effusion lymphoma (PEL) should be part of the differential diagnosis.

- *Plasma cells*: Plasma cells may be seen in chronic inflammatory processes, in plasmacytoma, multiple myeloma, and Hodgkin's lymphoma.
  - Normal plasma cells are larger than lymphocytes, they have eccentric nuclei, and a characteristic chromatin pattern (cart-wheel). The cytoplasm may contain immunoglobulins that may form eosinophilic granules or Russel's bodies, rarely seen in effusion specimens.
- *Other cells*: In addition to mesothelial cells, other benign epithelial cells such as hepatocytes (rarely) may be found in serous effusions (peritoneal washing specimens).
  - In female patients, columnar or cuboidal cells of Mullerian origin may be found in case of endosalpingiosis (the presence of ectopic epithelium resembling that of normal endosalpinx), and other benign conditions such as salpingitis, and tuboovarian abscess. The cells derived from these benign conditions may be associated with concentric calcification known as psammoma bodies (Figure 1.13). The presence of these epithelial inclusions may represent a source of false-positive diagnoses in peritoneal washings (a procedure usually performed for staging ovarian cancer). In peritoneal washings, endosalpingiosis consists of clusters of benign epithelial cells often surrounding a psammoma body.

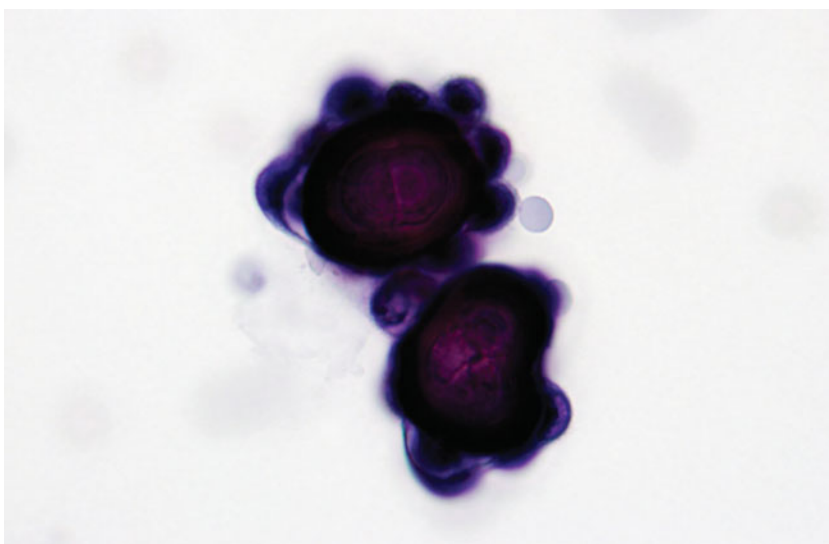

FIGURE 1.13 Peritoneal washings: Negative for malignancy. *Comment:* Presence of psammoma bodies alone does not necessarily indicate a neoplastic process (PAP, 100×).

The presence of psammoma bodies *alone* in cul-de-sac aspirates or peritoneal washing specimens does *not* represent a neoplastic process.

- These findings can be misinterpreted as suspicious or diagnostic of a serous carcinoma of the ovary. However, in the absence of any abnormal cellular proliferation accompanying psammoma bodies, the diagnosis of malignancy should be avoided. These cells lack any cellular pleomorphism, in contrast to ovarian neoplasms, including tumors of borderline malignancy that the cytoplasm is vacuolated and the nuclei are irregular and exhibit course chromatin and prominent nucleoli.

Suggested Readings

Das DK. Serous effusions in malignant lymphomas: A review. *Diagnostic Cytopathology*, 2006;*34*, 335–347.

Kalomenidis I, Rodriguez M, Barnette R et al. Patient with bilateral pleural effusion: Are the findings the same in each fluid? *Chest*, 2003;*124*, 167–176.

Koss LG, Melamed MR. *Koss's diagnostic cytology and its histopathologic bases* (5th ed.). Philadelphia, PA: Lippincott Williams & Wilkins, 2006.

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Martinez Garcia MA, Cases-Viedma E, Perpina Tordera M et al. Repeated thoracentesis: An important risk factor for eosinophilic pleural effusion? *Respiration*, 2003;*70*, 82–86.

Sidaway MK, Silverberg SG. Endosalpingiosis in female peritoneal washings: A diagnostic pitfall. *International Journal of Gynecology and Pathology*, 1987;*6*, 340–346.

2

General Diagnostic Criteria

Benign Versus Malignant

Adenocarcinomas are the most commonly seen malignancies in the body-cavity fluids.

The diagnosis of malignancy in effusions is first attempted on the basis of cellular pattern, usually determined on low-power magnification, and then based on morphologic characteristics of individual cells.

The diagnostic terminology commonly used as "atypical cells are present" must be avoided in reporting serous effusions. We believe that "atypical" diagnosis is meaningless to the clinicians.

- In cases with "cellular atypia" we use ancillary techniques, mainly immunocytochemistry (ICC) in order to reach a conclusive diagnosis.

Conventional Cytology

- Based on routine cytology, the first and the most important question to answer is whether the effusion sample under study represents a malignant process.
- An extremely simplistic approach to the diagnosis of a fluid specimen, is based on the following:
  - Cellular pattern
  - Cellular morphology

Cellular Pattern

When the majority of cells are in clusters:

- In these cases, the malignant cells can easily be detected, even at the low-power magnification (Figure 2.1A).

> The clusters of malignant cells are tight and compact, usually with smooth borders (Figure 2.1B).

- The cells mold, each taking the shape of the neighboring cell (Figure 2.2); a "cell within cell" pattern may be seen (Figure 2.3).

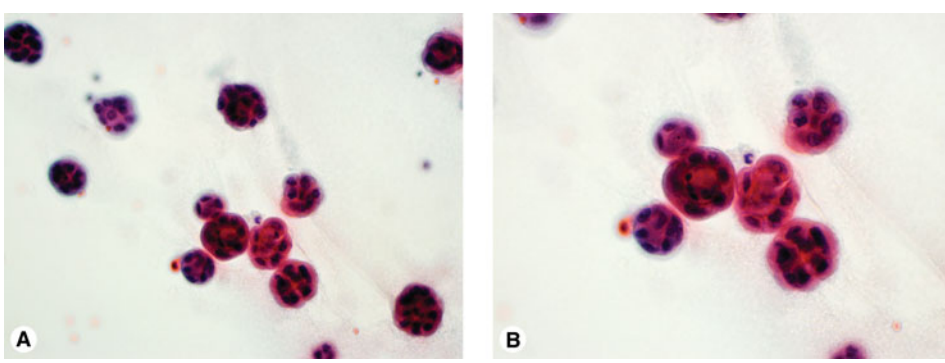

FIGURE 2.1 (A) Pleural fluid: Cellular evidence of adenocarcinoma. *Comment:* Tight clusters of malignant cells with smooth borders. The patient had a history of breast carcinoma (PAP, 40×). (B) Pleural fluid: Cellular evidence of adenocarcinoma. *Comment:* Tight clusters of malignant cells with smooth borders are commonly seen in patients with breast carcinoma (PAP, 100×).

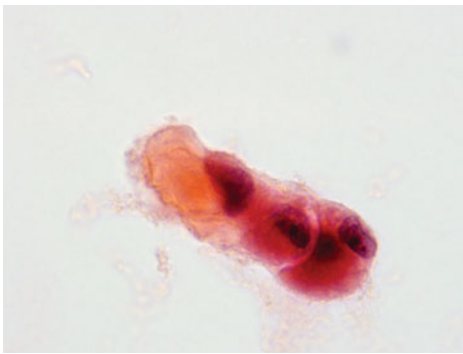

FIGURE 2.2 Pleural fluid: Cellular evidence of adenocarcinoma. *Comment:* Cellular molding is useful for diagnosis of malignancy. The patient had a history of lung carcinoma (PAP, 100×).

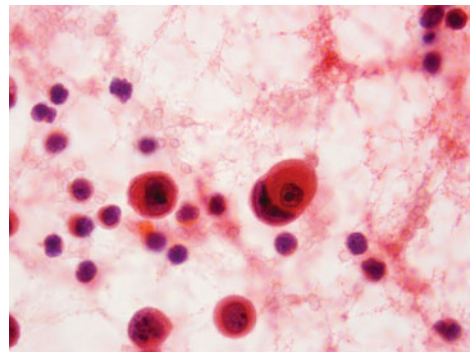

FIGURE 2.3 Peritoneal fluid: Cellular evidence of adenocarcinoma. Comment: "Cell within cell" pattern is shown here (PAP, 100×).

Two-Cell Type: In the majority of malignant effusions, low-power evaluation will show two distinct cell types: the "malignant cells" within tight clusters and the "reactive mesothelial cells" on the slide background, isolated, paired, or in small monolayer sheets (Figure 2.4).

- The reactive mesothelial cells may also group together, however, the grouping is usually loose, and without nuclear overlap. In such reactive conditions, nuclear features of the cells in clusters are identical to those of isolated or paired mesothelial cells seen in the smear background (Figure 2.5).

In patients with benign pericardial effusions, the reactive mesothelial cells may roll up (due to the constant cardiac movements), and they may be confused with adenocarcinoma cells. A careful clinical history and use of ICC will prevent an erroneous diagnosis in such conditions.

When the majority of malignant cells are isolated:

- The isolated malignant cells may be as a result of a variety of neoplastic conditions, but not commonly found in pleural, pericardial, and peritoneal effusions. In contrast, isolated cell pattern is more commonly observed in cerebrospinal fluid (CSF) specimens.

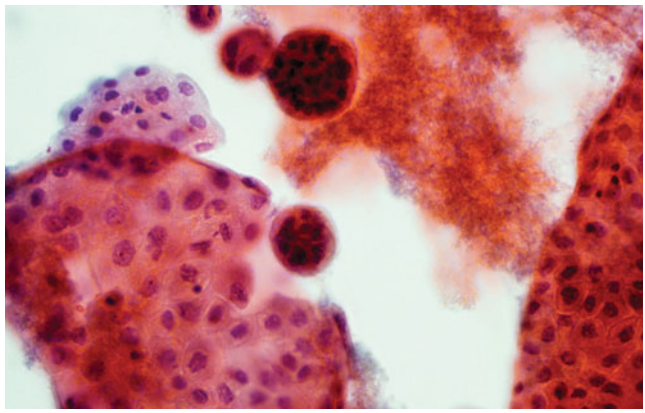

FIGURE 2.4 Pleural fluid: Cellular evidence of adenocarcinoma. *Comment:* "Two cell types"; malignant cells in tight clusters and reactive mesothelial cells in the background. The patient had a history of breast carcinoma (PAP, 100×).

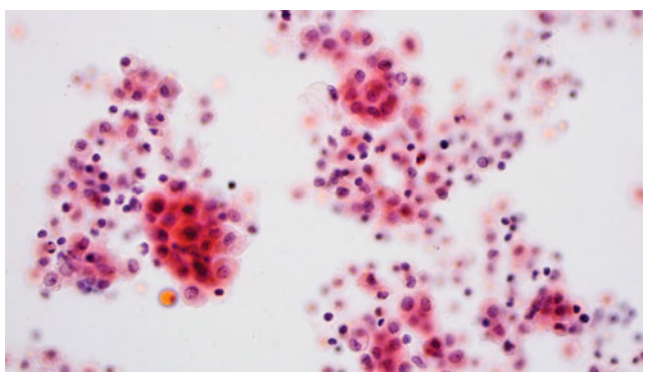

FIGURE 2.5 Pleural fluid: Negative for malignancy. Cellular evidence of reactive mesothelium. *Comment:* Benign mesothelial cells are in loose groups in contrast to adenocarcinoma cells seen in Figure 2.4 (PAP, 40×).

- In CSF specimens specifically, because of the general paucity of the neoplastic cells, cell clusters are uncommon and seen only in severe cases of meningeal carcinomatosis (see Chapter 6).
- When the isolated cells are obviously abnormal and show cytomorphologic characteristics of malignancy such as pleomorphism, high nucleocytoplasmic ratio, hyperchromasia, clumped, irregular chromatin, abnormal nucleoli, and irregular nuclear membrane, the diagnosis of malignancy can easily be rendered based on cytomorphologic criteria alone (Figure 2.6).
- A typical example of isolated cell pattern in effusion specimens is seen in metastatic breast carcinomas (usually lobular carcinomas) and in signet-ring-cell carcinomas of gastric or less commonly colonic origin.

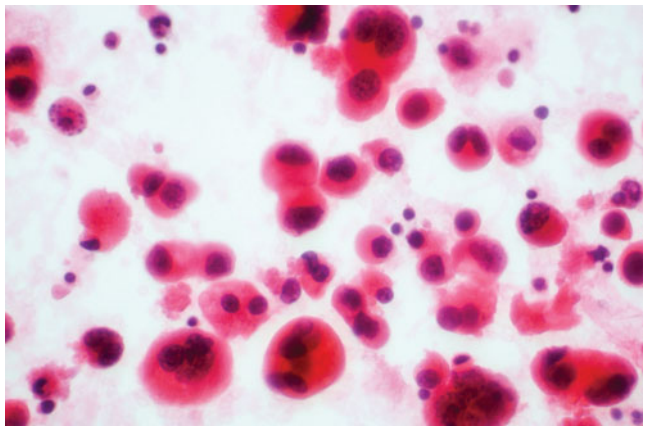

FIGURE 2.6 Peritoneal fluid: Cellular evidence of adenocarcinoma. *Comment:* Isolated pleomorphic malignant cells are present. The patient had a history of pancreatic carcinoma (PAP, 100×).

In the absence of cellular pleomorphism, the isolated malignant cells may be absolutely overlooked on low-power magnification. In such cases, especially in the presence of clinical history of malignancy, using ancillary techniques such as immunocytochemistry facilitates the detection of malignant cells (Figure 2.7A, B).

- In such cases, careful search for tightly grouped cell clusters not only will confirm the diagnosis of malignancy, but also will indicate the epithelial origin of the primary tumor.

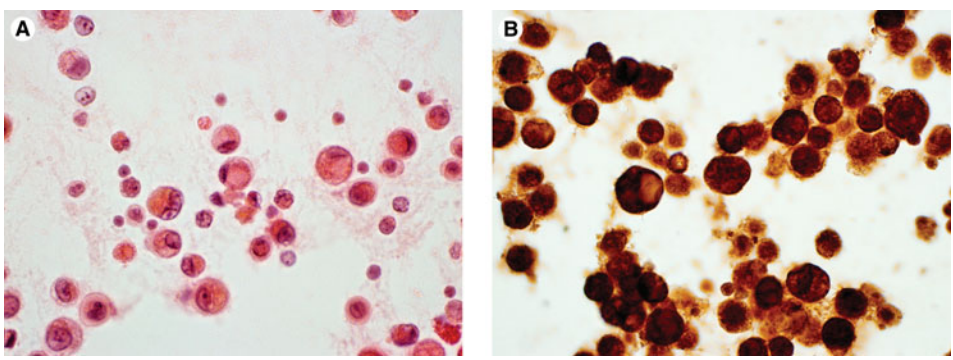

FIGURE 2.7 (A) Peritoneal fluid: "Preliminary diagnosis": Atypical cells of epithelial origin are present. Immunostains to follow. *Comment:* These isolated malignant cells lack pleomorphism. Therefore, immunocytochemistry is indicated. The patient had a history of gastric carcinoma (PAP, 60×). (B) Peritoneal fluid: "Final diagnosis": Cellular evidence of adenocarcinoma. *Comment:* The isolated malignant cells are strongly positive for EMA supporting the above diagnosis (EMA, (clone E29), 100×).

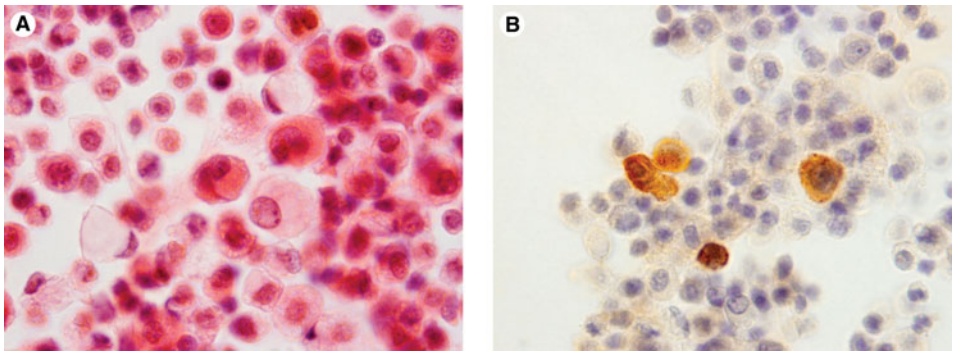

FIGURE 2.8 (A) Peritoneal fluid: Cellular evidence of a malignant neoplasm. Immunostains to follow for further sub-classification. *Comment:* The isolated cellular pattern is common in malignant melanomas. The patient had disseminated metastatic melanoma (PAP, 100×). (B) Peritoneal fluid: Cellular evidence of a malignant neoplasm consistent with malignant melanoma. *Comment:* HMB-45 positivity confirms the diagnosis of malignant melanoma (HMB-45, 100×).

- Less commonly encountered are malignant neoplasms that are known to produce isolated cells in cytologic material (usually in fine-needle aspiration cytology smears) including malignant melanomas (Figure 2.8A, B), malignant lymphomas (Figure 2.9A, B), and some other uncommonly seen neoplasms in effusions (Figure 2.10A, B).

Cellular Morphology

- Occasionally, the diagnosis of malignancy is easily made based on cytomorphologic abnormalities such as pleomorphism, multinucleation, high

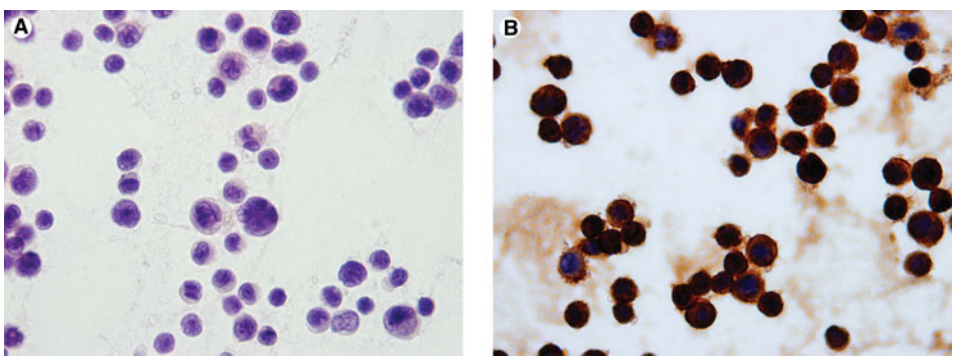

FIGURE 2.9 (A) Pleural fluid: Cellular evidence of malignant neoplasm with features suggestive of malignant lymphoma. Immunostains to follow. *Comment:* Malignant lymphomas typically present as an isolated cell pattern in effusions (PAP, 100×). (B) Pleural fluid: Cellular evidence of malignant lymphoma. *Comment:* CD 45 immunostain confirms the diagnosis of malignant lymphoma (CD45, 100×).

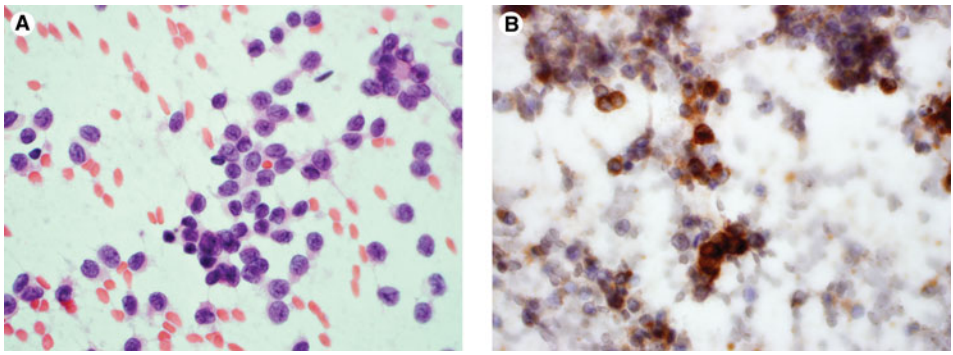

FIGURE 2.10 (A) Peritoneal fluid: Cellular evidence of malignant neoplasm. Immunostains to follow for further subclassification. *Comment:* The patient with recurrent granulosa cell tumor (PAP, 60×). (B) Peritoneal fluid: Cellular evidence of malignant neoplasm consistent with granulosa cell tumor. *Comment:* The positive inhibin immunostain confirms the diagnosis (Inhibin, 60×).

nucleo-cytoplasmic ratio, hyperchromasia, abnormal nucleoli, and clumped, irregular chromatin, regardless of the cellular arrangement (Figure 2.11).

The diagnostic problem is profound when no tight clusters are found on low-power magnification, and the cells show no convincing cytomorphologic characteristics of malignancy. This is especially common in cases of metastatic low-grade mammary carcinomas, when tumor cells are small with bland nuclei, and lack striking features of malignancy.

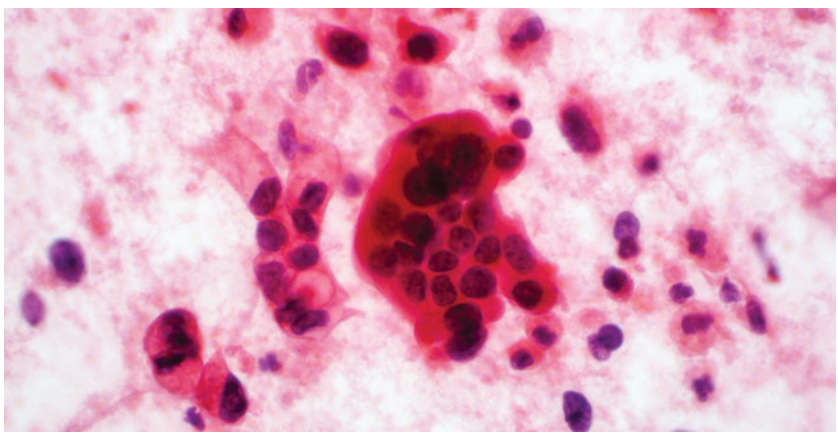

FIGURE 2.11 Peritoneal fluid: Cellular evidence of adenocarcinoma. *Comment:* Multinucleated cells with hyperchromasia, abnormal nuclei, and irregular chromatin are present. The patient with metastatic pancreatic carcinoma in the liver (PAP, 100×).

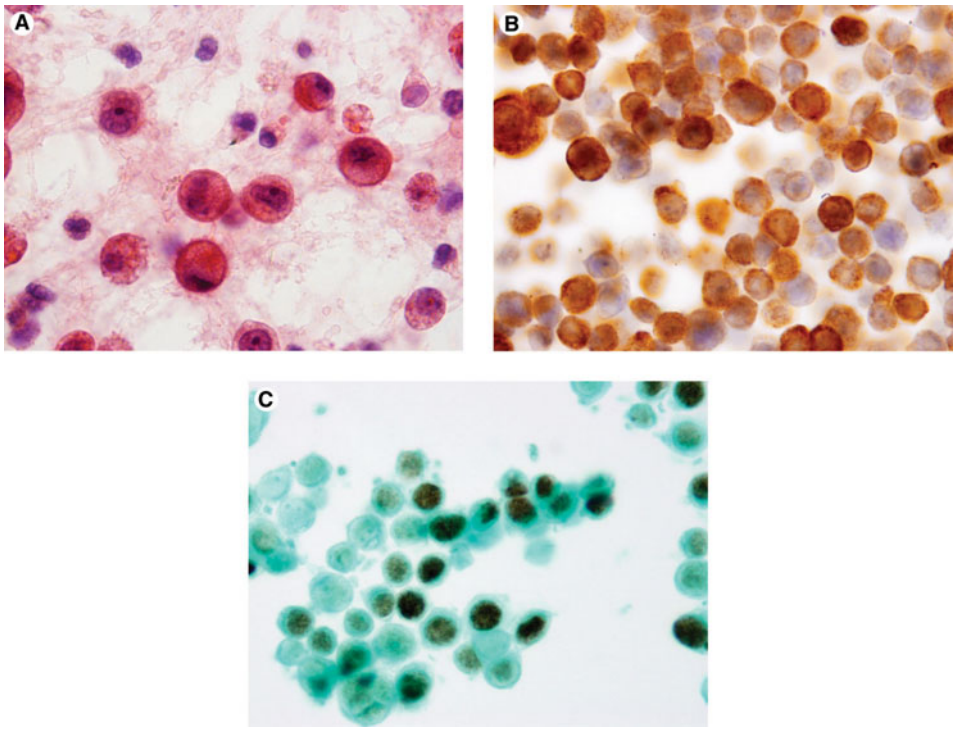

FIGURE 2.12 (A) Pleural fluid: "Preliminary diagnosis": Atypical cells of epithelial origin are present. Immunostains to follow. *Comment:* These isolated cells show no convincing cytomorphologic characteristics of malignancy. The patient with a remote history of breast carcinoma (PAP, 100×). (B) Pleural fluid: Cellular evidence of adenocarcinoma. *Comment:* EMA immunostain is strongly positive confirming the malignant nature of the cells (EMA, (clone E29), 100×). (C) Pleural fluid: "Final diagnosis" Cellular evidence of adenocarcinoma consistent with a breast primary. *Comment:* Nuclear staining for ER confirms the above diagnosis (ER, (clone 1D5), 100×).

- In such cases, the use of ICC is often very helpful for a definitive diagnosis. A diffuse intracytoplasmic staining for epithelial membrane antigen (EMA) is useful in such instances. In addition, when a patient presents with a remote history of a low-grade ER-positive breast carcinoma, immunostaining for estrogen receptor (ER) usually confirms the breast origin (Figures 2.12A, B, and C). Special caution should be observed in peritoneal specimens (peritoneal fluids and washings) of women (exclude endosalpingiosis).

Useful Findings

- Among many cytological characteristics described in the diagnosis of malignant cells in effusion specimens, we have found the following to be the most helpful in our experience.

Useful Findings	
Tight clusters with smooth borders	"Signet ring cells" in groups
Large tight papillary clusters	Cellular and nuclear molding
Two-cell-type pattern	Nuclear Abnormalities

- One of the most important diagnostic characteristics of epithelial malignancies in effusions is the presence of *tight groups of cells with a smooth border* easily observed on low-power magnification (Figure 2.13). On higher magnification, the cells show hyperchromasia, nuclear molding, and overlap. In contrast, benign reactive mesothelial cells may be seen in loosely grouped cell clusters with normochromasia and bland nuclear morphology on high magnification.
- *Large tight papillary clusters* are usually seen in adenocarcinomas (Figure 2.14) and malignant mesotheliomas. In contrast, benign reactive mesothelial cells in effusion samples are present in loose clusters.
- *The two-cell-type pattern*, as described by the presence of tight cell clusters (easily seen on low-power magnification) and the isolated or loosely cohesive

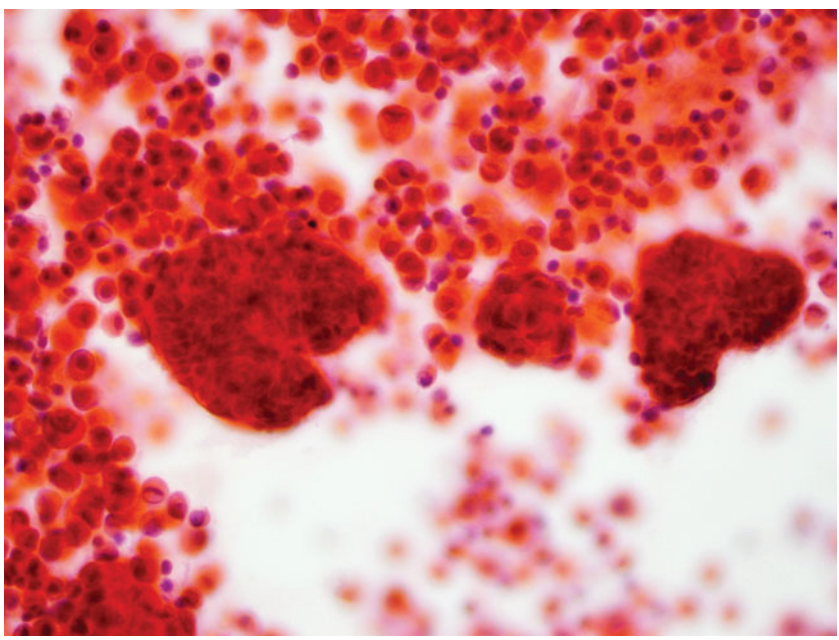

FIGURE 2.13 Pleural fluid: Cellular evidence of adenocarcinoma. *Comment:* Tight cell clusters with smooth borders easily observed in low-power magnification are an important diagnostic feature. The patient with nonsmall cell lung carcinoma (PAP, 40×).

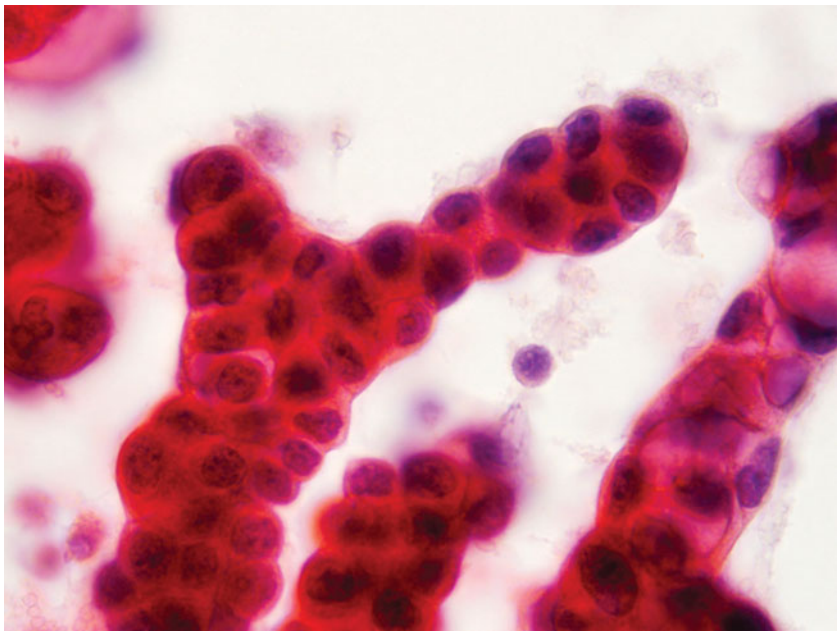

FIGURE 2.14 Pleural fluid: Cellular evidence of adenocarcinoma. *Comment:* Large tight papillary clusters are often seen in adenocarcinomas. The patient with breast carcinoma (PAP, 100×).

reactive mesothelial cells found on the smear background is a good indication of malignancy (Figure 2.4).

- Adenocarcinoma cells in effusions may show characteristic appearance of "signet ring cell," that is, nucleus pushed away by a finely or coarsely vacuolated cytoplasm. Isolated degenerated benign/reactive mesothelial cells may also show "signet ring cell" appearance. Therefore, one should only consider the presence of "*signet ring cells in groups*" as a criterion of malignancy. The "signet ring cells in clusters" often represent an adenocarcinoma. In these cases, nuclear abnormalities such as hyperchromasia and irregular nuclear membrane are evident on high magnification (Figure 2.15). We do not use the term "mucinous adenocarcinoma" in effusions unless the malignant cells are floating in a mucinous background.
- *Cellular and nuclear molding* is present in majority of epithelial malignancies in effusion specimens. The nuclear molding is commonly seen in cells derived from small-cell carcinomas (Figure 2.16). Occasionally, this finding facilitates the distinction of small carcinoma cells from inflammatory cells.
- The presence of *nuclear abnormalities* seen in malignant effusions is helpful to distinguish reactive mesothelial cells from epithelial malignancies and inflammatory cells from small-cell malignancies. Nuclear abnormalities

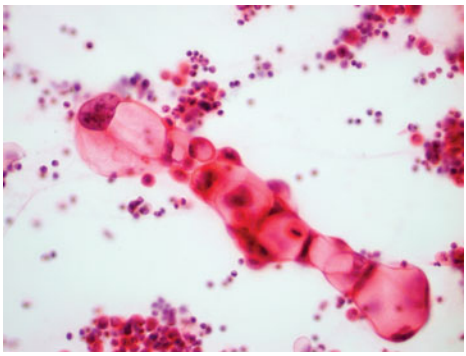

FIGURE 2.15 Peritoneal fluid: Cellular evidence of adenocarcinoma. *Comment:* Tight cluster of vacuolated cells with "signet ring" morphology. Note abnormal nuclear features. The patient with ovarian adenocarcinoma (PAP, 100×).

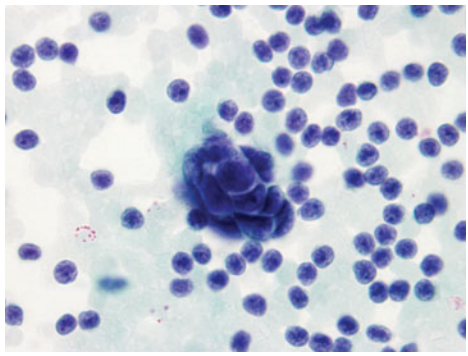

FIGURE 2.16 Pleural fluid: Cellular evidence of small-cell carcinoma. *Comment:* Nuclear molding is commonly seen in cells derived from small-cell carcinomas. The patient with histologically proven small-cell lung carcinoma (PAP, 100×).

include irregular nuclear membrane, unevenly distributed coarsely granular chromatin, and the presence of multiple nucleoli (Figure 2.17).

Useless Findings

- We have found the following cytologic findings of *no* specific diagnostic value in effusion cytology.

Useless Findings	
Cytoplasmic vacuoles	Multinucleated cells
Isolated "signet ring" cells	Psammoma bodies
Cell within cell	Mitosis
Prominent nucleoli	

- *Cytoplasmic vacuoles* may be seen as a result of mucin production by adenocarcinoma cells; however, it is more commonly seen as a result of cytoplasmic degeneration in reactive mesothelial cells (Figure 2.18).
- The presence of isolated "*signet ring cells*" alone is not an indication of adenocarcinoma. Reactive mesothelial cells with cytoplasmic degeneration may be present as isolated "signet ring cells." In such instances, the nuclei have a bland morphology, and frequently are normochromatic (Figure 2.18).
- *Cell within cell* may be seen in both benign reactive mesothelial cells (Figure 2.19) as well as in cases of malignancy in effusions; therefore, this finding is of *no* diagnostic value.

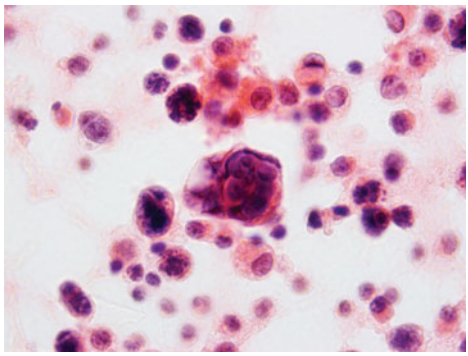

FIGURE 2.17 Peritoneal fluid: Cellular evidence of adenocarcinoma. *Comment:* Nuclear abnormalities including irregular nuclear membrane and unevenly distributed chromatin are present. The patient with fallopian tube adenocarcinoma (PAP, 100×).

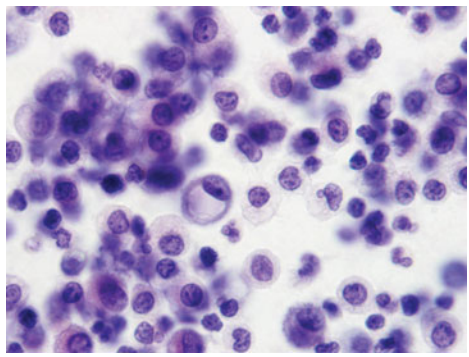

FIGURE 2.18 Pleural fluid: Negative for malignancy. *Comment:* Isolated cells with cytoplasmic vacuoles mimicking "signet ring" carcinoma are commonly seen as a result of mesothelial cells degeneration (PAP, 100×).

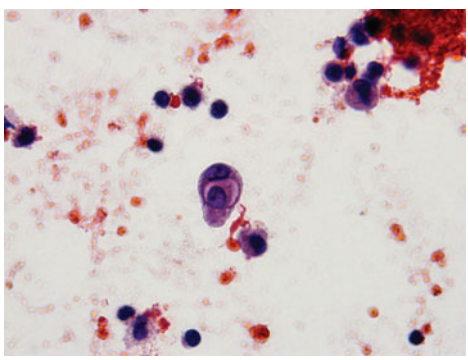

FIGURE 2.19 Pleural fluid: Negative for malignancy. *Comment:* Both benign reactive mesothelial cells and malignant cells may show "cell within cell" pattern. Attention should be paid to the nuclear features (PAP, 100×).

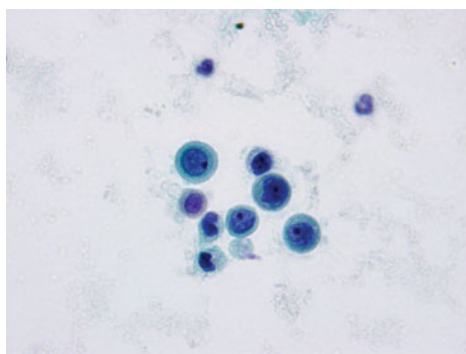

FIGURE 2.20 Peritoneal fluid: Negative for malignancy. *Comment:* Benign reactive mesothelial cells may show prominent nucleoli. The patient with cirrhosis (PAP, 100×).

- *Prominent nucleoli* are commonly seen in reactive mesothelial cells in effusions (Figure 2.20). This finding cannot be used in distinguishing between benign and malignant cells.
- *Multinucleated cells* may be seen in malignant effusions; however, they are more often present in nonspecific inflammatory/reactive conditions such as posttraumatic (Figure 2.21) and postradiation. Occasionally, multinucleated malignant cells are seen in cases of anaplastic carcinomas from the lung, thyroid, pancreas, and ovaries. Rarely pleomorphic binucleated or multinucleated sarcoma cells are found in effusions.

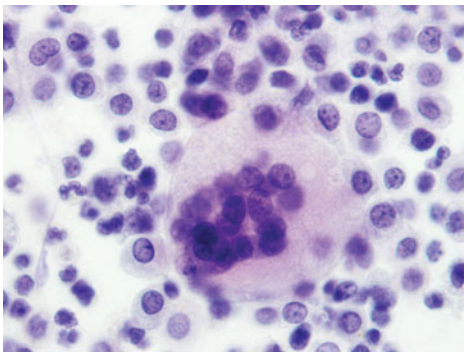

FIGURE 2.21 Pleural fluid: Negative for malignancy. *Comment:* Multinucleated giant cells may represent nonspecific inflammatory/reactive conditions. The patient with history of chest trauma (PAP, 100×).

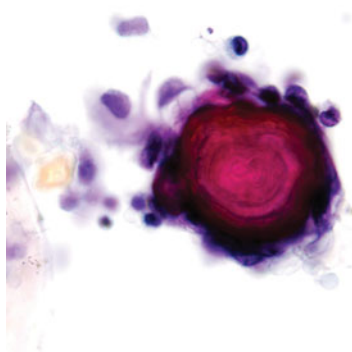

FIGURE 2.22 Cul-de-sac aspirate: Negative for malignancy. Psammoma bodies are present. *Comment:* In the absence of accompanying neoplastic cells, presence of psammoma bodies alone is not of diagnostic significance (PAP, 100×).

- *Psammoma bodies* may be seen in peritoneal effusions, especially in aspirates from cul-de-sac of women. In the absence of accompanying neoplastic cells, the presence of psammoma bodies alone is *not* of diagnostic significance (Figure 2.22).
- *Mitosis* can be seen in both benign and malignant effusions. Since reactive mesothelial cells may continue to divide while out of serosal cavities, the presence of mitosis in effusion samples does not necessarily indicate a neoplastic process. In the presence of abnormal mitosis, however, the possibility of a malignant process should be considered. In general, we do not consider the presence of mitotic figures in effusion samples of any diagnostic value.

Immunocytochemistry

- Accumulation of serous effusion in body cavities may be the first manifestation of a malignant condition. Since adenocarcinomas are the most commonly found malignancies in effusions, one of the major problems in the daily practice of cytology is to distinguish between carcinoma cells from commonly seen reactive mesothelial cells. This is especially true in patients with a history of radiation and/or chemotherapy.
- Overlapping cytologic features of adenocarcinoma, reactive mesothelial cells, and malignant mesothelioma have long been a diagnostic challenge to cytopathologists. ICC assists to reduce false-negative results of effusion cytology that is reported in over 50% of cases in routine cytology. Such errors in diagnosis usually are caused by misinterpretation of adenocarcinoma cells as reactive mesothelial cells. The false-positive diagnosis is less commonly seen and often caused by overinterpretation of reactive mesothelial cells as malignant cells.
- In this section, we will discuss the use of ICC in differentiating the most frequently found malignant conditions in serous effusions, that is, adenocarcinomas, and rare cases of malignant mesotheliomas from reactive processes.
- It should be emphasized that in current pathology practice, there is no single marker to differentiate benign from malignant cells.
- The following is a list of the most frequently used ICC markers—to be used in panels—to differentiate benign reactive mesothelial cells from the most commonly found epithelial malignancies in effusion specimens.

Commonly Used Immunocytochemical Markers

EMA: Malignant (adenocarcinoma, malignant mesothelioma)
CEA: Malignant (adenocarcinoma)
Ber-EP4: Malignant (adenocarcinoma)
B72.3: Malignant (adenocarcinoma)
LeuM1: Malignant (adenocarcinoma)
Desmin: Benign (reactive mesothelium)

Commonly Used Panels

- Since there is *no* single marker that can differentiate benign from malignant cells, ICC panels made of a variety of antibodies, are used in diagnostic cytology.
- The earliest panel used in this area was based on markers that are expressed by adenocarcinomas, such as EMA, CEA, Ber-EP4, B72.3, and LeuM1.

- In cases of adenocarcinomas, the most common pattern with a high sensitivity has been—EMA positive, CEA positive, Ber-EP4 positive, B72.3 positive, and LeuM1 positive. This panel detects the majority of adenocarcinoma cases in effusions. It should be noted, however, that there is a chance of false-positive results, especially in cases of peritoneal effusions in women since benign Mullerian-derived epithelial cells such as those in endosalpingiosis, may express some of these antigens.
- In cases of malignant mesotheliomas, the most common reactivity patterns are EMA positive, CEA negative, Ber-EP4 negative, B72.3 negative, and LeuM1 negative.
- In summary, benign and malignant profiles can be used as shown below.

Benign and Malignant Immunocytochemical Profiles

	Benign	Malignant
EMA	Negative	Positive
CEA	Negative	Positive
Ber-EP4	Negative	Positive
B72.3	Negative	Positive
LeuM1	Negative	Positive

- In our experience, the combination of EMA and CEA positivity has been the most helpful in detection of adenocarcinoma cells in effusions.

Use of Small Panels

- To differentiate benign reactive mesothelial cells from malignant cells, some authors recommend limited panels to include at least two negative mesothelial markers (CEA, Ber-Ep4, and B72.3) and two positive mesothelial markers (calretinin, CK 5/6, and WT1).
- At our laboratory, we use a small immunocytochemical panel of two antibodies which always includes EMA. This limited panel is especially helpful in effusions where reactive mesothelial cells are grouped together and raise the possibility of a malignant process. In our experience, this situation is frequently encountered in the evaluation of pericardial effusions, where because of the constant movement of the heart, the mesothelial cells may

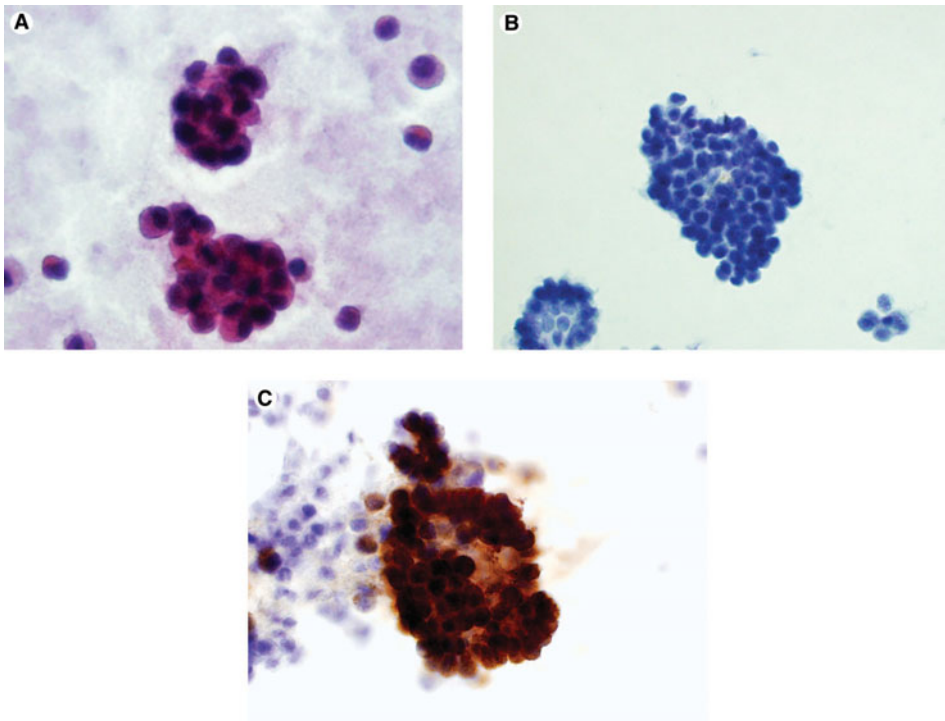

FIGURE 2.23 (A) Pericardial fluid: Negative for malignancy. Cellular evidence of reactive mesothelium. *Comment:* In pericardial effusions, mesothelial cells have the tendency to roll-up and raise the possibility of a malignant process (PAP, 60×). (B) Pericardial fluid: Negative for malignancy. Cellular evidence of reactive mesothelium. EMA immunostain is negative. *Comment:* EMA negativity in this pericardial effusion favors a benign process (EMA, (clone E29), 60×). (C) Pericardial fluid: Negative for malignancy. Cellular evidence of reactive mesothelium. Calretinin immunostain is positive. *Comment:* Calretinin positivity indicates the mesothelial origin of the cells seen in groups (Calretinin, 60×). The patient had heart failure with no malignancy.

roll up. A combination of a negative EMA and a positive calretinin is extremely helpful in this situation (Figure 2.23A, B, and C).

In our laboratory, EMA (clone E29) is the most frequently used antibody to differentiate benign versus malignant effusion.

- High sensitivity of EMA in the diagnosis of adenocarcinomas is documented by various studies, including ours.
- EMA is positive in both, adenocarcinomas and malignant mesotheliomas. In adenocarcinomas, its distribution is cytoplasmic (Figure 2.24), whereas in malignant mesotheliomas EMA is characteristically localized in the cell membrane (Figure 2.25).

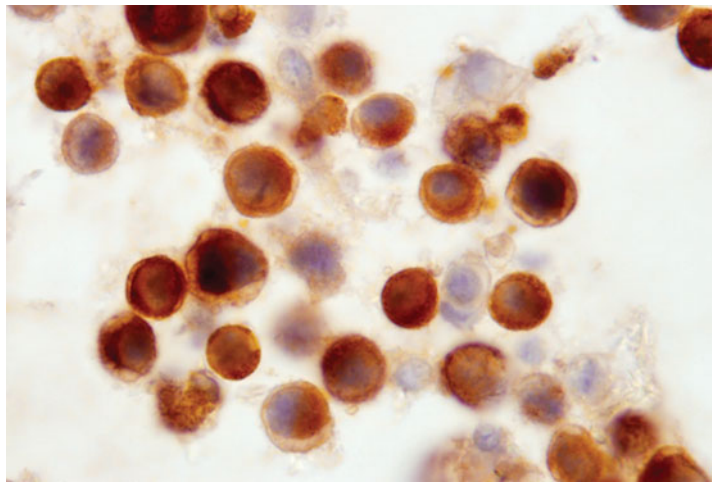

FIGURE 2.24 Pleural fluid: EMA immunostain is positive in adenocarcinomas. *Comment:* Strong intracytoplasmic EMA staining is characteristic of adenocarcinoma cells (EMA, (clone E29), 100×).

EMA immunostaining must be evaluated at low-power magnification where EMA-positive cells are easily detected. (Figure 2.26A, B). Reactive mesothelial cells are negative for EMA (clone E29). The presence of weak positivity in a few cells should not be regarded a positive result (Figure 2.27). Special attention should be made in peritoneal effusions of women, where cells derived from endosalpingiosis/endometriosis may be positive for EMA.

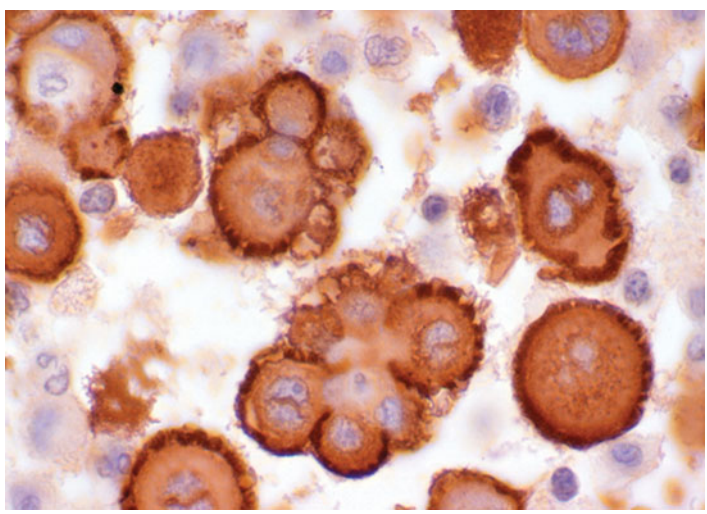

FIGURE 2.25 Pleural fluid: EMA immunostain is positive in malignant mesotheliomas. *Comment:* Strong membranous EMA staining is characteristic of malignant mesothelioma cells (EMA, (clone E29), 100×).

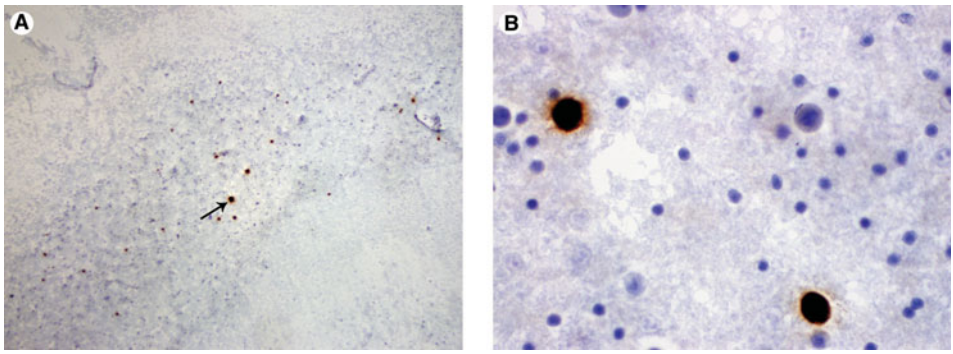

FIGURE 2.26 (A) Pleural fluid: EMA immunostain is positive in adenocarcinomas. *Comment:* Strong EMA positivity easily seen at low-power magnification (EMA, (clone E29), 10×). (B) Pleural fluid: EMA immunostain is positive in adenocarcinomas. *Comment:* Strong EMA positivity (EMA, (clone E29), 60×).

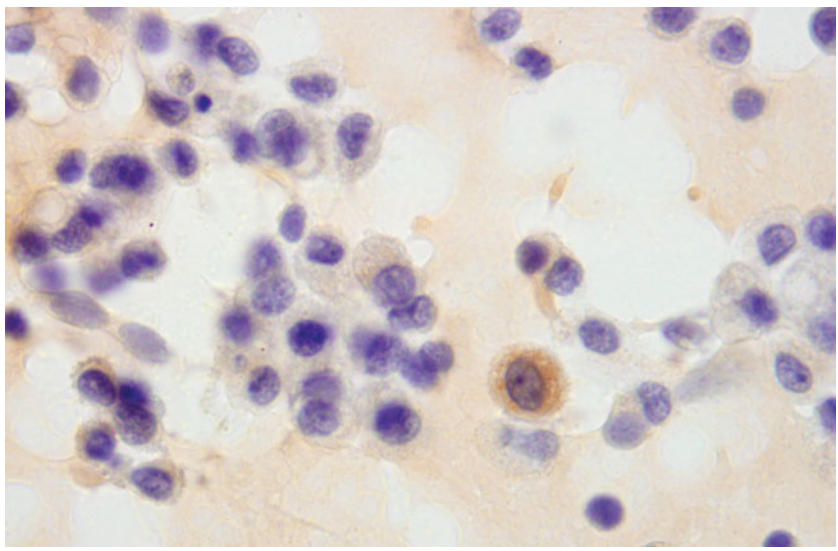

FIGURE 2.27 Pleural fluid: Negative for malignancy. *Comment:* A focal weak EMA staining should be considered a negative result (EMA, (clone E29), 100×).

- Some small panels include desmin as the marker for benign reactive mesothelial cells. Since desmin is not expressed in Mullerian–derived epithelium, it is potentially useful in women who present with peritoneal effusions, or in cases of peritoneal washings performed as a part of staging in female genital-tract malignancies. Desmin is expressed in reactive mesothelial cells (Figure 2.28A, B) and it is absent in benign Mullerian-derived epithelial cells (endosalpingiosis) and in Mullerian-derived adenocarcinomas.

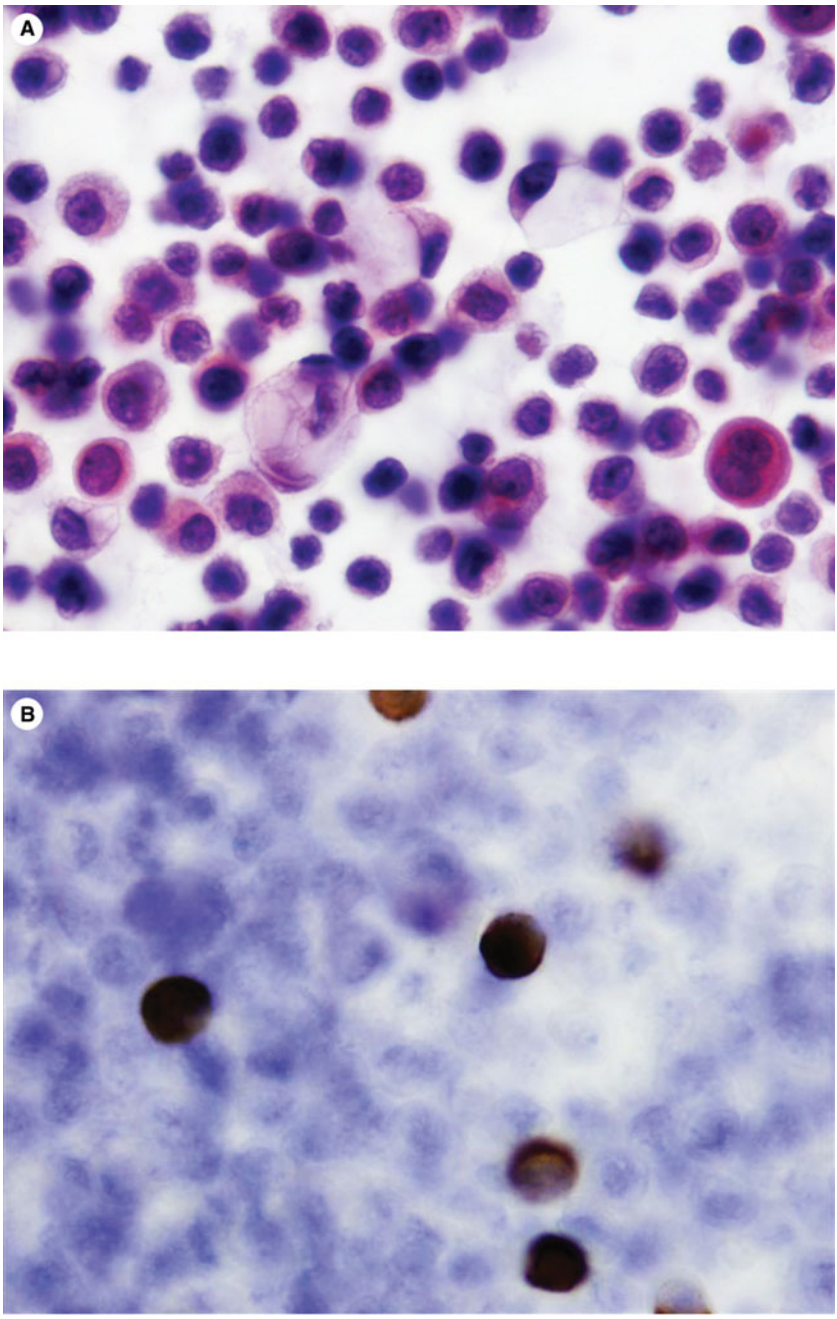

FIGURE 2.28 (A) Pleural fluid: "Preliminary diagnosis": Atypical cells of epithelial origin are present probably due to a reactive process. Immunostains to follow. *Comment:* The use of term "atypical cells" should be avoided in final interpretation of effusions (PAP, 100×). (B) Pleural fluid: "Final diagnosis": Negative for malignancy. Cellular evidence of reactive mesothelium. *Comment:* Desmin positivity is helpful to confirm presence of benign reactive mesothelial cells (Desmin, 100×).

Other Markers

- E-Cadherin is expressed in malignant mesotheliomas and some adenocarcinomas, whereas reactive mesothelial cells do not express this antibody.
- KOC also known as L523S and IMP3_is considered a useful marker by some investigators. KOC is expressed in majority of malignant effusions including adenocarcinomas and malignant mesotheliomas. Reactive mesothelial cells are negative for KOC.
- MOC-31 is an epithelial specific antigen that is considered to be a sensitive marker for adenocarcinomas.
- GLUT-1 is expressed in malignant effusions and is a useful marker to differentiate reactive mesothelial cells from adenocarcinomas, particularly those from ovarian and lung origins. GLUT-1 is also shown to be positive in adenocarcinomas from the gastrointestinal tract and breast origins.

Use of Immunocytochemistry in Common Diagnostic Problems in Effusions (Benign Versus Malignant)

	RMC	AdenoCa	MM
EMA	Negative	Positive	Positive
CEA	Negative	Positive	Negative
BER-EP4	Negative	Positive	Negative
B72.3	Negative	Positive	Negative
Leu-M1	Negative	Positive	Negative
Desmin	Positive	Negative	Negative
E-Cadherin	Negative	Positive	Positive
KOC	Negative	Positive	Positive
MOC-31	Negative	Positive	Negative
GLUT-1	Negative	Positive	Positive

RMC: Reactive mesothelial cells; AdenoCa: adenocarcinoma; MM: malignant mesothelioma.

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3

Types of Malignancy Based on Conventional Cytology and Immunocytochemistry

- Identification of the specific type of malignancy may carry important prognostic and therapeutic implications.
- In previous chapters we described diagnostic criteria for malignancy. In this chapter we will address the next commonly encountered question, that is, "*if malignant, what type?*"
- Determination of the specific type of malignancy based on cytomorphology alone can be a challenging and, on many occasions, an impossible task to achieve.

Cell-specific immunocytochemical markers offer an attractive means for confirming the cell of origin and may assist in the subclassification of malignancies.

Adenocarcinomas

Conventional Cytology

- In the majority of cases, establishing the diagnosis of adenocarcinoma in effusions is fairly easy.
- Common primary sites for adenocarcinomas seen in effusions include lung, breast, gastrointestinal tract, and female genital tract.
- There are several cytologic features that are helpful in suggesting the presence of adenocarcinoma cells in effusions.

Cytological Features of Adenocarcinomas	
Cell Balls	Signet Ring Cells
Papillary Clusters	Psammoma Bodies
Cytoplasmic Vacuoles	Indian Filing

- *Cell balls* are tightly grouped cell clusters with smooth borders, commonly seen in adenocarcinomas. "Cell balls" with smooth outlines are considered as characteristic for metastatic breast carcinomas in effusion samples; however, in addition to metastatic breast adenocarcinoma (Figure 3.1), we have seen cases of pulmonary carcinomas present as "cell balls" in pleural effusions.
- *Papillary clusters* in cytospin samples are not commonly found; however, their presence may indicate adenocarcinoma. Ovarian, lung, and kidney (Figure 3.2) carcinomas, and rarely thyroid carcinomas, may demonstrate this cytologic feature. Malignant mesotheliomas may also shed papillary clusters in the effusions. In our experience, benign reactive mesothelial cells do not present as papillary cell clusters in routinely prepared cytospin slides.
- *Cytoplasmic vacuoles* may be seen in adenocarcinoma cells (Figure 3.3). Cytoplasmic vacuoles are more commonly seen as a result of cellular degeneration. They may not necessarily indicate mucin production by adenocarcinoma cells.

The presence of cytoplasmic vacuoles in adenocarcinoma cells may not indicate mucin production by tumor cells, and therefore we do not use the term "mucinous adenocarcinoma" in the diagnosis of effusions.

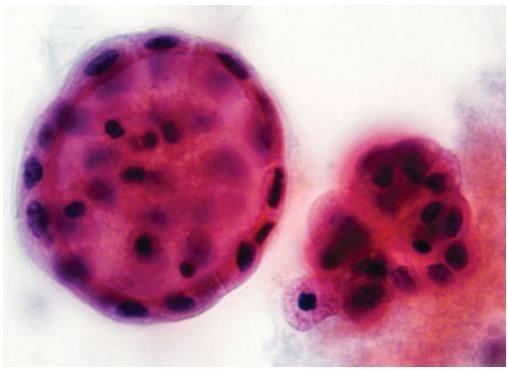


FIGURE 3.1 Pleural fluid: Cellular evidence of adenocarcinoma. *Comment:* "Cell balls" are commonly seen in metastatic breast carcinomas. The patient had a remote history of breast carcinoma and presented with pleural effusion (PAP, 100×).

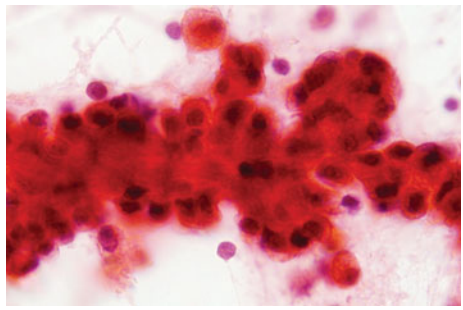


FIGURE 3.2 Pleural fluid: Cellular evidence of carcinoma with papillary features. *Comment:* Papillary cell clusters in effusions may indicate ovarian, lung, and kidney carcinomas and rarely carcinomas of thyroid origin. This patient had a history of renal cell carcinoma and metastatic nodules in the lung (PAP, 60×).

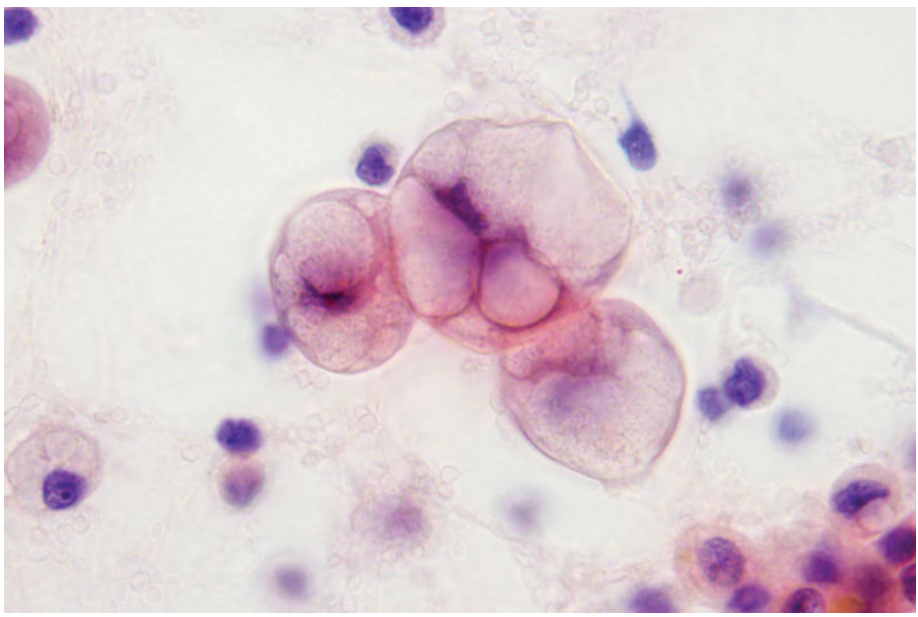


FIGURE 3.3 Peritoneal fluid: Cellular evidence of adenocarcinoma. *Comment:* Cytoplasmic vacuoles do not necessarily indicate mucin production by adenocarcinoma cells. This case represents a metastatic serous carcinoma of fallopian tube origin (PAP, 100×).

- Interestingly, typical cases of mucinous adenocarcinomas involving the peritoneal cavity, that is, pseudomyxoma peritonei, are rarely diagnosed cytologically, because of the paucity of cytologic material and a lack of cellular characteristics for malignancy in these peculiar mucinous tumors (Figure 3.4A, B).

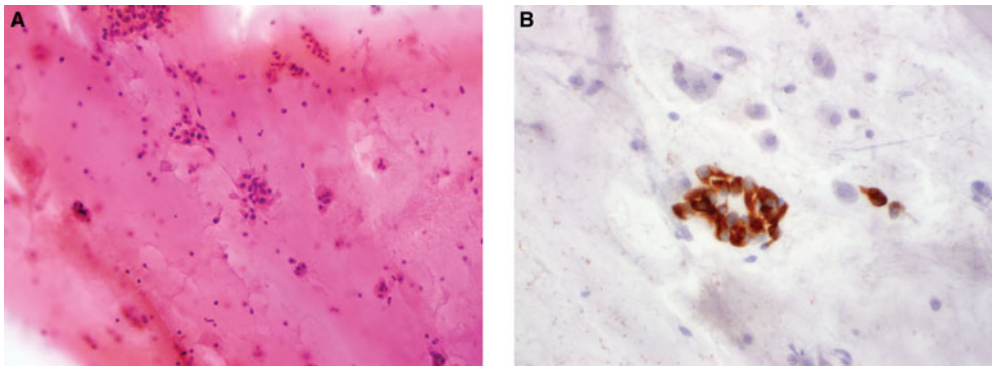


FIGURE 3.4 (A) Peritoneal fluid: Cellular evidence of mucinous neoplasm consistent with pseudomyxoma peritonei. *Comment:* Because of the paucity of cells and lack of cellular atypia, the diagnosis of a neoplastic process may be totally overlooked. This patient had a history of a low-grade mucinous adenocarcinoma of the ovary (PAP, 20×). (B) Peritoneal fluid: Cellular evidence of mucinous neoplasm consistent with pseudomyxoma peritonei. *Comment:* Immunocytochemistry for EMA is helpful to detect additional neoplastic cells, otherwise difficult to observe on Pap stain. The patient had a history of a low-grade mucinous adenocarcinoma of the ovary (EMA, (clone E29), 60×).

- *"Signet ring cells"* may be the result of cytoplasmic mucin pushing the nucleus against the cell membrane. The vacuoles may contain neutrophils.

> Only "signet ring" cells that are in tight clusters or show hyperchromasia or nuclear membrane abnormalities are considered to be of carcinomatous origin (Figure 3.5), more commonly from the breast, gastrointestinal tract, and ovary.

- Since degenerated mesothelial cells and macrophages may also appear as "signet ring" cells (Figure 3.6), the diagnosis of mucin-producing adenocarcinoma should not be made unless the "signet ring" cells are *in tight clusters* and demonstrate hyperchromasia and nuclear membrane abnormalities.
- *Psammoma bodies* are often seen in serous papillary neoplasms of the female genital tract, more commonly of ovarian origin (Figure 3.7). It should be emphasized, however, that the presence of psammoma bodies alone without accompanying cells (Figure 3.8) in peritoneal effusions may not represent a neoplastic process. These calcified structures are occasionally seen in women with endosalpingiosis involving peritoneal surfaces.

> The presence of psammoma bodies alone in serous effusions may not represent a neoplastic process.

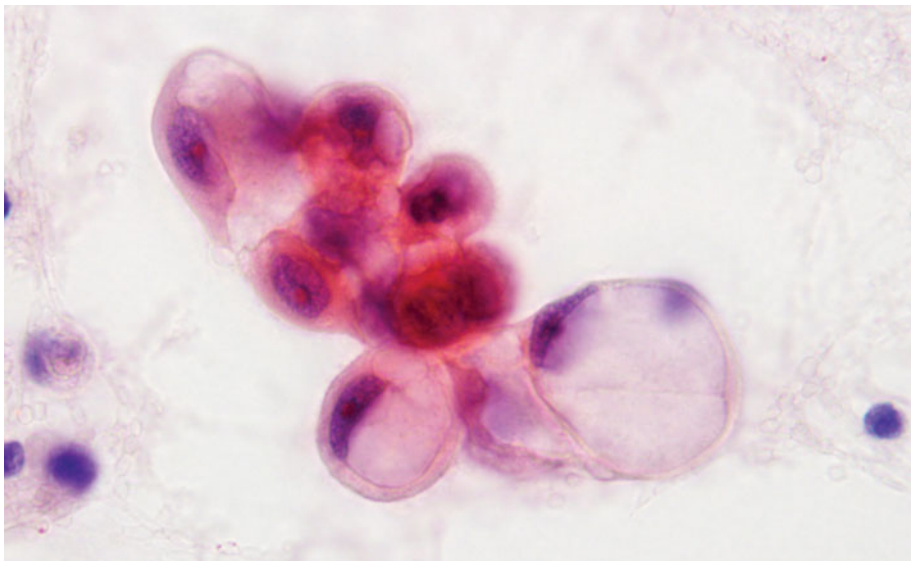


FIGURE 3.5 Pleural fluid: Cellular evidence of adenocarcinoma. *Comment:* "Signet ring" cells in groups are characteristic for adenocarcinoma (PAP, 100×).

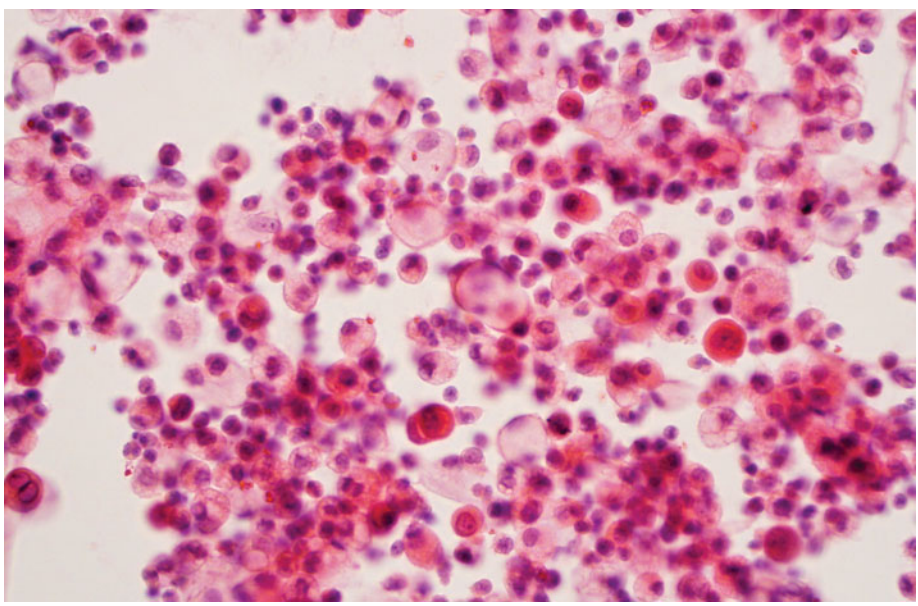


FIGURE 3.6 Pleural fluid: Negative for malignancy. Cellular evidence of reactive mesothelium. *Comment:* Single vacuolated cells with pushed-aside nuclei, that is, "signet ring" cells may be seen as a result of mesothelial cell degeneration. "Signet ring" cells must be seen in tight clusters with hyperchromasia and nuclear membrane abnormalities in order to be diagnostic of adenocarcinoma. The patient had prostatic and bladder carcinomas and long-standing bilateral pleural effusions due to heart failure (PAP, 60×).

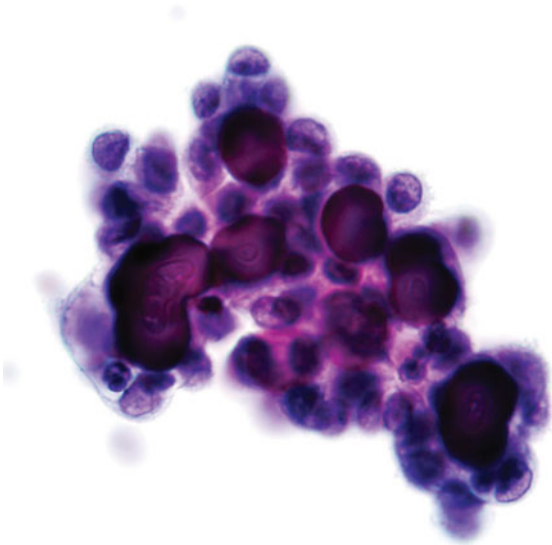


FIGURE 3.7 Peritoneal washings: Cellular evidence of serous neoplasm with psammoma bodies. *Comment:* The presence of benign-looking epithelial cells associated with psammoma bodies may represent a neoplastic process (benign or borderline malignancy). However, the lack of cellular abnormalities precludes the diagnosis of malignancy on cytologic samples. The patient had an ovarian serous borderline tumor (PAP, 60×).

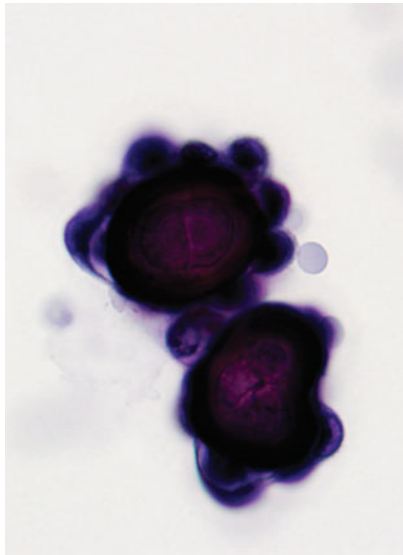


FIGURE 3.8 Peritoneal washings: Negative for malignancy. *Comment:* Psammoma bodies without a cellular component may be seen in non-neoplastic conditions such as endosalpingiosis (PAP, 100×).

- *Psammoma bodies* are also described in papillary carcinomas of thyroid and lung origin.
- *Indian filing* is referred to cells arranged in small chains, commonly seen in cases of breast, gastrointestinal tract, and pancreatic adenocarcinomas (Figure 3.9).

Immunocytochemistry

- In serous effusions, there are several cytomorphologic characteristics that are helpful in classifying malignant cells as adenocarcinoma cells. However, because of the low specificity of conventional cytology alone, today this diagnostic exercise is carried out with higher specificity utilizing commonly available immunocytochemical markers.
- Immunocytochemistry (ICC) can be applied as an adjunct to the conventional cytologic diagnosis. In our laboratory it is performed when the diagnostic difficulty is noticed at the time of initial cytologic examination, or when clinicians request the exclusion of a specific type of neoplasm

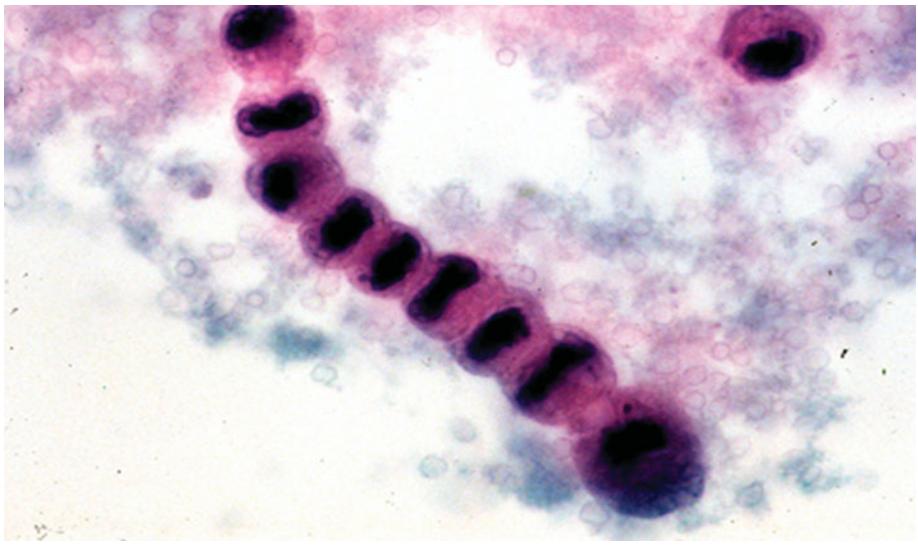
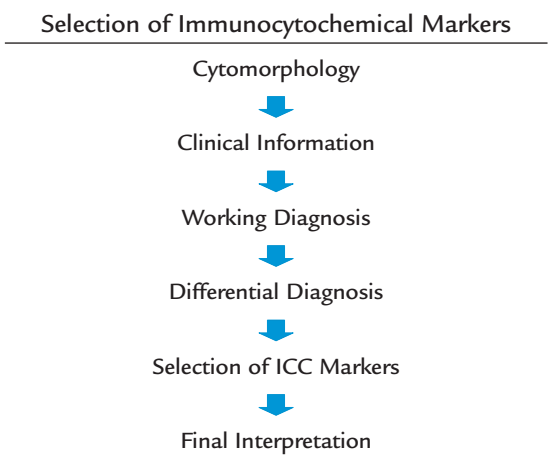


FIGURE 3.9 Pleural fluid: Cellular evidence of adenocarcinoma. *Comment:* "Indian file" cellular arrangements in effusions may be seen in metastatic carcinomas of the breast and gastrointestinal tract, and those of pancreatic origin. The patient had a remote history of breast cancer (PAP, 100×).

based on imaging findings such as exclusion of a primary pancreatic adenocarcinoma in a patient with a pancreatic mass and ascites or to rule out metastatic breast carcinoma in a patient with a remote history of breast carcinoma who presents with pulmonary nodules and pleural effusion.

- Since on many occasions there is no additional material available, at the sign-out time, we select the same alcohol-fixed Papanicolaou-stained slides with malignant cells that show the least amount of blood or inflammatory cells for subsequent immunocytochemical stains, and send it to our ICC laboratory (see Technique, Chapter 7).



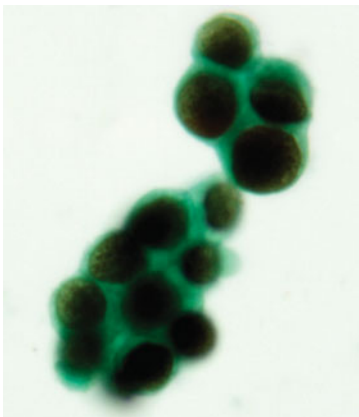


FIGURE 3.10 Peritoneal fluid: Cellular evidence of adenocarcinoma consistent with a lung primary. *Comment:* TTF-1 is positive in most nonsquamous carcinomas of the lung. The patient had a history of lung TTF-1-positive adenocarcinoma and presented with a pelvic mass (TTF-1, 100×).

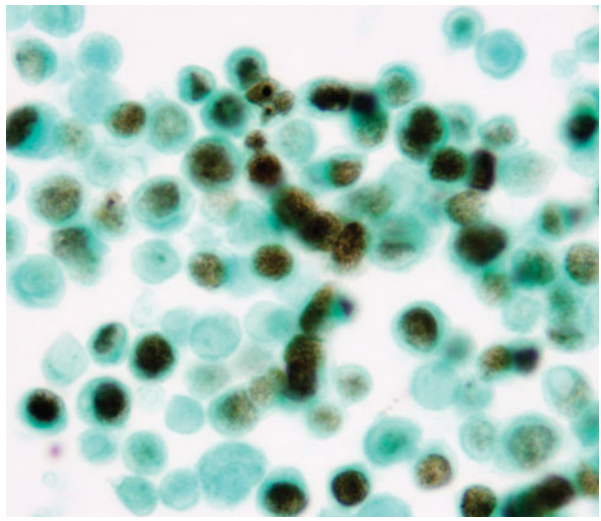


FIGURE 3.11 Pleural fluid: Cellular evidence of carcinoma consistent with a breast primary. *Comment:* The patient had a history of ER-positive breast carcinoma (ER, (clone 1D5), 100×).

Immunocytochemical Markers for the Diagnosis of Carcinomas

TTF1	CA-125 and WT-1
ER/PR	PSA
CK7 and CK20	TGB
CDx-2	

- *TTF1*: Thyroid transcription factor: for carcinomas of lung (Figure 3.10) and thyroid origin.
- *ER/PR*: Estrogen (Figure 3.11) and progesteron receptors for carcinomas of breast and female genital tract origins.
- *CK7 and CK20*: Cytokeratin 7 and 20 for gastrointestinal and gynecological tract neoplasms.
- *CDx-2*: Used for neoplasms of intestinal origin (Figure 3.12).
- *CA-125 and WT-1*: Used for adenocarcinomas of the female genital tract.
- *PSA*: Prostatic specific antigen, for prostatic adenocarcinomas.
- *TGB*: Thyroglobulin for carcinomas of thyroid origin.

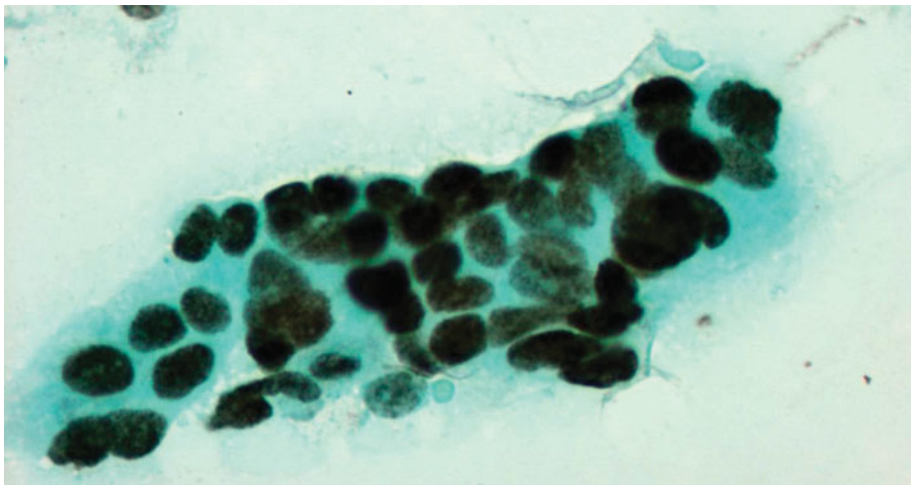


FIGURE 3.12 Peritoneal fluid: Cellular evidence of adenocarcinoma. *Comment:* Positive CDx-2 supports intestinal origin. The patient had a history of colonic carcinoma (CDx-2, 100×).

Squamous Cell Carcinomas

Conventional Cytology

- Squamous cell carcinomas are common primary tumors, arising mainly from the lung, female genital tract, upper respiratory tract, skin, and esophagus; however, they are rarely the cause of malignant effusions. The presence of squamous carcinoma cells in serous effusions has been an uncommon event until recently.
- We have experienced an increased number of metastatic squamous cell carcinomas, especially in pleural effusions in patients with head and neck malignancies. Perhaps this is due to the availability of more effective treatment and the prolonged course of their disease.

> It is generally believed that squamous cell carcinomas *do not* shed cells frequently, or when they do, the cells are mistaken for other malignancies such as poorly differentiated adenocarcinomas and malignant mesotheliomas.

Keratinizing Squamous Cell Carcinomas

- The identification of carcinoma cells in keratinizing squamous cell carcinomas can be relatively easy.
- Carcinoma cells may occur as tissue fragments, usually seen in cell blocks.
- Anucleated masses of keratin may also be seen in keratinizing squamous cell carcinomas.
- Squamous cells are round to oval, with dense cytoplasm and distinct, well-defined borders (Figure 3.13A).
- Pyknotic nuclei (Indian ink-like) may show coarsely granular chromatin.
- The nuclei are central and multinucleation is not uncommon.
- Except in poorly differentiated lesions, nucleoli are not prominent.
- Some cells may have cytoplasmic vacuoles. The vacuolization may be striking, causing the cells to simulate those of adenocarcinomas.
- Keratinized round/oval cells have a bright orangeophilic cytoplasm. However, the cytoplasmic staining may vary from acidophilic to cyanophilic or amphophilic.

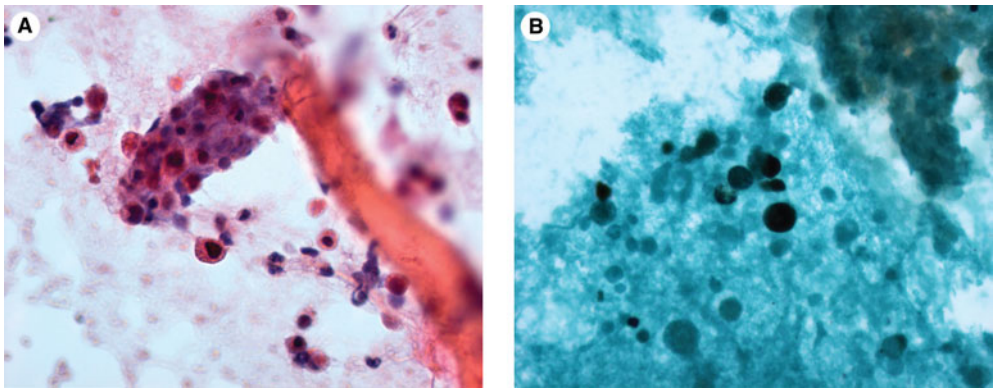


FIGURE 3.13 (A) Pleural fluid: Cellular evidence of carcinoma. *Comment:* The diagnosis of squamous cell carcinoma in effusions is difficult due to the usual paucity of diagnostic cells. This patient had a history of head and neck squamous cell carcinoma (PAP, 100×). (B) Pleural fluid: Cellular evidence of carcinoma consistent with metastatic squamous cell carcinoma. *Comment:* p63 immunostain facilitated the detection of carcinoma cells and confirmed the diagnosis of metastatic squamous cell carcinoma (p63, 100×).

- Less frequently seen in effusion samples are the following:
  - "Tadpole" cells, which are elongated orangeophilic cells with eccentric nuclei and long cytoplasmic tails.
  - "Fiber" cells, which are slender cells with central, thin, and elongated nuclei.
  - "Keratin pearl" cells, which are arranged in whorls as they wrap around each other.

Necrotic tumor cells from other types of malignancy may show orangeophilic dense cytoplasm, *not* to be confused with keratinizing squamous cells.

Nonkeratinizing Squamous Cell Carcinomas

- The cells do *not* demonstrate cytoplasmic keratinization. The cytoplasm is usually cyanophilic, mimicking adenocarcinoma cells.
- The nuclei are round to oval and vary in size.
- Nucleoli are prominent and frequently multiple, resembling adenocarcinoma cells.
- Nonkeratinizing squamous cell carcinomas in effusions are difficult to differentiate from metastatic adenocarcinomas on the basis of morphology alone.

In such cases the patient's age and sex, knowledge of previous cancer history and treatment, and use of ICC are important for proper diagnosis.

Immunocytochemistry

- In our laboratory, we use a small panel of two antibodies, that is, p63 and TTF-1, to distinguish poorly differentiated, nonkeratinizing squamous cell carcinoma cells (p63 positive/TTF-1 negative) from lung adenocarcinomas (p63 negative/TTF-1 positive). Both markers are extremely helpful in detecting rare isolated malignant cells in paucicellular effusions.
- In addition, p63 is a useful marker to differentiate squamous cell carcinomas from other non-small-cell carcinomas of the lung in cytologic samples (Figure 3.13B).
- TTF-1 is a helpful *negative* marker to differentiate lung poorly differentiated nonkeratinizing squamous cell carcinomas from poorly differentiated adenocarcinomas. TTF-1 is usually negative in most bronchogenic squamous cell carcinomas.
- A less commonly encountered differential diagnosis in cases of squamous cell carcinomas is malignant mesothelioma. Some investigators have used a combination of two positive mesothelial markers (WT1 and calretinin or mesothelin) with two negative mesothelioma markers (p63 and Moc-31) in histologic sections to distinguish between epithelioid mesotheliomas and squamous cell carcinomas.

Small-Cell Carcinomas

Conventional Cytology

- Most small-cell carcinomas found in serous effusions are of lung origin. However, patients with pulmonary small-cell carcinomas rarely present with pleural effusions.
- Cytologic characteristics of small-cell carcinomas in effusion samples (usually pleural) are well recognized and similar to other pulmonary specimens such as bronchial brushing (BB), bronchial washing (BW), and fine-needle aspiration (FNA).
- The cells are of small to medium size with high nuclear/cytoplasmic ratios.
- Nuclear molding is a characteristic finding, and is usually striking (Figure 3.14).
- The nuclei are round or angulated, and have coarse chromatin and a wrinkled nuclear membrane.
- Salt-and-pepper chromatin may be present, as described in cells of neuroendocrine neoplasms.

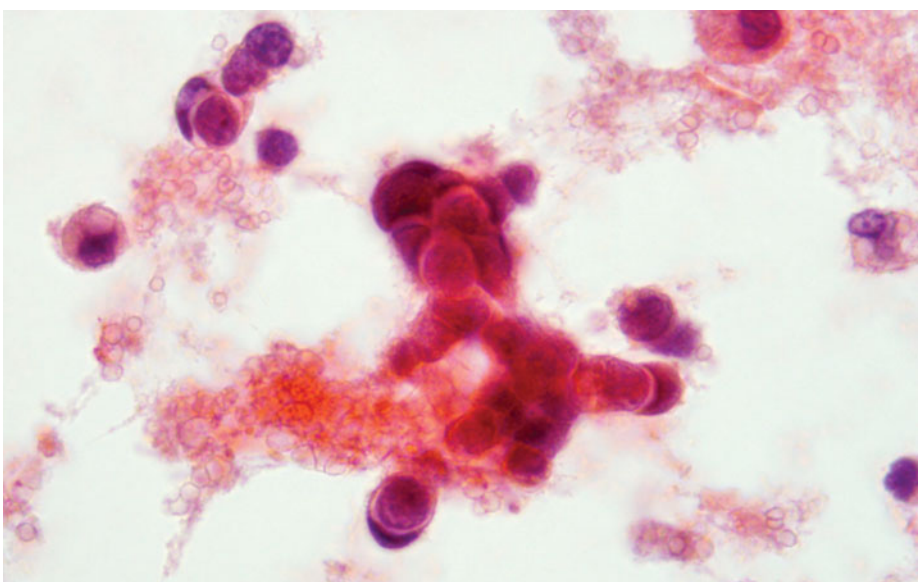


FIGURE 3.14 Pleural fluid: Cellular evidence of small-cell carcinoma. *Comment:* Nuclear molding is a characteristic finding of small-cell carcinomas. The patient was a heavy smoker and presented with a lung mass and pleural effusion (PAP, 100×).

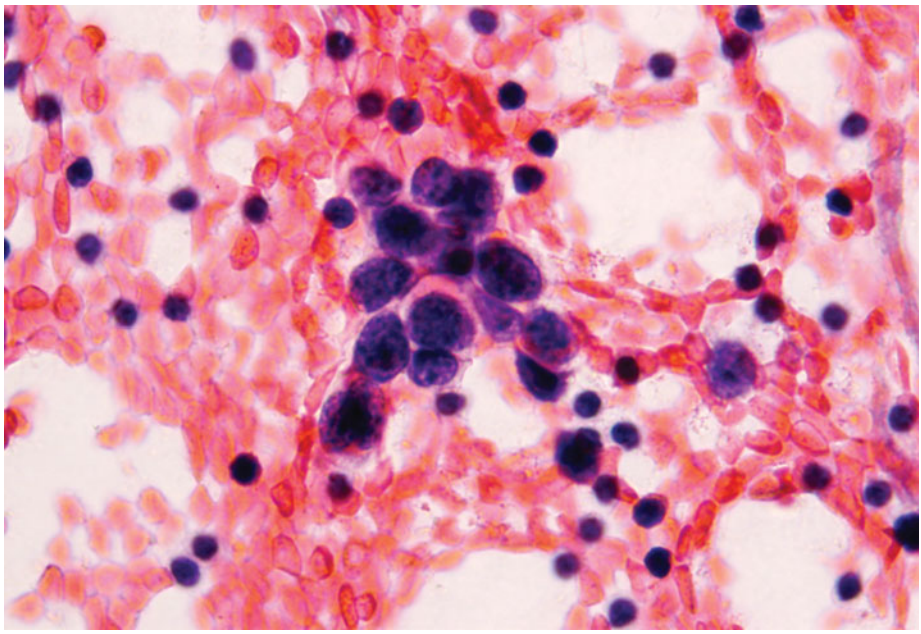


FIGURE 3.15 Pleural fluid: Cellular evidence of small-cell carcinoma. *Comment:* Tight aggregates of small blue cells are characteristic of small-cell carcinomas in the effusions of adult patients. The patient presented with a lung mass and pleural effusion (PAP, 100×).

- The cells commonly present as tight aggregates of small blue cells (Figure 3.15), cell balls, or small chains simulating the "*Indian file*" arrangement described in breast cancer (Figure 3.16).

When small carcinoma cells are found in isolated form, based on morphology alone, recognition of the cells may be difficult because of their cytologic resemblance to lymphocytes. In addition, the isolated tumor cells may be totally overlooked on low power magnification. A mixture of both isolated and cohesive cells, when present is of diagnostic value.

Immunocytochemistry

- In adults, the most common primary site for small-cell carcinomas found in effusions is the lung.
- The pathologic distinction of small-cell from non-small-cell lung carcinomas is of considerable therapeutic significance.

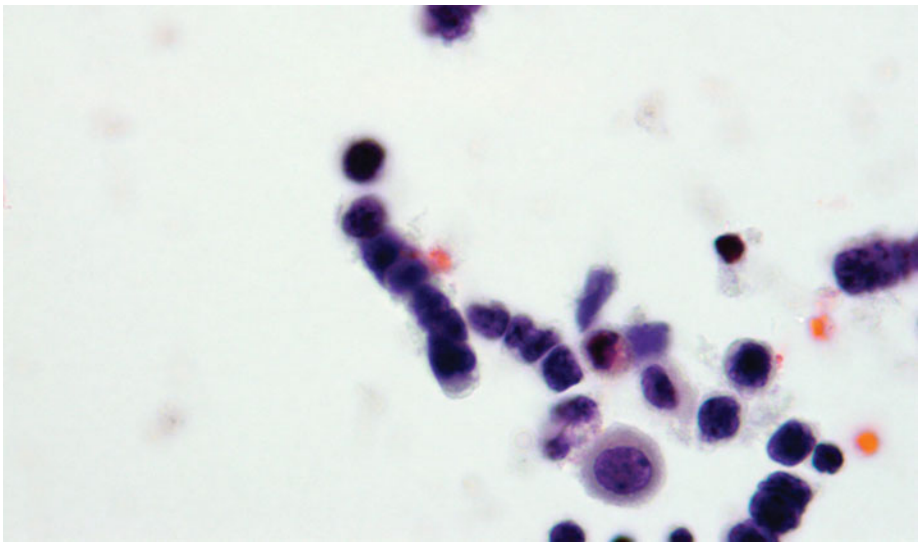


FIGURE 3.16 Peritoneal fluid: Cellular evidence of small-cell carcinoma. *Comment:* Small-Cell chains simulating the "Indian file" arrangement described in breast cancer may also be seen in effusions with small-cell carcinomas. This was an adult patient with recurrent small-cell carcinoma of the urinary bladder (PAP, 100×).

- The ability to distinguish poorly differentiated non-small-cell carcinomas from small-cell carcinomas is at times difficult based on morphology alone. Available immunocytochemical markers such as neuroendocrine markers are of limited utility in such instances.
- Using a cytokeratin cocktail, small-cell carcinomas show characteristic dot-like para-nuclear staining (Figure 3.17).

> Dot-like para-nuclear cytokeratin staining is a helpful marker for the diagnosis of small-cell carcinoma.

- Most cases of small-cell carcinomas are positive for synaptophysin and CD56, and a proportion of them are also positive for chromogranin A (Figure 3.18).
- Most small-cell carcinomas of the lung are also positive for TTF-1 (Figure 3.19).
- When the differential diagnosis is between basaloid squamous cell carcinoma and small-cell carcinoma, p63 is a helpful marker that is negative in small-cell carcinomas.

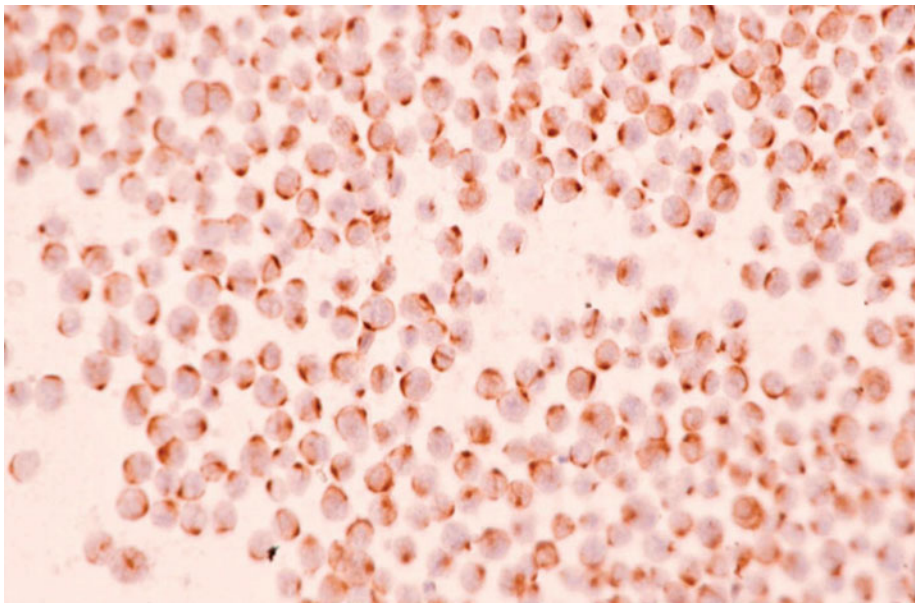


FIGURE 3.17 Pleural fluid: Cellular evidence of small-cell malignant neoplasm; the differential diagnosis includes malignant lymphoma and small-cell carcinoma. *Comment:* The characteristic dot-like para-nuclear staining of cytokeratin confirms the diagnosis of small-cell carcinoma. The patient presented with a lung mass and pleural effusion (CK, 100×).

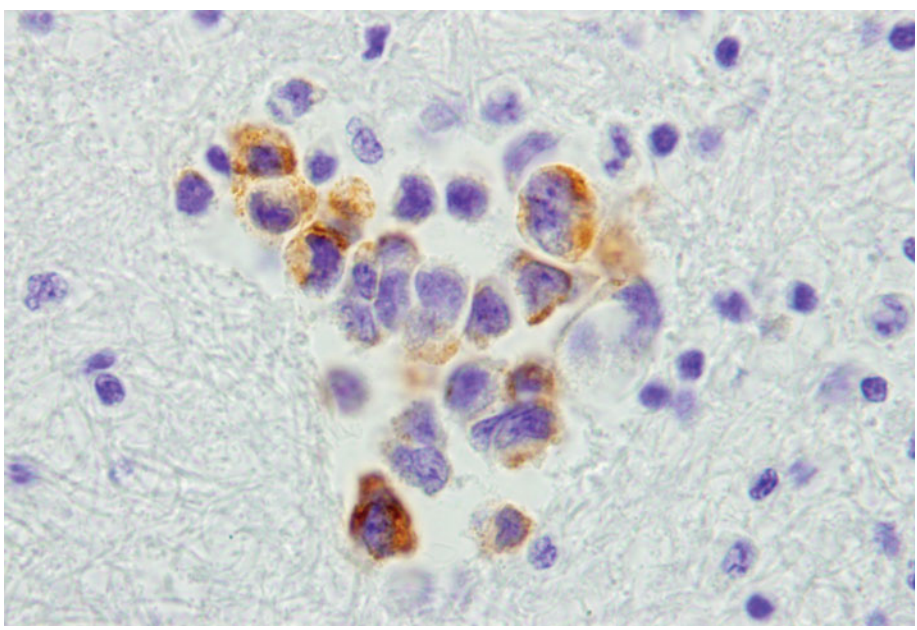


FIGURE 3.18 Pleural fluid: Cellular evidence of small-cell carcinoma. *Comment:* About one third of small-cell carcinomas express chromogranin (Chromogranin A, 100×).

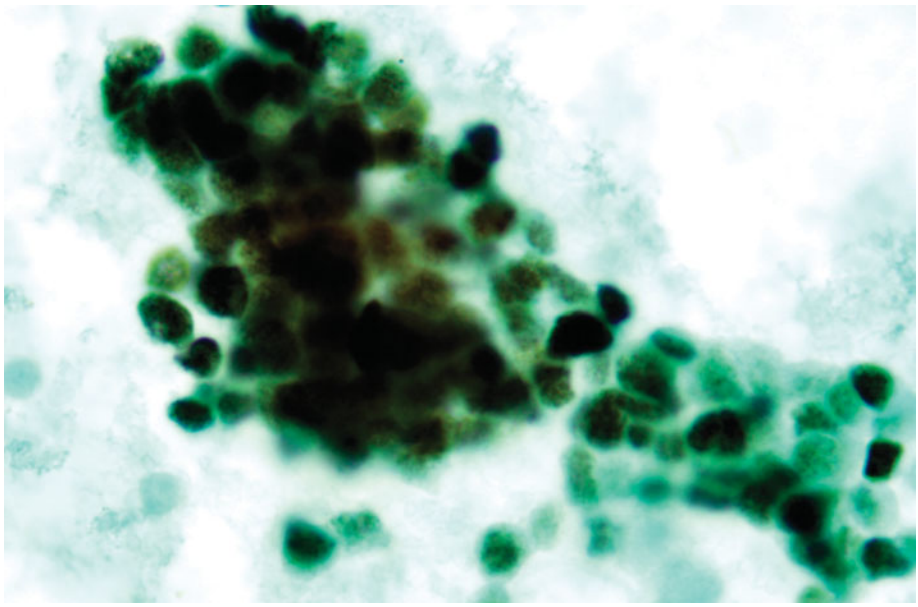


FIGURE 3.19 Pleural fluid: Cellular evidence of small-cell carcinoma. *Comment:* TTF-1 is a good marker for the diagnosis of small-cell carcinomas of lung origin (TTF-1, 100×).

- Most small-cell carcinomas of the lung are p63−/TTF-1+

Immunocytochemical Markers for Small-Cell Carcinomas

Positive	Negative
Cytokeratin (dot-like) Chromogranin A Synaptophysin CD-56 TTF-1	p63

- The following markers are used for the diagnosis of small-cell carcinomas in effusions.
  - *Cocktail cytokeratin*: Shows characteristic dot-like staining in small-cell carcinomas; the cocktail is composed of high molecular weight keratin, AE1/AE3, and CAM5.2.
  - *Chromogranin A*: Is a marker for neuroendocrine neoplasms, including small-cell carcinomas of the lung and gastrointestinal tract, and that of skin (Merkel cell tumor).

- *Synaptophysin*: Also a neuroendocrine marker, more sensitive and yet less specific than chromogranin.
- *CD 56*: A marker for neuroendocrine neoplasms.
- *TTF-1*: A marker for lung and thyroid carcinomas. Is useful especially in effusion specimens when there are only a few small tumor cells present. The dark nuclear staining facilitates their detection even on low-power magnification.
- *P63*: A marker for squamous cell carcinomas. It is negative in small-cell carcinoma and positive in basaloid variants of squamous cell carcinoma.

Small-Cell Malignancies in Young Patients

- In adults, small-cell carcinomas from other than pulmonary origin (those derived from metastatic skin or colorectal primaries) are rarely found in body cavity fluid specimens. However, in young patients, the presence of malignant small cells in effusions most likely represents childhood neoplasms, that is, small blue cell tumors. In such instances, a knowledge of the patient's age and other clinical information is important for a proper cytologic diagnosis.

In addition to the clinical information, accurate cytologic subclassification of small-cell malignancies heavily relies on the results of immunocytochemical studies.

- A small panel of 1–2 antibodies in the case of a known primary malignancy, and a larger panel including epithelial and lymphoid markers in the absence of a known primary tumor may prove to be extremely helpful in establishing the final diagnosis.

Immunocytochemical Markers for Small-Cell Malignancies in Young Patients

	CK	Des	Myogenin	CD99
Carcinoma	Positive	Negative	Negative	Negative
Rhabdomyo	Negative	Positive	Positive	Negative
Ewing/PNET	Negative	Negative	Negative	Positive
DSCT	Positive	Positive	Negative	Negative

CK: cytokeratins; Des: Desmin; Rhabdsarc: Rhabdomyosarcoma; DSCT: Desmoplastic small cell tumor

- The following markers are used for the diagnosis of small-cell malignancies in effusions of young patients.
  - *Cytokeratin*: Shows characteristic dot-like staining in small-cell carcinomas.
  - *Desmin*: Is a muscle marker (for both smooth and skeletal muscles).
  - *Myogenin*: Is a marker for skeletal muscle. Should be used following desmin positivity to confirm the diagnosis of rhabdomyosarcoma.
  - *CD99*: This is not a specific marker, yet very useful when all other small-cell markers are negative.

Malignant Lymphomas

Conventional Cytology

- Cytologic examination is an accurate method for the diagnosis of malignant lymphoma in effusions with a reasonably high overall diagnostic accuracy.
- In centers such as ours, with increasing numbers of HIV-positive patients, the diagnosis of malignant lymphoma in serous fluids has become a more demanding task for the cytopathologist.
- Malignant lymphomas are characterized by the presence of numerous *isolated* cells in the cytospin preparation of serous effusions associated with apoptotic bodies (Figure 3.20).
- The nuclei may show variations in size and shape. Nuclear indentation and convolution and the presence of prominent nucleoli are important diagnostic features for both B- and T-cell lymphomas (Figures 3.21 and 3.22).

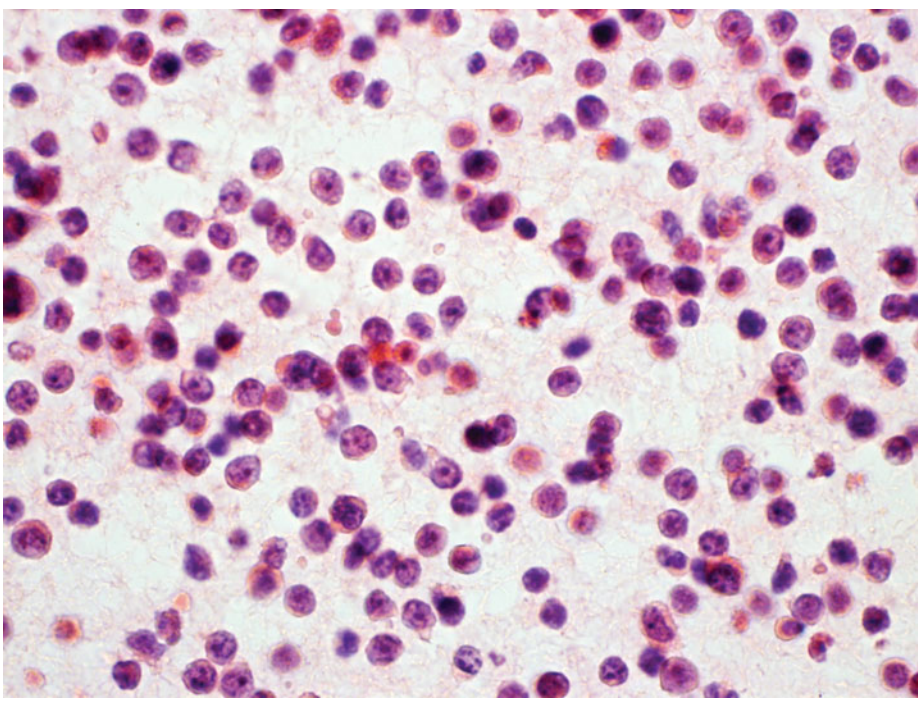


FIGURE 3.20 Pleural fluid: Cellular evidence of malignant lymphoma. *Comment:* Malignant lymphomas are characterized by the presence of numerous isolated lymphoid cells in the cytospin preparation of serous effusions. Note the presence of apoptotic bodies (PAP, 60×).

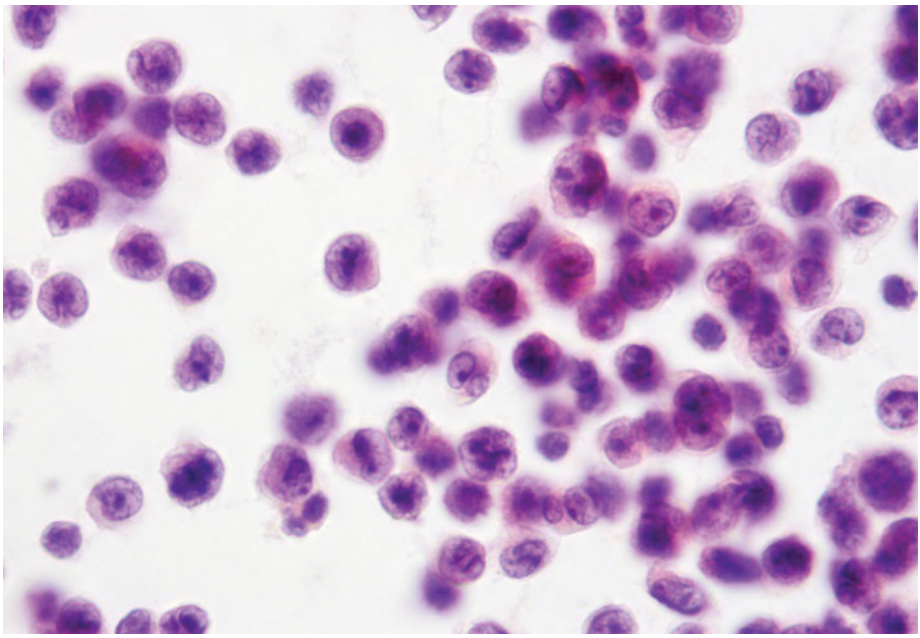


FIGURE 3.21 Peritoneal fluid: Cellular evidence of malignant lymphoma. *Comment:* Note the isolated cellular pattern. The nuclei show variations in size and shape. The presence of prominent nucleoli is commonly seen and is an important diagnostic feature for malignant lymphomas. The patient had a history of B-cell lymphoma (PAP, 100×).

- Individual cell necrosis (apoptotic bodies) is a peculiar and, in our experience, a very useful finding in the diagnosis of lymphoma in serous effusions (Figure 3.23).
- Apoptosis may be the result of previous chemotherapy; however, in our experience it may also be seen in the absence of treatment history.

When apoptotic cells are numerous, on low-power magnification, they can be confused with neutrophils that may result in an erroneous diagnosis of acute inflammation.

- The cytoplasm of lymphoma cells is usually scant, basophilic, and rarely well preserved.
- Small-Cell lymphocytic lymphoma, chronic lymphocytic leukemia (CLL), and Waldenstrom's macroglobulinemia may present with a large number of "mature-looking" lymphocytes. In such cases, unequivocal separation of these neoplastic processes from chronic nonspecific inflammation

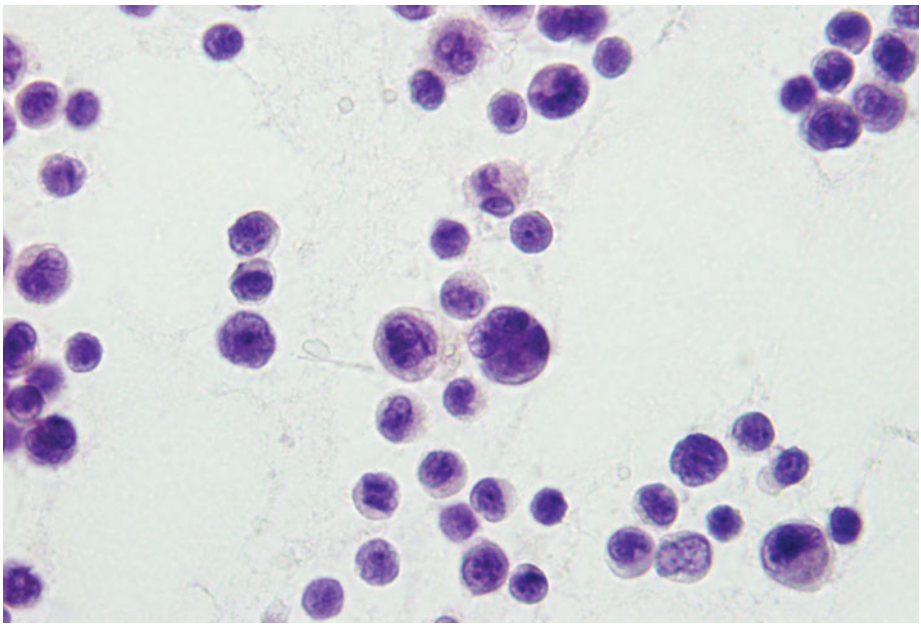


FIGURE 3.22 Pleural fluid: Cellular evidence of malignant lymphoma. *Comment:* Isolated lymphoid cells with nuclear membrane indentation and convolution are characteristic findings of T-cell lymphomas. The patient had a history of mediastinal T-cell lymphoma (PAP, 100×).

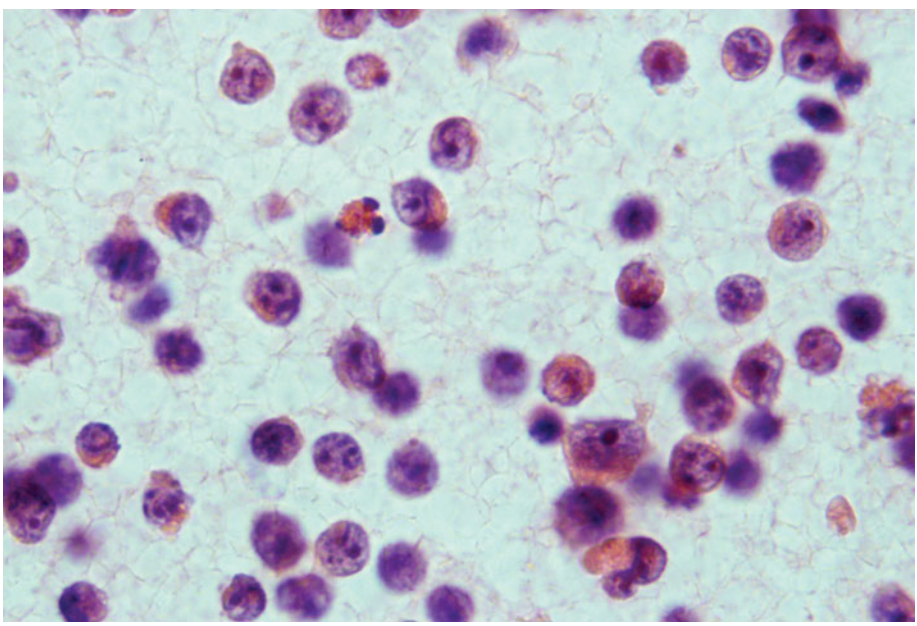


FIGURE 3.23 Pleural fluid: Cellular evidence of malignant lymphoma. *Comment:* Individual cell necrosis (apoptotic bodies) is a peculiar and useful finding in the diagnosis of lymphomas in serous effusions. Note that the apoptotic bodies may be mistaken for segmented leukocytes on low-power magnification (PAP, 100×).

involving serous effusions or tuberculosis is not possible on the basis of cytomorphology alone.

- Monoclonal proliferation of T or B lymphocytes can be determined by immunocytochemical stains, flow cytometry, or gene rearrangement studies.

> At our laboratory, based on conventional cytology alone we do *not* render a diagnosis of malignant lymphoma in serous effusions, unless the cells have nuclear characteristics of malignancy and a large number of malignant cells are present.

- When the number of abnormal lymphoid cells is not considered to be sufficient for the unequivocal diagnosis of a malignant lymphoproliferative process, the following diagnosis is given: "*Cellular evidence of atypical lymphoproliferative process; recommend further studies to rule out malignancy.*"

> In equivocal cases a follow-up specimen is sent for flow cytometry and/or for gene rearrangement studies. In any case of potential lymphoma we share the case with our hematopathologists and seek their opinion.

- When a large number of mature lymphocytes are present in a patient with a known well-differentiated small-cell lymphoma or chronic lymphocytic leukemia, we make a descriptive diagnosis of: "*numerous lymphocytes are present, consistent with…*" In such instances, ancillary studies are recommended for confirmation.

> Large-cell anaplastic lymphomas should be differentiated from other large-cell malignancies with isolated cell patterns such as anaplastic carcinomas, mainly of lung origin.

- Primary effusion lymphoma (PEL) is a rare type of lymphoma that presents as an effusion, seldom evidence of a solid neoplasm; therefore, cytology is a basic diagnostic method. PEL usually occurs in HIV-positive males with a history of Kaposi's sarcoma. PEL is associated with Epstein–Barr virus (EBV) and human herpes virus type 8 (HHV-8). The cytologic

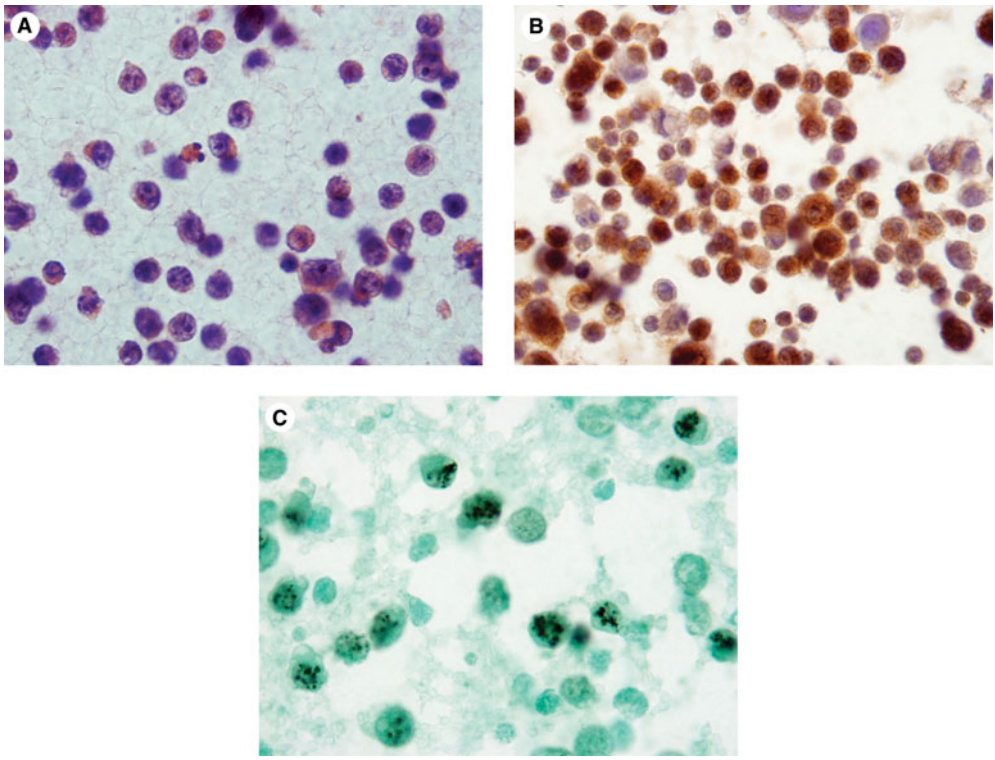


FIGURE 3.24 (A) Peritoneal fluid: Cellular evidence of malignant lymphoma. *Comment:* Abnormal nuclear membrane and apoptotic bodies are present. This HIV-positive patient presented with peritoneal effusion and no known history of lymphoma (PAP, 60×). (B) Peritoneal fluid: Cellular evidence of malignant T-cell lymphoma consistent with primary effusion lymphoma (PEL). *Comment:* Malignant lymphoid cells are positive for CD3 (CD3, 100×). (C) Peritoneal fluid: Malignant cells are positive for HHV8 by in situ hybridization (HHV8, 100×).

characteristics are similar to other lymphomas secondarily affecting serous membranes (Figure 3.24A–C), such as monomorphic populations of atypical medium- to large-sized lymphoid cells, with mono- or binucleated hyperchromatic nuclei and a small-to-moderate amount of basophilic cytoplasm containing cytoplasmic vesicles.

- Although malignant germ cell tumors rarely metastasize to serous membranes, in younger patients seminomas/germinomas should always be considered in the differential diagnosis of large-cell lymphomas. In such cases, a small panel of immunocytochemical markers can be conclusive.
- Hodgkin's lymphomas rarely involve serous membranes. The cytologic picture is usually nonspecific and composed of a mixture of small mature lymphocytes and occasional larger mononuclear cells.

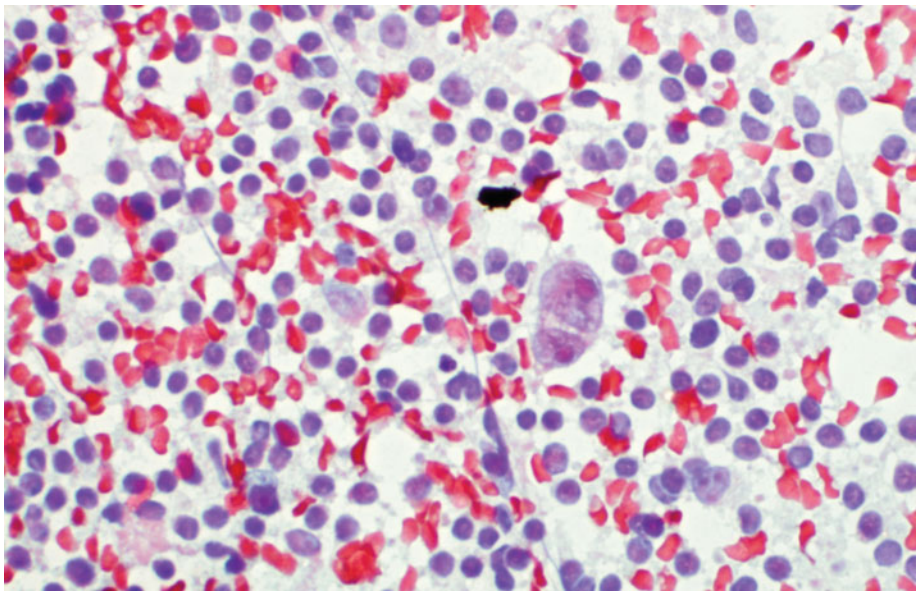


FIGURE 3.25 Pleural fluid: Cellular findings consistent with Hodgkin's lymphoma. *Comment:* Hodgkin's lymphomas are rarely diagnosed in effusions. The presence of abnormal mono-binucleated Reed–Sternberg cells and the patient's clinical history are essential for the diagnosis. This patient had recurrent mediastinal Hodgkin's lymphoma (PAP, 60×).

- The diagnosis of Hodgkin's lymphoma can only be made by finding abnormal mono-multinucleated Reed–Sternberg cells. The patient's previous history and use of immunocytochemical markers may prove to be helpful in these cases (Figure 3.25).

Immunocytochemistry

- In adults, the most common type of lymphoma found in effusion samples is B-cell type.
- The pathologic distinction between B-cell and T-cell lymphomas is of considerable therapeutic significance.
- The ability to distinguish B-cell from T-cell lymphomas is practically impossible based on morphology alone. Furthermore, and specifically in cases of small-cell lymphoma, due to bland cytologic features, cytomorphology alone may not be sufficient for the diagnosis of the malignant lymphoreticular process. ICC, flow cytometry, and molecular tests play an essential role in the diagnosis of malignant lymphomas in effusions and their cell of origin.

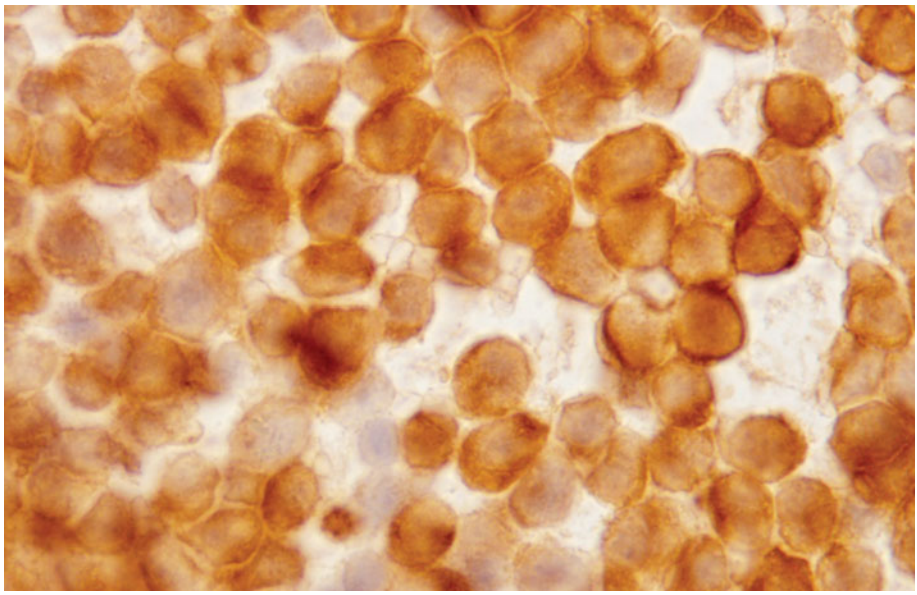


FIGURE 3.26 Peritoneal fluid: Cellular evidence of malignant B-cell lymphoma. *Comment:* Malignant cells are positive for CD20. This patient had a history of retroperitoneal lymphoma and presented with peritoneal effusion (CD20, 100×).

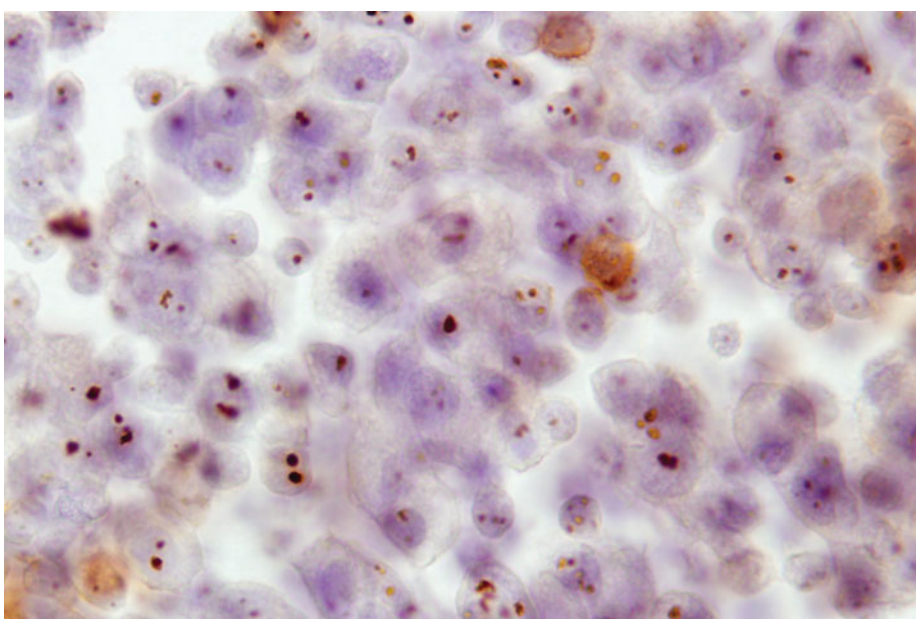


FIGURE 3.27 Pleural fluid: Cellular evidence of malignant lymphoma. *Comment:* Malignant cells with dot-like-artifact CD20 immunostain (CD20, 100×).

Immunocytochemical Markers for Malignant Lymphomas

Positive	Negative
CD45	Cytokeratin
CD20/CD79a	S100/HMB45
CD3	PLAP
CD30	
CD15	

- The following markers are used for the diagnosis of malignant lymphomas:
  - *CD45*: A common marker for lymphoid cells.
  - *CD20*: A marker for B-lymphocytes (Figure 3.26).
  - *CD3*: A marker for T-lymphocytes.
  - *CD30*: A marker for anaplastic lymphomas and Hodgkin's lymphomas.
  - *CD15*: A marker for Hodgkin's lymphomas.
  - *Cytokeratin*: A common epithelial marker.
  - *S100/HMB45*: A marker for malignant melanoma.
  - *PLAP (placental alkaline phosphatase)*: A marker for seminomas/germinomas.

CD20 immunostain in B-cell lymphomas may show a staining artifact characterized by intranuclear dot-like staining (Figure 3.27). In such cases, we use CD-79a immunocytochemical stain to confirm the B-cell lineage.

Malignant Melanomas

Conventional Cytology

- Metastatic melanoma in effusions presents predominantly as isolated cells or cells in small loose clusters. Binucleation and multinucleation are common. Nucleoli are large and may be multiple (Figure 3.28A, B).
- Intranuclear cytoplasmic inclusions and intracytoplasmic melanin, if present, are of diagnostic value.

Immunocytochemistry

- In amelanotic melanomas, in the absence of intracytoplasmic melanin, the diagnosis of malignant melanoma based on conventional cytology is practically impossible.
- Melanoma cells may be confused with other large-cell malignancies such as large-cell lymphomas, undifferentiated carcinomas and less commonly found in effusions, seminomas/germinomas. The distinction in such cases is facilitated by using ICC.

Immunocytochemical Markers for Malignant Melanoma

Positive	Negative
HMB-45	Cytokeratin
S100	EMA
Tyrosinase	Calretinin
Melan-A (MART-1)	CD45

- The following markers are used for the diagnosis of malignant melanomas:
  - *S100*: A commonly used marker for malignant melanoma.
  - *HMB45*: The best marker for malignant melanoma in alcohol-fixed cytologic material (Figure 3.28B).
  - *Tyrosinase*: An additional marker for malignant melanoma.
  - *Melan-A (MART-1)*: An additional marker for malignant melanoma.
  - *Cytokeratin*: A common epithelial marker.
  - *EMA (epithelial membrane antigen)*: Epithelial marker.
  - *Calretinin*: The best marker for mesothelial cells.
  - *CD45*: A common marker for lymphoid cells.

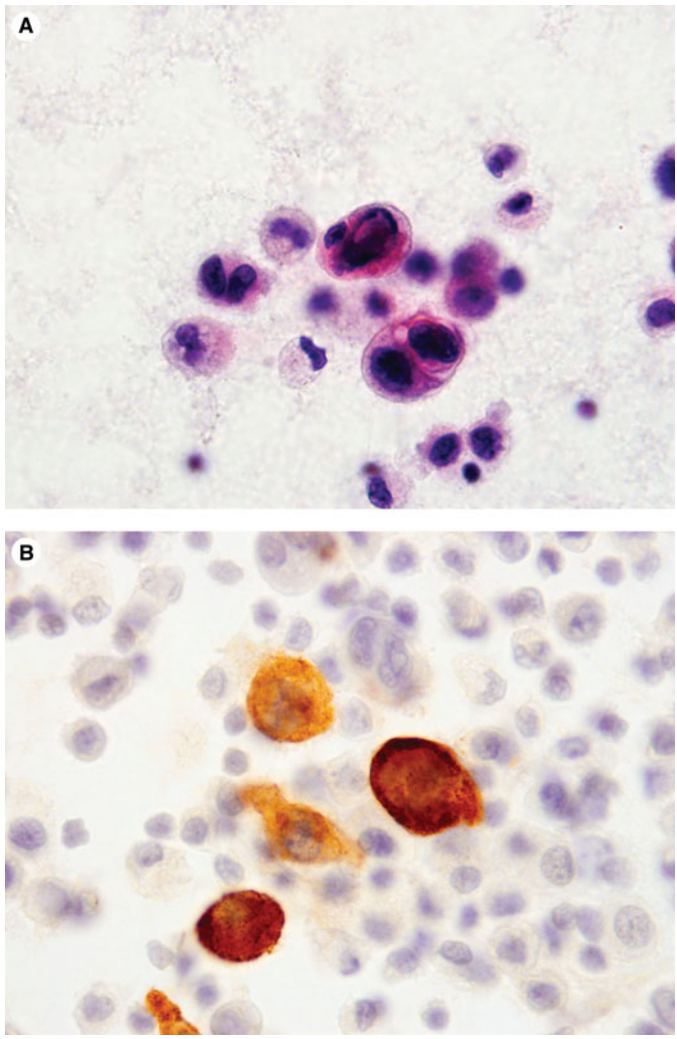


FIGURE 3.28 (A) Pleural fluid: Cellular evidence of a malignant neoplasm. *Comment:* In a patient with a history of disseminated malignant melanoma, these isolated pleomorphic cells represent pleural involvement by amelanotic malignant melanoma. Immunostains with melanoma markers should follow (PAP, 100×). (B) Pleural fluid: Cellular evidence of malignant neoplasm consistent with metastatic melanoma. *Comment:* The HMB-45-positive immunostain confirms the diagnosis of malignant melanoma (HMB-45, 100×).

Sarcomas

Conventional Cytology

- Sarcomas are rarely found in serous effusions. Cytologic findings depend on the morphology of the primary tumor. Sarcomas may be divided into spindle cell, round cell, and pleomorphic subtypes.
- High-grade sarcomas might be confused with epithelial malignancies.
- High-grade spindle cell sarcomas present as isolated, pleomorphic, and/or spindle cells with irregular nuclear contours and large nucleoli.

> Low-grade spindle cell sarcomas are difficult to diagnose in effusion samples due to their bland cellular morphology. Such cases are usually diagnosed as *"spindle cell lesion, not further classified,"* and followed by a panel of immunostains.

- Round cell sarcomas should be differentiated from undifferentiated carcinomas and melanomas. A knowledge of clinical history is extremely important in order to make the appropriate diagnosis of these uncommon tumors in effusions.
- Differential diagnosis of embryonal rhabdomyosarcoma, mesenchymal chondrosarcoma, and Ewing's sarcoma/PNET with more commonly found small-cell carcinomas in the body cavity fluid is mainly carried out based on clinical history and ICC.

Immunocytochemistry

- In contrast to advanced immunocytochemical panels of epithelial markers, antibodies against mesenchymal-derived cells are few, and, in general, ICC in sarcomas is not as helpful as it is in the diagnosis of epithelial malignancies in effusion cytology.

Immunocytochemical Markers for Diagnosing Sarcomas

Positive Markers	Type of Sarcoma
Desmin	Leiomyosarcoma Rhabdomyosarcoma
H-Caldesmon	Leiomyosarcoma
Myogenin	Rhabdomyosarcoma
S100	Chondrosarcoma/Nerve sheath tumors
CD31	Angiosarcoma/Kaposi sarcoma
CD34	Angiosarcoma/Kaposi sarcoma/GIST
D2-40	Lymphangiosarcoma
C kit (CD117)	GIST

GIST: gastrointestinal stromal tumor.

The following markers are used for the diagnosis of sarcomas:

- *Desmin*: A muscle marker for both smooth and skeletal muscles.
- *H-Caldesmon*: A smooth muscle marker.
- *Myogenin*: A marker for skeletal muscle. Myogenin is used following a desmin positivity to confirm the diagnosis of rhabdomyosarcoma.
- *S100*: Marker for malignant melanoma and some sarcomas.
- *CD31*: Endothelial marker.
- *CD34*: Endothelial and GIST marker.
- *D2-40*: Lymphatic endothelial and mesothelial marker.
- *C-kit (CD117)*: GIST marker.
- *Cytokeratin*: Epithelial marker.
- *EMA (epithelial membrane antigen)*: Epithelial marker.
- *Calretinin*: Mesothelial cell marker.
- *CD45*: A common marker for lymphoid cells.

Negative Immunocytochemical Markers for Sarcomas

Cytokeratin	Calretinin
EMA	CD45

Malignant Mesotheliomas

- More than 80% of all patients with malignant mesothelioma develop body cavity effusion at an early stage of the disease.
- There is an increasing number of cases of malignant mesotheliomas referred to the courts of law all around the world for the expert opinion of pathologists for compensation reasons in subjects exposed to asbestos, that in many instances rely on cytologic material from serous effusions, usually pleural.

> The consequence of a diagnosis of malignant mesothelioma is usually an immediate litigation act.

- Benign mesotheliomas are fibrous and are classified as fibromas of serous membranes. They usually do not produce an effusion.
- Malignant mesotheliomas are divided into epithelioid, sarcomatoid, and mixed types.
- The epithelioid and mixed types are commonly associated with malignant effusions.

> The term "mesothelioma" is used here to refer to the malignant epithelioid type.

- Microscopic diagnosis of malignant mesothelioma is usually a difficult task and cytologic changes may be subtle in some cases.

> The morphological features are not always reliable for differentiation between neoplastic and non-neoplastic mesothelial cells or between mesothelioma cells and cells from metastatic adenocarcinomas. With the current availability of specific mesothelial markers, we always use immunocytochemistry to confirm the diagnosis of malignant mesothelioma in effusions. Currently, however, there is no available marker that accurately can differentiate benign from malignant mesothelial processes.

- If the cells show cytomorphologic characteristics of malignancy as described in Chapter 2, and at the same time resemble "mesothelial cells," the diagnosis of malignant mesothelioma can be made with certainty.

- Some mesotheliomas exfoliate only small numbers of bland-looking cells, which are impossible to differentiate from benign mesothelial cells based on cytologic criteria. These cases may be diagnosed as *"atypical mesothelial cells,"* whereas the clinical findings may support a diagnosis of malignant mesothelioma (Figure 3.29).

Diagnosis	Malignant Cell Morphology	Resemblance to Mesothelial Cells
Mesothelioma	Yes	Yes
Reactive mesothelial cells	No	Yes
Adenocarcinoma	Yes	No

One should be extremely careful in making a diagnosis of malignant mesothelioma based on cytomorphology alone. Imaging findings such as pleural-based mass and relevant clinical findings such as relapsed effusions must be taken into consideration, and support a cytologic diagnosis of malignant mesothelioma.

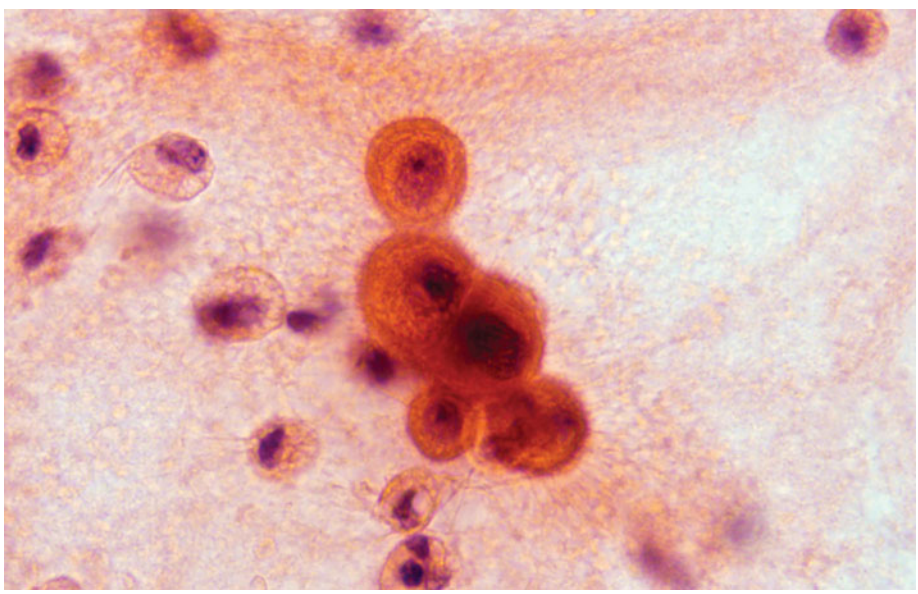


FIGURE 3.29 Pleural fluid: Atypical cells of mesothelial origin are present. Recommend clinicopathological correlation and further studies if indicated. *Comment:* Some malignant mesotheliomas exfoliate only small numbers of bland-looking cells, which are impossible to differentiate from reactive mesothelial cells. This patient had a history of repeated pleural effusions and histologically proven malignant epithelioid mesothelioma (PAP, 100×).

Conventional Cytology

- In body cavity fluid specimens, cells of mesothelioma occur singly or in small or large three-dimensional clusters.
- Cells are cuboidal or polygonal, usually larger than reactive mesothelial cells, and vary in size.
- Individual cell morphology resembles those of reactive mesothelial cells, including dense, two-tone cytoplasm, peripherally located vacuoles (blebs), and brush borders.
- The nuclei of mesothelioma cells are usually centrally located. Because the nuclei and amount of cytoplasm are both increased, the N/C ratio is usually unchanged. This is in contrast to cases of adenocarcinomas.
- Nucleoli may be single or multiple and prominent. Intranuclear inclusions may be seen. In contrast to the smooth border clusters of adenocarcinoma cells, mesothelioma cells usually group with an irregular ("knobby" surface) (Figure 3.30A, B).
- Dense cytoplasm with small peripheral blebs suggests cells of mesothelial origin; however, this is not a specific finding and may occasionally be seen in adenocarcinomas and squamous cell carcinomas.
- The "cell within a cell" pattern that is frequently seen in mesotheliomas may also be seen in adenocarcinomas. In fact, this finding may also be seen in non-neoplastic cells of mesothelial origin.

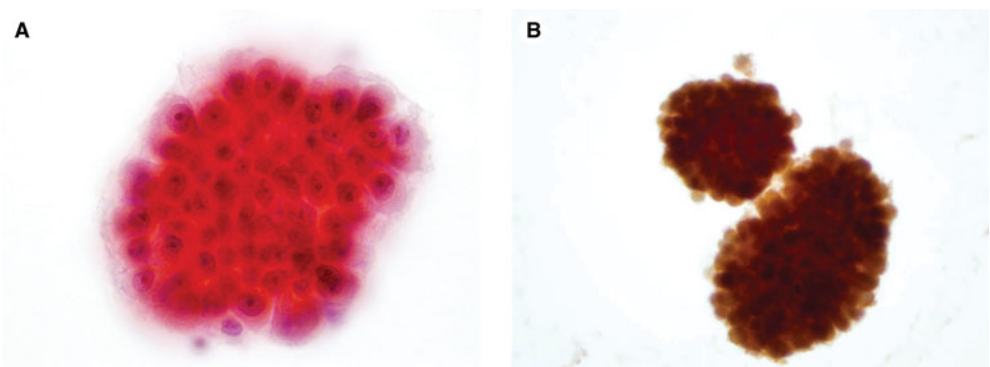


FIGURE 3.30 (A) Pleural fluid: Cellular evidence of a malignant epithelial neoplasm. *Comment:* The tight cell cluster with an irregular "knobby" surface suggests the possibility of malignant mesothelioma. The patient had a pleural-based lesion on CT scan (PAP, 100×). (B) Pleural fluid: Cellular evidence of malignant epithelial neoplasm consistent with mesothelioma. *Comment:* Calretinin was performed in this case to confirm the diagnosis of malignant mesothelioma. Nuclear and cytoplasmic staining is evident. The patient had a pleural-based lesion on CT scan (Calretinin, 100×).

- Binucleation and multinucleation are commonly seen in mesotheliomas; however, they may also be seen in adenocarcinomas.
- Cells in mesotheliomas are cuboidal or polygonal with a relatively constant N/C ratio, whereas adenocarcinoma cells are usually cuboidal or columnar with more evident nuclear molding and pleomorphism.
- In cell block preparations, the presence of papillary tissue fragments with a fibrovascular core is more commonly seen in mesotheliomas, whereas true acinar formations are more common in adenocarcinomas.
- If the cells are malignant by cytologic criteria, and they do not resemble "mesothelial cells"; most likely they represent malignancies other than malignant mesothelioma, mainly adenocarcinomas.

Immunocytochemistry

- Overlapping cytologic features of adenocarcinoma, reactive mesothelial cells, and malignant mesothelioma have long been a diagnostic challenge to cytopathologists, and the role of ICC in this distinction has been addressed in Chapter 2.
- Currently there are several mesothelial markers in the market, and calretinin is considered as the most specific one. Calretinin positivity of malignant cells in effusion samples confirms the diagnosis of mesothelioma. None of the mesothelial markers, however, are helpful in differentiating benign from malignant processes.

Immunocytochemical Markers for Malignant Mesothelioma

Positive	Negative
Calretinin	CEA
D2-40	BER-ep4
EMA	B72.3
Mesothelin	LeuM-1
E-Cadherin	Desmin
CK5/6	
Podoplanin	
HBME-1	
WT-1	
Thrombomodulin	

- The following markers are used for the diagnosis of malignant mesothelioma:
  - *Calretinin*: The best marker for mesothelial cells that shows nuclear and cytoplasmic staining (Figure 3.30B). Also present in ovarian and testicular stromal tumors and in adrenocortical neoplasms.
  - *D2-40*: Mesothelial and lymphatic endothelial marker.
  - *EMA*: Epithelial membrane antigen expressed in adenocarcinomas and mesotheliomas. A membranous staining is characteristic for malignant mesothelioma (Figure 3.31). Adenocarcinoma cells show strong intracy toplasmic staining.
  - *Mesothelin*: Antigen present on normal mesothelial cells and overexpressed in several human tumors, including mesothelioma and ovarian and pancreatic adenocarcinomas.
  - *E-Cadherin*: Expressed in malignant mesotheliomas and some adenocarcinomas whereas reactive mesothelial cells do not express this antibody.
  - *CK5/6*: Expressed in malignant mesothelioma and in many types of nonpulmonary carcinomas.

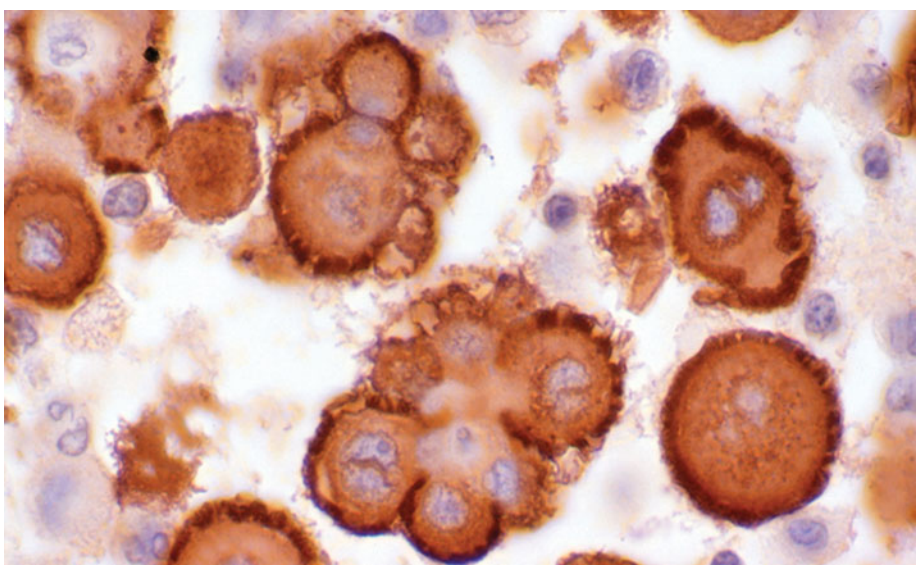


FIGURE 3.31 Peritoneal fluid: Cellular evidence of malignant epithelial neoplasm. *Comment:* EMA is expressed in cells from epithelial malignancies (both adenocarcinomas and malignant mesotheliomas) in effusion samples. A membranous staining, however, is characteristic of malignant mesotheliomas. This patient had a known history of peritoneal mesothelioma (EMA, (clone E29) 100×).

- *Podoplanin*: Mesothelial and lymphatic endothelial marker.
- *HBME-1*: Marker of malignant mesothelioma and papillary thyroid carcinoma.
- *WT-1*: Marker of malignant mesothelioma and also seen in ovarian carcinomas.
- *Thrombomodulin*: Marker of malignant mesothelioma and endothelial neoplasms.
- *CEA*: Positive in many adenocarcinomas and negative in malignant mesotheliomas. (The best negative marker for mesotheliomas).
- *BER-ep4*: Positive in many adenocarcinomas and negative in malignant mesotheliomas.
- *B72.3*: Positive in many adenocarcinomas and negative in malignant mesotheliomas.
- *LeuM-1*: Positive in many adenocarcinomas and negative in malignant mesotheliomas.
- *Desmin* is expressed in reactive mesothelial cells; also a marker of tumors of muscle origin.

Undifferentiated Large-Cell Malignant Neoplasms

Conventional Cytology

- Undifferentiated malignant neoplasms in effusions usually present in an isolated cell pattern.
- The cells are usually large with high N/C ratios and typical morphologic characteristics of malignancy. Therefore, rendering a diagnosis of malignancy is usually not difficult.
- The dilemma is to subclassify malignant cells into anaplastic carcinoma, malignant lymphoma, sarcoma, or amelanotic melanoma.
- The use of both clinical information, including site of origin, patient's age and sex, and prior history of cancer or related treatments, and ICC markers is extremely important in the appropriate subclassification of these malignant neoplasms.

Immunocytochemistry

- In adults the most common origin of undifferentiated malignant neoplasm in effusions is undifferentiated carcinoma, especially from the lung (in pleural effusion) and pancreas (in peritoneal effusion).
- The subclassification of undifferentiated malignancy in effusions is of considerable therapeutic significance.
- The ability to distinguish undifferentiated carcinomas from malignant mesotheliomas and the less commonly found neoplasms in effusions such as malignant melanomas, malignant lymphomas, sarcomas, and germ cell tumors based on conventional cytology may prove to be an impossible task. However, ancillary techniques, such as ICC and flow cytometry, are helpful in this differential diagnosis.

Immunocytochemical Markers for Undifferentiated Malignancies

Large-cell Undifferentiated Carcinomas	Malignant Mesotheliomas	Malignant Melanomas	Malignant Large-Cell Lymphomas	Malignant Germ Cell Tumors	Pleomorphic Sarcomas
EMA	EMA	S100	CD45	PLAP	Desmin
CEA Ber-EP4	Calretinin D2-40	HMB45 Tyrosinase	CD20 CD79a	CD30 AFP	H-Caldesmon Myogenin
B72.3 (TAG-72)	HBME-1	Melan-A (MART-1)	CD3	c-kit	S100
Leu-M1	Podoplanin		CD30		CD31
E-Cadherin					CD34
IMP-3 (KOC)					D2-40
MOC-31					c-Kit (CD117)
GLUT-1					CD99
					bcl2

Summary of Markers for Undifferentiated Malignancies

Large-cell Undifferentiated Carcinomas

- *EMA*: Epithelial membrane antigen expressed in epithelial malignant effusions; a membranous staining is characteristic for malignant mesothelioma.
- *CEA*: Positive in many adenocarcinomas and negative in malignant mesotheliomas.
- *Ber-EP4*: Positive in many adenocarcinomas and negative in malignant mesotheliomas.
- *B72.3(TAG-72)*: Positive in many adenocarcinomas and negative in malignant mesotheliomas.
- *LeuM-1*: Positive in many adenocarcinomas and negative in malignant mesotheliomas.
- *E-Cadherin*: Expressed in malignant mesotheliomas and some adenocarcinomas whereas reactive mesothelial cells do not express this antibody.
- *IMP-3 (KOC)*: Also known as L523S, mainly expressed in adenocarcinoma cells.
- *MOC-31*: Expressed in adenocarcinomas and malignant mesotheliomas but not in reactive mesothelial cells.
- *GLUT-1*: Expressed in adenocarcinomas and malignant mesotheliomas but not in reactive mesothelial cells.

Malignant Mesothelioma

- *EMA*: Epithelial membrane antigen expressed in epithelial malignant effusions; a membranous staining is characteristic for malignant mesothelioma.
- *Calretinin*: Best marker for mesothelial cells. Nuclear and cytoplasmic staining. Also present in ovarian and testicular stromal tumors and adrenocortical neoplasms.
- *D2-40*: An endothelial lymphatic marker positive in the majority of mesotheliomas.
- *HBME-1*: A highly sensitive and specific marker for mesothelial cells and used in a panel to distinguish between malignant mesotheliomas and adenocarcinomas.
- *Podoplanin*: A marker for malignant mesothelioma.

Malignant Melanoma

- *S-100*: A commonly used marker for malignant melanoma.
- *HMB45*: The best marker for malignant melanoma in alcohol-fixed cytologic material.
- *Tyrosinase*: Additional marker for malignant melanoma.
- *Melan-A (MART-1)*: Additional marker for malignant melanoma.

Malignant Large-cell Lymphomas

- *CD45*: A common marker for lymphoid cells.
- *CD20*: A marker for B-lymphocytes.
- *CD79a*: Immunoglobulin-associated alpha is a B-lymphocyte antigen receptor.
- *CD3*: A marker for T-lymphocytes.
- *CD30*: A marker for anaplastic lymphomas and Hodgkin's lymphomas.

Malignant Germ Cell Tumors

- *PLAP*: Placental alkaline phosphatase is a marker of germ cell tumors and is diffusely expressed in seminomas/disgerminomas.
- *CD30*: A marker for anaplastic lymphomas and Hodgkin's lymphomas.
- *AFP*: Alpha-feto protein is an oncofetal antigen diffusely expressed in the majority of yolk sac tumors. Some embryonal carcinomas may show focal positivity.

- *c-kit (CD117)*: A receptor tyrosine kinase, present in mast cell tumors, gastrointestinal stromal tumors and seminomas.

Pleomorphic Sarcomas

- *Desmin*: A muscle marker for both smooth and skeletal muscles.
- *H-Caldesmon*: A smooth muscle marker.
- *Myogenin*: A marker for skeletal muscle. Myogenin is used following desmin positivity to confirm the diagnosis of rhabdomyosarcoma.
- *S100*: A marker for malignant melanoma and some sarcomas.
- *CD31*: Endothelial marker.
- *CD34*: Endothelial marker for gastrointestinal stromal tumors.
- *D2-40*: Lymphatic endothelial and mesothelial marker.
- *C-kit (CD117)*: A receptor tyrosine kinase, present in mast cell tumors, gastrointestinal stromal tumors, and seminomas.
- *CD99*: Not a specific marker, but is useful for the diagnosis of synovial sarcoma when coexpressed with cytokeratin and/or EMA.
- *bcl2*: A marker for follicular cell lymphoma. Also useful for the diagnosis of synovial sarcoma when is coexpressed with cytokeratin and/or EMA.

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Major Differential Diagnoses

- In previous chapters, we described diagnostic criteria for malignancy and reviewed different types of malignancies based on conventional cytomorphology and immunocytochemistry (ICC). In this chapter, we will address the differential diagnosis on each encountered diagnostic category.
- Differential diagnosis between specific neoplastic categories based on cytomorphology alone can be challenging and in many times an impossible task to achieve.
- On many occasions, differential diagnosis is encountered at the time of preliminary microscopic review of routinely prepared cytologic material, that is, Papanicolaou-stained, alcohol-fixed slides.
- In our daily practice, we select previously Papanicolaou-stained, alcohol-fixed slides in order to perform ICC, we work out the differential diagnosis, and we reach the final cytopathologic interpretation.
- New immunocytochemical antibodies are continuously developed, studied, and recommended to be used in these major differential diagnoses. Other ancillary techniques such as FCM, molecular assays, and more recently flow ICC may also facilitate the diagnosis.

Adenocarcinomas

Adenocarcinomas versus Reactive Mesothelium

- This differential diagnosis is a major diagnostic problem in effusion cytology.

> The diagnostic terminology commonly used as "atypical cells are present" must be avoided in reporting of serous effusions.

- More than 50% of immunocytochemical tests performed in our laboratory on cytologic samples are intended to differentiate "atypical" cells from adenocarcinoma cells.

> Adenocarcinomas are the most commonly seen malignancies in body-cavity fluids.

- ICC assists to reduce false-negative results which are reported in over 50% of cases in routine cytology. Such errors usually are caused by misinterpretation of adenocarcinoma cells as reactive mesothelial cells.
- False-positive diagnoses are less commonly seen in effusion cytology and often are caused by overinterpretation of reactive mesothelial cells as malignant cells. In this section, we will discuss the use of ICC in these differential diagnoses.
- In current practice of pathology, there is no single marker to differentiate benign from malignant cells. Therefore, ICC panels are frequently used in diagnostic cytology.
- The following is a list of ICC antibodies most commonly used to differentiate benign reactive mesothelial cells from adenocarcinoma cells.

Commonly Used Immunocytochemical Markers

EMA: Malignant (adenocarcinoma)
CEA: Malignant (adenocarcinoma)
Ber-EP4: Malignant (adenocarcinoma)
B72.3: Malignant (adenocarcinoma)
LeuM1: Malignant (adenocarcinoma)
Desmin: Benign (reactive mesothelium)
Calretinin: Benign and malignant (cells of mesothelial origin)

In our experience, the most useful limited panel of ICC includes EMA, CEA, and calretinin. Nuclear and intracytoplasmic positivity for calretinin and negativity for EMA confirms a reactive mesothelial proliferation.

- In cases of adenocarcinomas, the most common immunocytochemical pattern with high sensitivity has been—EMA positive, CEA positive, Ber-EP4 positive, B72.3 positive, and LeuM1 positive. This panel detects the majority of adenocarcinoma cases in effusions. It should be noted, however, that there is a chance of false-positive results, especially in cases of peritoneal effusions in women since benign Mullerian-derived epithelial cells such as those in endosalpingiosis, may express some of these antigens.
- Some small panels include desmin as the marker for benign reactive mesothelial cells. Since desmin is not expressed in Mullerian-derived epithelium, it is potentially useful in women who present with peritoneal effusions, or in cases of peritoneal washings performed as a part of the staging process for female genital-tract malignancies. Desmin is expressed in reactive mesothelial cells and it is absent in benign Mullerian-derived epithelial cells (endosalpingiosis) and in Mullerian-derived adenocarcinomas.
- There are specific markers of cell lineage that are expressed in both benign and malignant cells such as calretinin. Therefore, calretinin cannot distinguish between benign and malignant mesothelial cells. In such instances, cytomorphologic criteria should be strictly followed, as described in Chapter 2.

In summary, benign and malignant profiles can be used as shown below.

Benign and Malignant Immunocytochemical Profiles

	Benign	Malignant
EMA	Negative	Positive
CEA	Negative	Positive
Desmin	Positive	Negative
Ber-EP4	Negative	Positive
B72.3	Negative	Positive
LeuM1	Negative	Positive

- At our laboratory we use a small immunocytochemical panel of at least two antibodies which always includes EMA and calretinin.
- EMA (epithelial membrane antigen) is an antibody to a human milk fat globule (HMFG) membrane protein, secreted by mammary epithelial cells.
- EMA is seen in a variety of simple and glandular epithelia, including respiratory tract, pancreas, intestine, and endometrium. EMA is also shown on the surface of mesothelial cells.
- Reactive mesothelial cells in effusions may show a weak EMA immunostaining in a limited number of cells in contrast to the strong intracytoplasmic staining seen in adenocarcinoma cells.
- Discordant results have been reported in the efficacy of EMA expression in distinction between benign reactive and adenocarcinoma cells. These conflicting results could be attributed to differences in data analysis, scoring system, and/or staining techniques using different antibodies and antigen retrieval methods.
- EMA clone Mc5 stains reactive and malignant mesothelial cells as well as adenocarcinoma cells. Therefore, it is not a reliable marker for this differential diagnosis.
- EMA clone E29 is usually negative or weakly positive (not detected on low-power magnification), in cases of reactive mesothelium, strongly positive in majority of adenocarcinomas and malignant mesotheliomas, and therefore, is a reliable marker for differentiating reactive mesothelium from adenocarcinomas.
- CEA (carcinoembryonic antigen) is an oncofetal antigen that is expressed in large number of carcinomas. It is negative in mesothelial cells. Therefore, this antibody is extremely helpful in distinction of adenocarcinomas from reactive mesothelium.

In our laboratory, EMA (clone E29) is the most frequently used antibody to differentiate benign versus malignant effusions. EMA immunostaining must be evaluated at low-power magnification where EMA-positive cells are easily detected. Reactive mesothelial cells are negative for EMA (clone E29). The presence of weak positivity in a few cells only, should not be regarded as a positive result.

- Calretinin is a calcium-binding protein expressed normally in neurons of the central and peripheral nervous system. It is a useful marker in the differential diagnosis between reactive mesothelium and adenocarcinomas.
- Positive staining for calretinin is defined as nuclear and cytoplasmic staining. Some adenocarcinomas may show cytoplasmic staining only.
- It should be noted that a cytokeratin (CK) cocktail cannot differentiate between reactive mesothelial cells and adenocarcinoma cells, therefore of no diagnostic utility.
- Other markers used in the differential diagnosis between reactive mesothelium and adenocarcinomas are listed below.
    - *EMA*: Epithelial membrane antigen is expressed in epithelial malignant effusions. It is negative in reactive mesothelial cells.
    - *CEA*: Positive in many adenocarcinomas. It is negative in mesothelial derived cells.
    - *Ber-EP4*: Positive in many adenocarcinomas. It is negative in mesothelial derived cells.
    - *B72.3*: Positive in many adenocarcinomas. It is negative in mesothelial derived cells.
    - *LeuM-1*: Positive in many adenocarcinomas. It is negative in mesothelial derived cells.
    - *Calretinin*: It is the best marker for mesothelial cells. Nuclear and cytoplasmic staining is present. Also present in ovarian and testicular stromal tumors and adrenocortical neoplasms.
    - *Desmin*: It is expressed in reactive mesothelial cells; also a marker of tumors of muscle origin.
    - *E-Cadherin*: It is expressed in malignant mesotheliomas and some adenocarcinomas; it is negative in reactive mesothelial cells.
    - *IMP-3*: Insulin-like growth factor-II mRNA-binding protein 3, also known as KOC or L523S, mainly expressed in adenocarcinoma cells.
    - *MOC-31*: It is expressed in adenocarcinomas and malignant mesotheliomas but not in reactive mesothelial cells.
    - *GLUT-1*: It is expressed in adenocarcinomas and malignant mesotheliomas but not in reactive mesothelial cells.

Use of Immunocytochemistry in Differential Diagnosis of Adenocarcinomas versus Reactive Mesothelium

	RMC	AdenoCa
EMA	Negative	Positive
CEA	Negative	Positive
Ber-EP4	Negative	Positive
B72.3 (TAG-72)	Negative	Positive
Leu-M1	Negative	Positive
Calretinin	Positive	Negative
Desmin	Positive	Negative
E-Cadherin	Negative	Positive
IMP-3 (KOC)	Negative	Positive
MOC-31	Negative	Positive
GLUT-1	Negative	Positive

RMC: reactive mesothelial cells; AdenoCa: adenocarcinoma cells

Adenocarcinoma versus Malignant Mesothelioma

- Adenocarcinomas are the most commonly diagnosed malignancies in effusion samples. In contrast, malignant mesotheliomas are rare.
- The differential diagnosis between malignant mesotheliomas and adenocarcinomas which are metastatic to serous membranes may be difficult based on cytologic material. Clinical information and imaging results are always required for this distinction.
- The differential diagnosis between adenocarcinomas and malignant mesotheliomas can be facilitated by the use of diagnostic tools such as, electron microscopy, histochemical stains (histochemistry), and more effectively, by ICC.
- *Electron microscopy (EM)* remains the gold standard for the differentiation between adenocarcinoma and malignant mesotheliomas. The most common ultrastructural characteristics of the malignant mesotheliomas are the presence of long, slender, often branching, undulating microvilli on the apical surface of the cuboidal cells lining the tubules or papillae, dilated intercellular spaces, and intracellular lumens.
- EM is not a practical technique and it cannot be recommended as a standard procedure because of its cost, its availability, and the time involved.
- *Histochemistry* has been commonly used prior to the ICC era. Malignant mesothelioma is characterized by accumulation of abundant intercellular

hyaluronic acid (HA), a feature that is not seen in adenocarcinomas. However, glycogen and HA within mesothelioma cells, may mimic mucin found in adenocarcinoma cells that may create diagnostic difficulty.

- Adenocarcinomas often produce epithelial mucins that are either neutral or acidic. These tumors can be identified using mucicarmine or periodic acid-Schiff-diastase stains.
- Cells of mesothelial origin, on the other hand, produce acidic mucins of the connective tissue type, namely HA.
- HA can be detected by various histochemical stains such as Alcian blue at pH 2.5 and Hale's colloidal iron.
- Hyaluronidase predigestion largely removes the HA from cells of mesothelial origin but not the mucin of adenocarcinomas.
- The determination of HA by histochemical stains has been shown to be a highly specific but relatively insensitive method of identification of malignant mesotheliomas, because it can be detected only in about one third to one half of the cases.

Histochemical Stains used for the Differential Diagnosis Between Adenocarcinoma and Malignant Mesothelioma

	HA	Mucicarmine	PAS-D
Adenocarcinoma	Negative	Positive	Positive
Mesothelioma	Positive	Negative	Negative

HA: hyaluronic acid; PAS-D: periodic acid-Schiff-diastase

In recent years because of availability of ICC in most laboratories and high sensitivity of new mesothelial markers, electron microscopy and histochemistry techniques are not frequently used in distinction between adenocarcinomas and malignant mesotheliomas.

- *Immunocytochemistry* plays an important role in distinction between malignant mesotheliomas and adenocarcinomas.
- The use of antibody panels is highly recommended since no single antibody has been demonstrated to be absolutely sensitive and specific for the diagnosis of malignant mesothelioma. Furthermore, use of extensive antibody panels may still leave some cases unresolved.

No single antibody is able to reliably differentiate between adenocarcinomas and malignant mesotheliomas. Various antibody panels have been recommended for this distinction with no overall consensus about how many and which markers should be used. We recommend the use of at least 2–3 antibodies for this distinction.

- The earliest antibody panels were based on markers that are expressed by adenocarcinomas such as CEA, Ber-EP4, and LeuM1. Later, mesothelial markers have been added.
- The most common reactivity pattern in mesotheliomas (EMA positive, CEA negative, and Ber-EP4 negative) is found in most cases with up to 100% specificity.
- *EMA* is expressed mainly in epithelioid mesotheliomas, which are the most frequently found mesotheliomas in effusions.
- EMA staining seen in mesotheliomas is at the periphery of cell clusters and circumferentially around individual cells, exhibiting a thick membrane staining. This staining pattern is different from the one seen in adenocarcinomas, characterized by strong intracytoplasmic staining.
- *CEA* is an oncofetal glycoprotein that is not expressed in malignant mesotheliomas. It is, however, commonly expressed by a large number of adenocarcinomas.
- The introduction of monoclonal CEA has increased its diagnostic sensitivity and specificity, and it remains one of the most robust and useful antibodies in diagnostic-effusion cytology.
- *LeuM1 (CD15)* is a monomyelocytic marker with high specificity and sensitivity for the diagnosis of adenocarcinoma. It is negative in malignant mesothelioma.
- *B72.3* is a monoclonal antibody raised in mice against a tumor-associated glycoprotein complex expressed in breast carcinoma cell lines. This antibody has high specificity and sensitivity for the diagnosis of adenocarcinoma. It is negative in malignant mesothelioma.
- *Ber-EP4* is an antibody that reacts with two glycoproteins that are on the surface and in the cytoplasm of epithelial cells. Ber-EP4 does not react with mesothelial cells. Majority of adenocarcinomas express this antigen.

- *MOC-31* is a monoclonal antibody that recognizes an epithelial-associated transmembrane glycoprotein of unknown function that is often expressed in epithelial tumors. Moc-31 is a highly sensitive marker for adenocarcinomas from a broad range of primary sites. Moc-31 is a useful marker to differentiate malignant mesotheliomas from metastatic adenocarcinomas in effusion samples.
- *Calretinin* is a calcium-binding protein, which is expressed normally in neurons of the central and peripheral nervous system. It is a useful marker in differentiating epithelioid mesotheliomas from adenocarcinomas.
- Positive staining for calretinin is defined as nuclear and cytoplasmic staining. Some adenocarcinomas may show cytoplasmic staining to be considered as negative.
- Calretinin is both, a sensitive and specific marker of reactive and neoplastic mesothelial cells in cytologic preparations and thus useful in the differential diagnosis of malignant mesothelioma and metastatic adenocarcinomas in malignant effusions.
- In our laboratory, calretinin is the most commonly used mesothelial cell marker.

> Although calretinin is helpful in distinguishing malignant mesotheliomas from adenocarcinomas, it is not useful in differentiating benign *"atypical"* from malignant mesothelial cells.

- *D2-40* is an endothelial lymphatic marker that is also expressed in mesothelial cells as a thick membranous staining. D2-40 is positive in majority of mesotheliomas.
- Compared to calretinin, D2-40 is shown to be a more sensitive marker, but less specific for mesothelial cells. D2-40 is expressed in some adenocarcinomas as cytoplasmic (nonmembranous) staining.
- *HBME-1* is an antibody against cultured mesothelioma cells that recognizes an antigen on the microvillus surface. It has a high sensitivity and specificity for mesothelial cells and it is used in antibody panels to distinguish between malignant mesotheliomas and adenocarcinomas.
- *Podoplanin* is considered to be useful in the diagnosis of malignant mesothelioma. It appears to be a sensitive and specific marker for malignant mesothelioma.

Immunocytochemical Markers for the Differential Diagnosis Between Adenocarcinoma and Malignant Mesothelioma

	Adenocarcinoma	Malignant Mesothelioma
EMA	Positive[a]	Positive[b]
CEA	Positive	Negative
Leu-M1	Positive	Negative
B72.3	Positive	Negative
Ber-EP4	Positive	Negative
MOC-31	Positive	Negative
Calretinin	Negative	Positive
D2-40	Negative	Positive
HBME-1	Negative	Positive
Podoplanin	Negative	Positive

[a]Intracytoplasmic staining pattern

[b]Membranous staining pattern.

- Other markers have been investigated for the diagnosis of mesothelioma in serous effusions including: CK 5/6, HBME-1, Leu-M1 (CD15), B72.3, E-cadherin, podoplanin, mesothelin, thrombomodulin, blood group related antigen BG-8 ($Lewis^Y$), and WT-1.
- *CK 5/6* are intracytoplasmic intermediate filaments expressed by both epithelial and mesothelial cells. CK5/6 is mainly expressed in squamous epithelium and in the basal-myoepithelial cells of prostate, breast and salivary gland, and their neoplasms. Epithelioid or biphasic malignant mesothelioma also usually expresses CK5/6.
- Small panel of calretinin and CK 5/6 maybe useful for the detection of mesothelial cells in effusions. Positive staining with both antigens is indicative of cells of mesothelial origin. This panel does not help discriminate reactive from malignant processes as both antigens are expressed by both benign and malignant mesothelial cells. Since CK5/6 is also expressed in a number of adenocarcinoma cells in effusions, CK5/6 seems to be of very limited value as an additional marker in effusion cytology.
- *E-Cadherin is* expressed in malignant mesotheliomas and it has been used to differentiate mesothelioma cells from their reactive counterpart. However, E-cadherin is not helpful in distinction between adenocarcinomas from malignant mesotheliomas.

- *Mesothelin* is a cell surface antigen of unknown function that is strongly expressed in mesothelial cells. It is a less sensitive and specific marker for mesothelial cells in cytologic material when compared with calretinin. Despite the low specificity of mesothelin for discriminating between malignant mesotheliomas and adenocarcinomas, immunostaining for this marker may have some utility in those instances in which the results of a limited antibody panel are equivocal. Due to its high sensitivity for the diagnosis of malignant mesothelioma, negativity for this marker may be useful to exclude the diagnosis of malignant mesothelioma.
- *Thrombomodulin* is a glycoprotein expressed by the endothelium, mesothelium, synovium, and placental syncytiotrophoblasts. It is also expressed in malignant mesothelioma. Due to its low sensitivity and specificity, utility of thrombomodulin is limited in this differential diagnosis.
- *Blood group-related antigen (BG8)* ($Lewis^Y$) is an antibody against the $Lewis^Y$ antigen. It has been shown to be highly sensitive for the diagnosis of adenocarcinomas in effusions.
- *Wilms' tumor susceptibility gene 1 (WT1)* is a useful marker to distinguish malignant mesothelioma from lung adenocarcinoma cells in pleural effusions. However, its utility in peritoneal effusions is limited. WT-1 nuclear staining in peritoneal fluid is highly suggestive of a primary ovarian carcinoma.

Large-Cell Carcinomas

- When malignant cells found in effusion samples are in isolated (single) cell pattern (with no cell balls/clusters), cytologic differential diagnoses usually includes undifferentiated large-cell carcinomas, malignant melanomas, malignant lymphomas, germ-cell tumors, and sarcomas. In such instances, using cytomorphologic diagnostic criteria alone may not be sufficient for proper classification of malignant cells.
- In addition, correlation of clinical and imaging findings is recommended along with the use of limited ICC panels as described here.

> Although cytokeratin is the most commonly used epithelial marker in histologic and some cytologic samples, its utility in effusion cytology is limited due to expression of cytokeratins by both mesothelial and non-mesothelial epithelial cells.

Large-Cell Carcinoma versus Malignant Melanoma

- Metastatic melanoma in effusions is an extremely rare event.
- Malignant melanoma cells present as predominantly isolated cells or in small loose clusters.
- Binucleation and multinucleation are common. Nucleoli are large and may be multiple. Intranuclear cytoplasmic inclusions and intracytoplasmic melanin, if present, are of diagnostic value.
- The diagnosis of malignant melanoma based on conventional cytology alone is practically impossible. Clinical correlation is important.
- In effusions with isolated malignant cells ("isolated cell pattern"), ICC is used to detect melanoma cells in a patient with a known history of malignant melanoma.

The following markers are used for the diagnosis of malignant melanoma

- *S100*: Commonly used marker for malignant melanoma.
- *HMB45*: A best marker for malignant melanoma in alcohol-fixed cytologic material.
- *Tyrosinase*: An additional marker for malignant melanoma.
- *Melan-A (MART-1)*: An additional marker for malignant melanoma.

Positive Immunocytochemical Markers for Malignant Melanoma	
S100	Tyrosinase
HMB-45	Melan-A (MART-1)

- In effusion cytology, major differential diagnosis of malignant melanoma is with the more commonly found neoplasms, that is, carcinomas. That can be accomplished by using a limited ICC panel composed of one epithelial marker and one or two melanoma markers. In contrast to histologic sections where CK is the epithelial marker of choice, in effusion samples, EMA should be used since CK is expressed in both mesothelial and nonmesothelial epithelial (adenocarcinoma) cells.

Limited Immunocytochemical Panel Differentiating Large-cell Carcinomas from Malignant Melanoma

	Large-Cell Carcinoma	Malignant Melanoma
EMA	Positive	Negative
S100	Negative	Positive
HMB45	Negative	Positive

Large-Cell Carcinomas versus Malignant Large-Cell Lymphomas

- Cytologic examination is an accurate method for the diagnosis of large-cell malignant lymphoma in effusions with a reasonably high overall diagnostic accuracy.
- Large-cell malignant lymphomas are characterized by the presence of numerous isolated large cells in the cytospin preparation of the serous effusions that may be associated with apoptotic bodies.
- The nuclei are characterized by variation in size and shape, nuclear indentations, and presence of prominent nucleoli.
- Individual cell necrosis (apoptotic bodies) is a peculiar and, in our experience, a very useful finding in the diagnosis of lymphoma in serous effusions.
- The cytoplasm is usually scant, basophilic, and rarely well preserved.
- Monoclonal proliferation of T or B lymphocytes can be determined by immunocytochemical stains, FCM or by gene rearrangement studies.

Large-Cell lymphomas should be differentiated from other large-cell malignancies with isolated cell pattern such as large-cell carcinomas.

The following markers are used for the diagnosis of malignant lymphomas.

- *CD45*: A common marker for lymphoid cells.
- *CD20*: A marker for B-lymphocytes.
- *CD79a*: Immunoglobulin-associated alpha is a B lymphocyte antigen receptor.
- *CD3*: A marker for T-lymphocytes.
- *CD30*: A marker for anaplastic lymphomas and Hodgkin's lymphomas.

Immunocytochemical Markers for Large-Cell Lymphomas

CD45	CD3
CD20/CD79a	CD30

Limited Immunocytochemical Panel Differentiating Large-cell Carcinomas from Large-cell Lymphoma

	Large-Cell Carcinoma	Large-Cell Lymphoma
EMA	Positive	Negative[a]
CD45	Negative	Positive
CD20/CD79a	Negative	Positive
CD3	Negative	Positive

[a]Some lymphomas may be positive.

Large-Cell Carcinoma versus Germ-Cell neoplasms

- Malignant germ-cell tumors rarely metastasize to the serous membranes. However, in young patients, seminomas/germinomas should always be considered in the differential diagnosis of large-cell carcinomas.

The following markers are used for the diagnosis of germ-cell neoplasms.

- *PLAP*: Placental alkaline phosphatase is a marker of germ-cell tumors and it is diffusely expressed in seminomas/disgerminomas.

- *CD30*: A marker for anaplastic lymphomas and Hodgkin's lymphomas.
- *AFP*: Alpha-feto protein is an oncofetal antigen diffusely expressed in majority of yolk-sac tumors. Some embryonal carcinomas may show focal positivity.
- *c-kit (CD117)*: Is a receptor tyrosine kinase, present in mast-cell tumors, gastrointestinal stromal tumors, and in seminomas.

Immunocytochemical Markers for Malignant Germ-Cell Tumors

PLAP	AFP
CD30	c-KIT

Limited Immunocytochemical Panel Differentiating Large-Cell Carcinomas from Malignant Germ-Cell Tumors

	Large-Cell Carcinoma	Seminoma	Embryonal Carcinoma	Yolk-sac Carcinoma
EMA	Positive	Negative	Positive	Positive
PLAP	Negative	Positive	Negative	Negative
CD30	Negative	Negative	Positive	Negative
AFP	Negative	Negative[a]	Negative[a]	Positive
c-kit	Negative	Positive	Negative	Negative

[a]May be expressed in isolated cells

Large-Cell Carcinoma versus Pleomorphic Sarcomas

- Sarcomas rarely metastasize to serosal surfaces. Pleomorphic-type sarcomas present as isolated malignant cells in effusions that should be differentiated from more commonly seen large-cell carcinoma.
- In contrast to advanced immunocytochemical panels of epithelial markers, antibodies against mesenchymal derived cells are few, and in general, ICC in sarcomas is not as helpful as it is in the diagnosis of epithelial malignancies in effusion cytology.

The following markers are used for the diagnosis of sarcomas.

- *Desmin*: A muscle marker for both smooth and skeletal muscles.
- *H-Caldesmon*: A smooth muscle marker.

- *Myogenin*: A marker for skeletal muscle. Myogenin is used following a desmin positivity to confirm the diagnosis of rhabdomyosarcoma.
- *S100*: A marker for malignant melanoma and some sarcomas.
- *CD31*: A endothelial marker.
- *CD34*: A endothelial and GIST marker.
- *D2-40*: A lymphatic endothelial and mesothelial marker.
- *C-kit (CD117)*: It is a receptor tyrosine kinase, present in mast cell tumors, gastrointestinal stromal tumors, and in seminomas.
- *CD99*: Is not a specific marker, however is useful for the diagnosis of synovial sarcoma when co-expressed with cytokeratin and/or EMA.
- *BCl2*: Is a marker for follicular cell lymphoma. It is also useful for the diagnosis of synovial sarcoma when it is coexpressed with cytokeratin and/or EMA.

Immunocytochemical Panel Differentiating Large-Cell Carcinomas from Pleomorphic Sarcomas

Positive Markers for Sarcomas	Type of Sarcoma
Desmin	Leiomyosarcoma/Rhabdomyosarcoma
H-Caldesmon	Leiomyosarcoma
Myogenin	Rhabdomyosarcoma
S100	Chondrosarcoma/Nerve-sheath tumors
CD31	Angiosarcoma/Kaposi sarcoma
CD34	Angiosarcoma/Kaposi sarcoma/GIST
D2-40	Lymphangiosarcoma
C kit (CD117)	GIST
Keratin, EMA, CD99, and Bcl2	Synovial sarcoma

Immunocytochemical Markers Used for the Differential Diagnosis Between Large-Cell Carcinoma and Sarcomas

	Large-cell Carcinoma	Leiomyo-sarcoma	Rhabdo-myosarcoma	Chondrosarcoma Liposarcoma Malignant Nerve-sheath Tumor	Endothelial Sarcomas	GIST	Synovial Sarcoma
EMA	Positive	Negative	Negative	Negative	Negative	Negative	Positive
Desmin	Negative	Positive	Positive	Negative	Negative	Negative[b]	Negative
H-Caldesmon	Negative	Positive	Negative	Negative	Negative	Negative[b]	Negative
Myogenin	Negative	Negative	Positive	Negative	Negative	Negative	Negative
S100	Negative	Negative	Negative	Positive	Negative	Negative[b]	Negative
CD31	Negative	Negative	Negative	Negative	Positive	Negative	Negative
CD34	Negative	Negative	Negative	Negative	Positive	Positive	Negative
D2-40	Negative	Negative	Negative	Negative	Negative Positive[a]	Negative[c]	Negative
c-Kit	Negative	Negative	Negative	Negative	Negative	Positive	Negative
CD99 & Bcl-2	Negative	Negative	Negative	Negative	Negative	Negative	Positive

[a]Positive in lymphangiosarcoma

[b]It may be positive

[c]D2-40 is also expressed in mesothelial cells and should be used with caution in effusions.

Small-Cell Carcinoma

- Cytologic characteristics of small-cell carcinomas in effusion samples are well recognized and similar to those of other cytologic samples.
- The cells are of small to medium size with high nuclear/cytoplasmic ratio, nuclear molding, and occasionally salt and pepper chromatin.
- Using a CK cocktail, small-cell carcinomas show a characteristic dot-like para-nuclear staining.

> Dot-like para-nuclear cytokeratin staining is a characteristic immunocytochemical finding of small-cell carcinomas.

- Other immunocytochemical markers to distinguish small-cell carcinomas from other small-cell malignancies include chromogranin A, synaptophysin, and CD56. p63 immunostain is usually negative in small-cell carcinomas, therefore useful to differentiate them from basaloid squamous cell carcinomas.

> When small carcinoma cells are found in isolated form, based on morphology alone, recognition of the cells may be difficult because of their cytologic resemblance to lymphocytes. In addition, the isolated tumor cells may be totally overlooked on low-power magnification. A mixture of both isolated and cohesive cells, when present is of diagnostic value.

The following markers are used for the diagnosis of small-cell carcinomas in effusions.

- *Cocktail cytokeratin*: Shows characteristic dot-like staining in small-cell carcinomas; the cocktail is composed of high-molecular-weight keratin, AE1/AE3, and CAM5.2.
- *Chromogranin A*: Is a marker for neuroendocrine neoplasms, including small-cell carcinomas of the lung and GI tract, and that of skin (Merkel-cell tumor)
- *Synaptophysin*: Also a neuroendocrine marker, more sensitive and yet less specific than chromogranin.
- *CD 56*: A marker for neuroendocrine neoplasms.

Positive Immunocytochemical Markers for Small-Cell Carcinoma

Cytokeratin (dot-like)	Synaptophysin
Chromogranin A	CD-56

Small-Cell Carcinoma versus Malignant Small-Cell Lymphomas

- The major differential diagnosis of small-cell carcinoma in effusions is with malignant small-cell lymphoma.
- Conventional cytology of effusions is not considered to be an optimal diagnostic method for small-cell-malignant lymphomas.
- In general, malignant lymphomas are cytologically characterized by the presence of numerous isolated cells in the cytospin preparation of the serous effusions.
- In contrast to malignant large-cell lymphomas, cytoplasm is usually scant, or totally undetectable. Nuclear abnormalities, if present, are not easily detectable. Individual cell necrosis (apoptotic bodies) may be present.
- In addition to small-cell-malignant lymphomas, other small-cell lymphocytic proliferative processes such as chronic lymphocytic leukemia, and Waldenstrom's macroglobulinemia, may present with a large number of "mature looking" lymphocytes in effusions. In such cases, an unequivocal separation of these neoplastic processes from chronic nonspecific inflammation involving serous effusions or tuberculosis is not possible on the basis of cytomorphology alone.
- Monoclonal proliferation of T- or B-lymphocytes can be determined by ICC, FCM, or by gene rearrangement studies.
- The following ICC markers are used for the diagnosis of small-cell lymphomas.
  - *CD45*: A common marker for lymphoid cells.
  - *CD20*: A marker for B-lymphocytes.
  - *CD79a*: Immunoglobulin-associated alpha is a B-lymphocyte antigen receptor.
  - *CD3*: A marker for T-lymphocytes.
  - *cyclin D1*: It is a nuclear marker overexpressed in mantle-cell lymphoma.

- *CD23*: It is a B-cell antigen useful to distinguish small-cell lymphocytic lymphoma/chronic lymphocytic leukemia (positive) from mantle-cell lymphoma (negative).
- *CD10 (CALLA)*: Is a B-cell precursor marker and it may be present in a subset of B-cell lymphomas (follicular center cell and mantle-cell lymphomas).
- *CD5*: It is expressed in all mature T-cells as well as in a subset of B-cell lymphomas (mantle cell and small lymphocytic lymphoma).

Immunocytochemical Markers for Small-Cell Malignant Lymphomas

CD45	CD23
CD20/CD79a	CD10
CD3	CD5
Cyclin D1	

- When a large number of mature looking lymphocytes are present in a patient with a known well-differentiated small-cell lymphoma or chronic-lymphocytic leukemia, a descriptive diagnosis of: "*numerous lymphocytes are present, consistent with…*" is rendered and followed by ancillary studies.

Limited Immunocytochemical Panel Differentiating Small-Cell Carcinomas from Malignant Small-Cell Lymphoma

	Small-Cell Carcinoma	Small-Cell Lymphoma
EMA	Positive	Negative
CD45	Negative	Positive
CD20/CD79a	Negative	Positive
CD3	Negative	Positive

The diagnosis of small-cell lymphocytic proliferative processes including small-cell lymphoma in serous effusions should not be rendered based on conventional cytology alone. In such cases, the following diagnostic terminology is suggested: "*Cellular evidence of atypical lymphoproliferative process; recommend further studies to rule out malignancy.*"

Flow cytometry and/or gene rearrangement studies should be used for the diagnosis of small-cell lymphoma.

Small-Cell Carcinoma versus Small-Cell Sarcomas

- Small-Cell carcinoma versus small-cell sarcoma is rarely encountered in differential diagnosis, especially in pediatric population.
- In young individuals, the presence of malignant small cells in effusions most likely represents a small blue-cell tumor of childhood or a malignant lymphoreticular process. Therefore, the knowledge of patient's age and other clinical information is important for a proper diagnosis that may be facilitated by ICC.

Accurate cytologic subclassification of small-cell malignancies in effusions of young patients heavily relies on the results of immunocytochemical studies.

Immunocytochemical Markers Used in Small-Cell Malignant Neoplasms of Young Patients

Positive Markers for Small-Cell Sarcomas	Type of Small-Cell Sarcoma
Desmin	Rhabdsarc
Myogenin	Rhabdsarc
CD99	Ewing/PNET
Desmin, Cytokeratin	DSRCT

CK: cytokeratins; Des: desmin; Rhabdsarc.: rhabdomyosarcoma; DSRCT: desmoplastic small round cell tumor; PNET: peripheral neuroectodermal tumor.

- *Desmin*: Is a muscle marker (for both smooth and skeletal muscles).
- *Myogenin*: Is a marker for skeletal muscle. It should be used following a desmin positivity to confirm the diagnosis of rhabdomyosarcoma.
- *CD99*: Is not a specific marker, however is useful for the diagnosis of Ewing's sarcoma/PNET (membranous staining). It is also expressed in synovial sarcoma.

- *Cytokeratin*: Shows characteristic dot-like staining in small-cell carcinomas.

Limited Immunocytochemical Panel Differentiating Small-Cell Carcinomas from Small-Cell Sarcomas

	CK	Desmin	Myogenin	CD99
Small-Cell Carcinoma	Positive	Negative	Negative	Negative
Rhabdomyosarcoma	Negative	Positive	Positive	Negative
Ewing/PNET	Negative	Negative	Negative	Positive
DSCT	Positive	Positive	Negative	Negative

PNET: peripheral neuroectodermal tumor; DSRCT: desmoplastic small round cell tumor.

Immunocytochemical panels for detection of small-cell sarcomas along with cytogenetic studies may be used to achieve a specific diagnosis.

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5

Detection of the Primary Site of Carcinomas

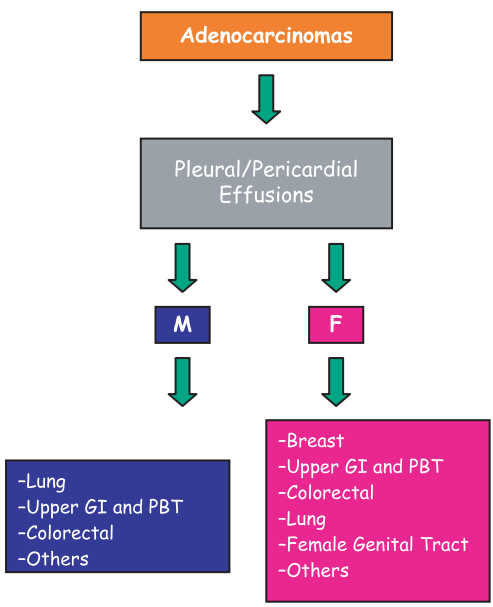
- Metastases from carcinomas of an unknown primary site in serous membranes are common. In addition, cytologic evaluation of effusion samples may be equally challenging as significant due to its impact on patients' management.
- In previous chapters, we have discussed cytologic and immunocytochemical characteristics that are used to reach the diagnosis of malignancy based on the examination of effusions collected from chest (pleural), abdomen (peritoneal/ascitic), or pericardium (pericardial).
- In this chapter, we will discuss the stepwise approach in formulating a final diagnosis that includes potential primary site of the most commonly found malignancies in effusion, that is, carcinomas.
- The search for the detection of the primary site of carcinomas will be based on the major cytologic types of neoplasms including: adenocarcinoma, squamous cell carcinoma, small-cell carcinoma, and undifferentiated large-cell carcinoma.

Cytologic Type, Source of Sample, and Patient's Gender

- Determination of the primary site of carcinomas based on cytomorphology and immunocytochemistry may be a difficult assignment in many occasions. The source of effusion (pleural/pericardial or ascetic/peritoneal washings) and the knowledge of patient's clinical information, especially age and gender, are essential for achieving this clinically important task.
- The following tables indicate the most common primary sites of carcinomas based on cytologic diagnosis depending on the type of sample in male and female patients.

Adenocarcinomas

- Adenocarcinomas are the most commonly seen carcinomas in effusion samples.
- Common primary sites for adenocarcinomas detected in pleural/pericardial effusions include the lungs, breast, upper gastrointestinal tract (GI) and pancreatobiliary tract (PBT), colorectal, and female genital tract.



Immunocytochemical Markers Used for Adenocarcinomas Found in Pleural/Pericardial Effusions in Males

	Lung	Upper GI and PBT	Colorectal
TTF-1	Positive	Negative	Negative
CDX-2	Negative[a]	Negative[a]	Positive
CK 7	Positive	Positive	Negative
CK 20	Negative	Negative	Positive

[a]Positive in intestinal-type carcinomas

Upper GI: upper gastrointestinal tract, PBT: pancreatobiliary tract

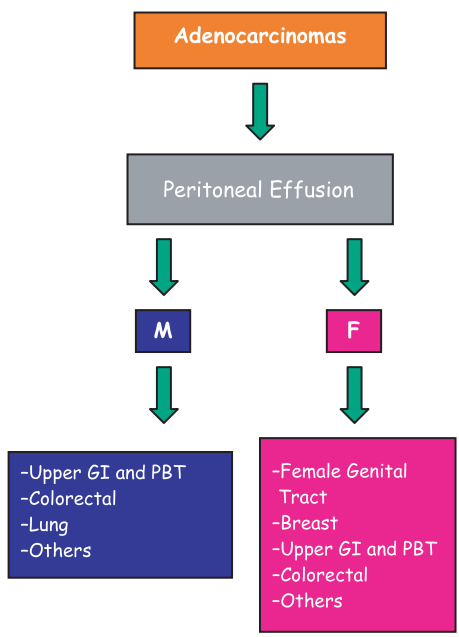
Immunocytochemical Markers Used for Adenocarcinomas Found in Pleural/Pericardial Effusions in Females

	Breast	Lung	Female Genital Tract
ER	Positive	Negative	Positive[a]
Mammaglobin	Positive	Negative	Negative
TTF-1	Negative	Positive	Negative
CA-125	Negative	Negative	Positive
WT-1	Negative	Negative	Positive[b]

[a]With exception of mucinous type

[b]Mainly in serous subtype

- Common primary sites for adenocarcinomas detected in peritoneal fluid/peritoneal washings include upper GI and PBT, colorectal, breast, female genital tract, and less commonly in lung.



Immunocytochemical Markers Used for Adenocarcinomas Found in Peritoneal Fluid in Males

	Upper GI and PBT	Colorectal	Lung
CDX-2	Negative	Positive	Negative[a]
CK-7	Positive	Negative	Positive
CK-20	Negative	Positive	Negative
TTF-1	Negative	Negative	Positive

[a]With the exception of mucinous-type carcinoma

Upper GI: upper gastrointestinal tract, PBT: pancreatobiliary tract

Immunocytochemical Markers Used for Adenocarcinomas Found in Peritoneal Fluid/Peritoneal Washings in Females

	Female Genital Tract	Upper GI and PBT	Colorectal	Breast
CK-7	Positive	Positive	Negative	Positive
CK-20	Negative	Negative	Positive	Negative
CA-125	Positive	Negative	Negative	Negative
WT-1	Positive[a]	Negative	Negative	Negative
P16	Positive	Negative	Negative	Negative
CDX-2	Negative	Positive	Positive	Negative
ER	Positive[b]	Negative	Negative	Positive
Mammaglobin	Negative	Negative	Negative	Positive

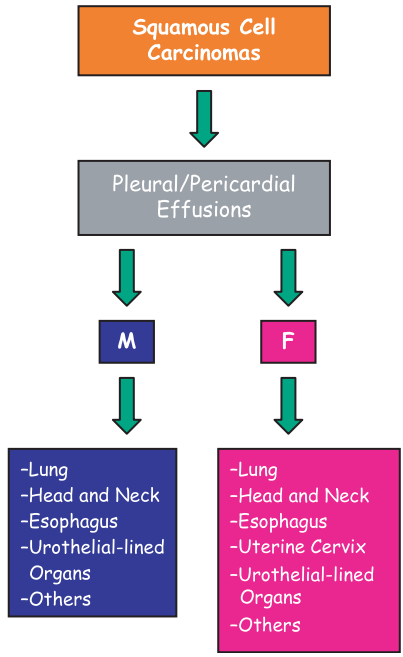
[a]Mainly in serous subtype

[b]With the exception of mucinous subtypes

Upper GI: upper gastrointestinal tract; PBT: pancreatobiliary tract

Squamous Cell Carcinomas

- Squamous cell carcinomas are rarely the cause of malignant effusions. Primary sites for squamous cell carcinomas seen in pleural/pericardial effusions include the lungs, head and neck, esophagus, urothelial-lined organs, and uterine cervix.
- With more advanced treatment modalities in recent years, metastatic squamous cell carcinomas especially in pleural effusions have more commonly been detected, probably due to prolonged survival of patients with head and neck malignancies.



Immunocytochemical Markers Used for Squamous Cell Carcinomas Found in Pleural/Pericardial Effusions in Males

	Lung	Head & Neck	Esophagus	Urothelial-lined Organs
TTF-1	Negative	Negative	Negative	Negative
P63	Positive	Positive	Positive	Positive
P16	Negative	Positive[a]	Negative	Negative

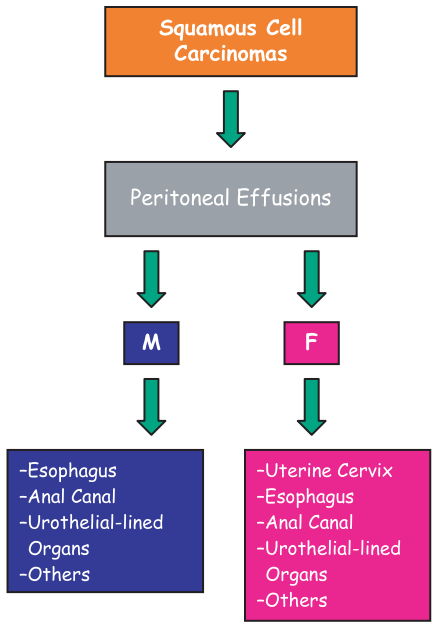
[a]Usually associated with human papilloma virus infection

Immunocytochemical Markers Used for Squamous Cell Carcinomas Found in Pleural/Pericardial Effusions in Females

	Lung	Head & Neck	Esophagus	Urothelial-lined Organs	Uterine Cervix
TTF-1	Negative[a]	Negative	Negative	Negative	Negative
P63	Positive	Positive	Positive	Positive	Positive
P16	Negative	Negative[a]	Negative	Negative	Positive

[a]Some are positive

- Common primary sites for squamous cell carcinomas seen in peritoneal fluid/peritoneal washings include the esophagus, urothelial-lined organs, anal canal, and uterine cervix.



Immunocytochemical Markers Used for Squamous Cell Carcinomas Found in Peritoneal Effusions/Peritoneal Washings in Males

	Esophagus	Anal Canal	Urothelial-lined Organs
P63	Positive	Positive	Positive
P16	Negative	Positive	Negative[a]

[a]Some carcinomas may be positive

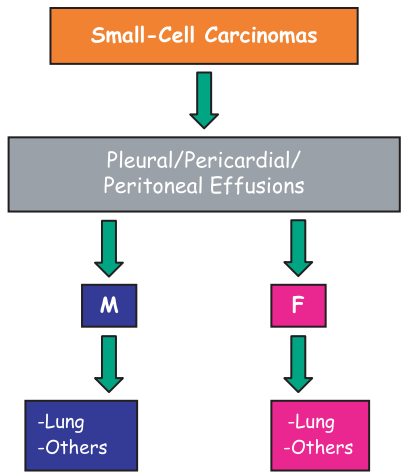
Immunocytochemical Markers Used for Squamous Cell Carcinomas Found in Peritoneal Fluid/Peritoneal Washings in Females

	Uterine Cervix	Esophagus	Anal Canal	Urothelial-lined Organs
P63	Positive	Positive	Positive	Positive
P16	Positive	Negative	Positive	Negative[a]

[a]Some may be positive

Small-Cell Carcinomas

- Small-Cell carcinomas are rarely found in effusion samples. Primary sites are similar for male and female and in all effusion types (pleural, pericardial, and peritoneal) and in most cases are of lung origin.



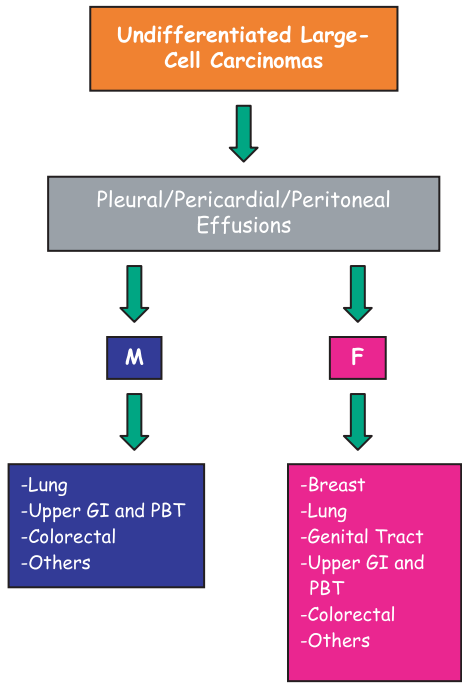
Immunocytochemical Markers Used for Small-Cell Carcinomas Found in Effusions in Both Males and Females

	Lung	Skin (Merkel Cell)	Urothelial-lined Organs	Prostate
TTF-1	Positive	Negative	Negative[a]	Negative[a]
Ck 20	Negative	Positive	Negative	Negative
P63	Negative	Negative	Negative[a]	Negative
PSA	Negative	Negative	Negative	Negative[a]

[a]Few carcinomas may be positive

Undifferentiated Large-Cell Carcinomas

- Undifferentiated large-cell carcinomas in effusions are usually present in an isolated cell pattern.
- Primary sites are similar in all effusion types (pleural, pericardial, ascitic, and peritoneal) and in most cases are of lung, breast, and upper GI and PBT, and colorectal origin.



Immunocytochemical Markers Used for Undifferentiated Large-Cell Carcinomas Found in Pleural, Pericardial, and Peritoneal Effusions in Males

	Lung	Upper GI and PBT	Colorectal
TTF-1	Positive	Negative	Negative
CDX-2	Negative	Positive	Positive
CK-7	Positive	Positive	Negative
CK-20	Negative	Negative	Positive

Upper GI: Upper gastrointestinal tract; PBT: Pancreatobiliary tract

Immunocytochemical Markers Used for Undifferentiated Large-Cell Carcinomas Found in Pleural/Pericardial Effusions, Ascitic Fluid, and Peritoneal Washings in Females

	Lung	Upper GI and PBT	Colorectal	Breast	Female Genital Tract
TTF-1	Positive	Negative	Negative	Negative	Negative
CDX-2	Negative[a]	Positive	Positive	Negative	Negative
CK-7	Positive	Positive	Negative	Positive	Positive
CK-20	Negative	Negative	Positive	Negative	Negative
ER	Negative	Negative	Negative	Negative[b]	Positive
Mammaglobin	Negative	Negative	Negative	Positive	Negative
CA-125	Negative	Negative	Negative	Negative	Positive
WT-1	Negative	Negative	Negative	Negative	Positive[c]

[a]With the exception of the mucinous type

[b]With the exception of ER-positive high-grade carcinomas

[c]Mainly in the serous subtype

Upper GI: upper gastrointestinal tract; PBT: pancreatobiliary tract

Major Primary Sites of Carcinomas

Carcinomas of Lung Origin

- In 2009, American Cancer Society statistics considered lung cancer to be the most frequent cause of cancer death and the second in incidence in both males and females.
- Lung cancer is classified as non-small-cell carcinomas (80%), small-cell carcinomas (17%), and others (3%).
- The distinction between non-small-cell carcinoma and small-cell carcinoma carries prognostic and predictive implications.
- The two main subtypes of non-small-cell carcinomas are adenocarcinomas and squamous cell carcinomas.
- The distinction between adenocarcinomas and squamous cell carcinomas of the lungs has therapeutic implications that may be facilitated by ICC.

Immunomarkers

TTF-1 (Thyroid transcription factor-1)

- TTF-1 is a commonly used positive marker for lung carcinomas with the exception of the squamous type.
- Sensitivity of up to 76% for adenocarcinomas and 83% for small-cell carcinomas of lung origin are reported.

> Based on cytomorphology alone, subtypes of lung carcinoma cannot be differentiated from those metastatic to the serous surfaces. TTF-1 as a nuclear marker for lung carcinomas is proven to be helpful in this distinction.

- TTF-1 is a sensitive immunomarker for distinguishing between pulmonary and nonpulmonary carcinomas; however, some nonpulmonary small-cell carcinomas have shown positivity for this antigen. Merkel cell carcinoma, however, has been consistently negative for this marker.
- The use of TTF-1 in cytologic samples is well established. A sensitivity of 54% is reported in lung adenocarcinomas using cytologic material (Figure 5.1A, B).

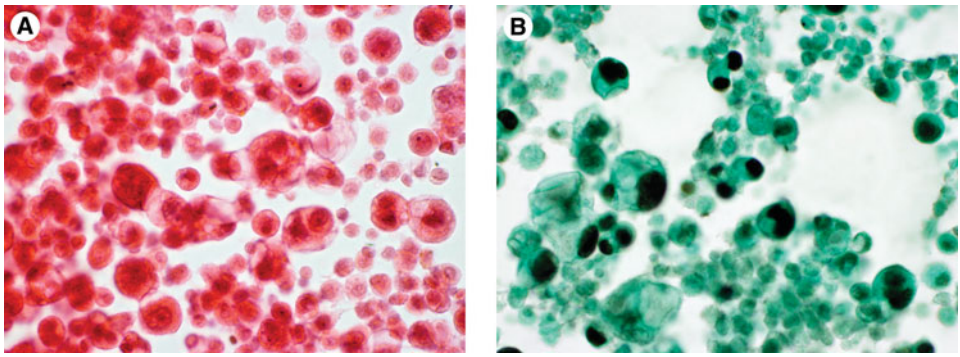


FIGURE 5.1 (A) Pleural fluid: Cellular evidence of adenocarcinoma. *Comment:* The patient had remote history of breast carcinoma who presented with pleural effusion and a lung mass (PAP, 60×). (B) Pleural fluid: Cellular evidence of adenocarcinoma consistent with a lung primary. *Comment:* The patient had remote history of breast carcinoma who presented with pleural effusion and a lung mass. TTF-1-positive nuclear immunostaining was used for detection of the site of origin. ER immunostain was negative (TTF-1, 60×).

- Due to the low sensitivity of TTF-1 expression in pulmonary squamous cell carcinomas, this marker is not helpful in establishing this site of origin in squamous cell carcinomas of the lungs.

> Although TTF-1 is not a specific marker for small-cell lung carcinoma, it may assist in distinguishing these tumors from some nonpulmonary small-cell carcinomas, particularly Merkel cell carcinoma, especially when it is used in conjunction with CK20.

- TTF-1 is also expressed in thyroid carcinomas. However, since thyroid carcinomas rarely metastasize to the serosal surfaces, TTF-1 can be used as a useful marker to distinguish carcinomas of lung origin from metastatic carcinomas of other organs.
- Caution should be exercised in patients with a known or suspected thyroid carcinoma who have a lung mass and a malignant effusion. In such patients, the immunostaining results for TTF-1 should be evaluated in conjunction with the thyroglobulin, to exclude the rare possibility of thyroid carcinoma metastatic to the serosal surfaces.

p63

- p63 is another marker which is well studied in cytologic samples. It is a member of the p53 family, normally expressed in squamous and urothelial cells, and basal layers of prostatic glands, breast, and bronchial epithelia.

Likewise, carcinomas of squamous and urothelial origin express this nuclear antigen.

- p63 has been used to distinguish basaloid squamous cell carcinomas from small-cell carcinomas of pulmonary origin.
- The use of p63 significantly increases the sensitivity for the identification of squamous component of non-small-cell carcinomas of the lung (from 35% to 88%).
- The use of p63 in patients with non-small-cell carcinoma of the lung improves therapeutic selection of these patients by the detection of the squamous component.
- p63 immunocytochemistry maybe used in pulmonary cytologic samples of non-small-cell carcinomas to identify squamous differentiation.
- A strong well-defined nuclear staining for p63 in carcinoma cells is characteristic for squamous cell carcinomas.
- In noneffusion cytologic samples such as bronchial specimens, the interpretation of p63 should be done with caution. Normal bronchial reserve cells may be present in cytologic samples. They are naturally p63 positive, and therefore the interpretation of p63 results should be restricted to the cells in groups previously identified as morphologically malignant and marked with a diamond pen.

Based on cytomorphology alone, subtypes of lung carcinomas cannot be differentiated from those metastatic to the serous surfaces. TTF-1 as a nuclear marker for lung carcinomas is proven to be helpful.

CK7

- Cytokeratin 7 is expressed in majority of carcinomas including those of lung origin, and thus its use is of no practical value in the detection of cancers of lung origin.

Carcinomas of Breast Origin

- Based on 2009 American Cancer Society statistics, breast cancer is the most common type of cancer in women and the second most common cause of cancer death after lung cancer.
- In patients with pleural or peritoneal effusions and remote history of breast carcinoma, late serosal metastases should be investigated.

- Breast metastasis to the ovaries is usually a late manifestation of the disease and may present as bilateral ovarian tumors and malignant peritoneal effusions. In addition, patients with breast carcinoma have an increased risk of primary ovarian cancer, and so these two entities may overlap clinically.

> Based on cytomorphology alone, distinction between breast carcinoma from other metastatic adenocarcinomas to the serosal surfaces may be impossible.

Immunomarkers

ER (estrogen receptor)

- It may be used as a diagnostic marker to indicate breast origin. Special attention should be given, however, to the type of antibody used for this purpose (ER-1D5, Dako) in our daily practice with reliable results (Figure 5.2A, B).
- In metastatic breast carcinoma cells, nuclear staining with ER is usually diffuse and easily found on low-power magnification.
- In contrast, ovarian carcinoma cells show focal nuclear staining with ER.

> ER should be used with caution in peritoneal fluid specimens, since cells derived from benign epithelial inclusions (endosalpingiosis) may stain with ER and result in an erroneous diagnosis.

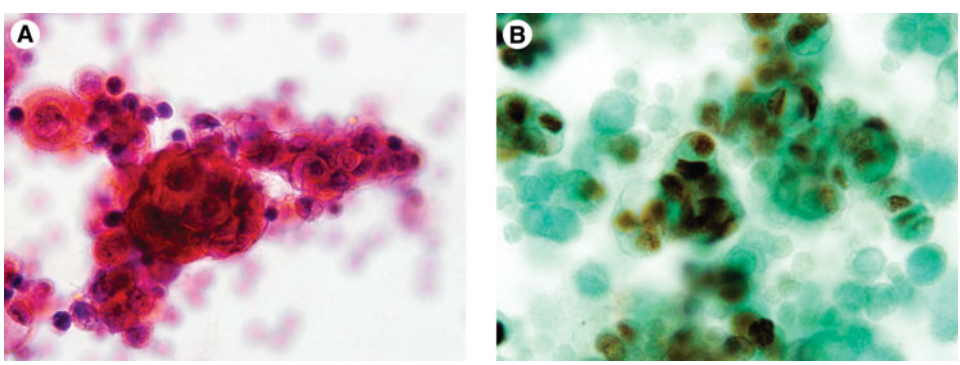


FIGURE 5.2 (A) Pleural fluid: Cellular evidence of adenocarcinoma. *Comment:* The patient had a history of breast carcinoma and multiple lung lesions (PAP, 100×). (B) Pleural fluid: Cellular evidence of adenocarcinoma consistent with a breast primary. *Comment:* Nuclear staining for ER in this sample confirms the mammary origin of the carcinoma cells (ER, (clone 1D5), 100×).

- The use of ER in effusions outside of the abdominal cavity, that is, pleural, pericardial, and CSF, for the detection of metastatic cells from breast and female genital tract carcinomas, is considered a safe practice in our experience using the ER-1D5 antibody.

Mammaglobin

- This glycoprotein is used to identify metastatic breast carcinomas in histologic samples. Its use in cytologic material is limited.

CK7

- Cytokeratin 7 is expressed in majority of carcinomas including those of mammary origin, and therefore, its use is practically of no value in the detection of breast carcinoma cells in effusions.

Carcinomas of the Upper Gastrointestinal Tract and Pancreato-biliary Tract

- In general, following lung and breast carcinomas, those of the gastrointestinal tract are the most common solid neoplasms metastasizing to the serosal surfaces.
- Malignant effusions in such cases are usually adenocarcinomas and less commonly squamous cell carcinomas (from the esophagus).

> Carcinomas of pancreas and biliary tract cannot be differentiated from other adenocarcinomas, based on cytomorphology.

- Cells from hepatocellular carcinomas are rarely found in effusion samples.

Immunomarkers

CK7

- Cytokeratin 7 is a general epithelial marker that is expressed in majority of carcinomas from different sites. CK7 negativity, however, is more helpful than its positivity in the diagnosis of some metastatic carcinomas such as those of colorectal and hepatocellular origins.

CK20

- Cytokeratin 20 is negative in majority of upper GI tract neoplasms; however, CK20 can be co-expressed with CK7 in PBT carcinomas.

Carcinomas of Colorectal Origin

- Colorectal cancer is the third most common cancer and the third most common cause of cancer mortality in both men and women in the United States.
- Malignant effusion in patients with colorectal carcinomas is a poor prognostic indication; 20% of patients die within 1 month, and the median survival is only a few months.
- Cytologically metastatic colorectal adenocarcinomas cannot be differentiated from other adenocarcinomas involving serosal surfaces. Thus, a proper identification of the site of origin, based on cytology of effusions may be crucial.

Immunomarkers

CK7

- Cytokeratin 7 is positive in majority of carcinomas. Therefore, its negativity in colorectal carcinomas is of diagnostic significance.

CK20

- Cytokeratin 20 is positive in some carcinomas including those of colorectal origin. However, a panel of CK 7 negative and CK 20 positive is practically diagnostic of a carcinoma of colorectal origin (Figure 5.3A, B).

CDX-2

- It is a useful marker for neoplasms with intestinal-type differentiation, including colorectal carcinomas (Figure 5.3C).

Carcinomas of Female Genital Tract

- Ovarian cancer is the leading cause of death from gynecological cancers in western countries.
- Carcinomas of the ovary are the most common female genital-tract malignancies associated with the accumulation of fluid-containing malignant cells commonly in the peritoneal cavity.
- The presence of carcinoma cells in peritoneal effusion is commonly the initial presentation of stage III ovarian cancer. However, ovarian carcinomas rarely metastasize to other body cavities.

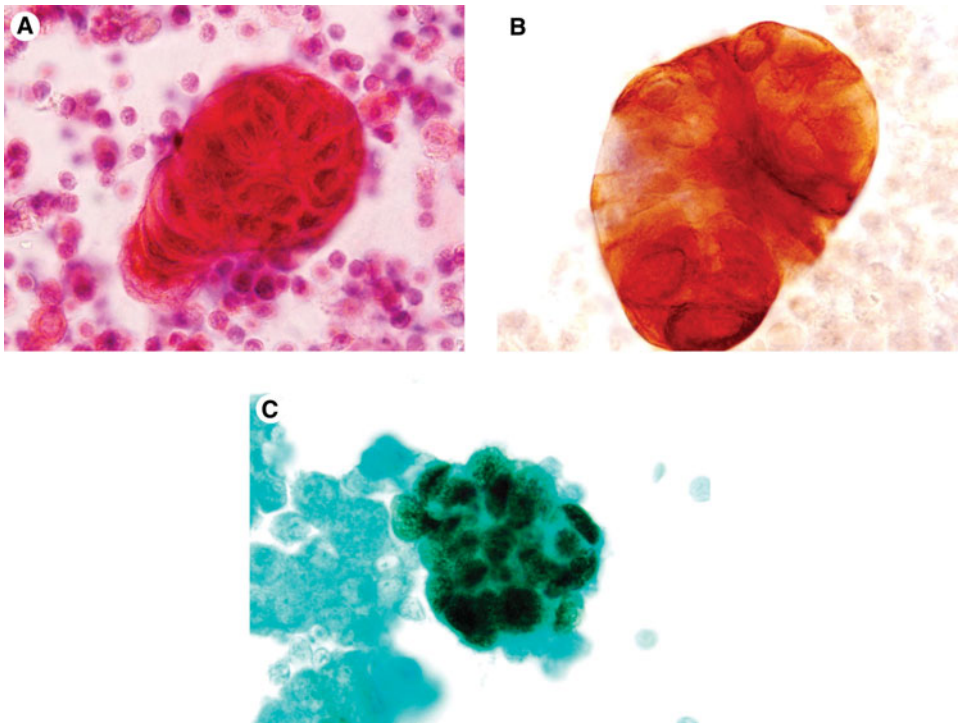


FIGURE 5.3 (A) Pleural fluid: Cellular evidence of adenocarcinoma. *Comment:* This patient presented with disseminated colonic carcinoma. Although colonic carcinomas are rarely found in pleural samples, this adenocarcinoma clinically proved to be of colonic origin (PAP, 100×). (B) Pleural fluid: Cellular evidence of adenocarcinoma consistent with a colonic primary. *Comment:* We used a small panel of CK20, CK7, and CDX-2 to confirm the colonic origin of this tumor. CK7 was negative. CK20 was positive (shown here). This pattern of staining is characteristic of colorectal carcinomas (CK20, 100×). (C) Pleural fluid: Cellular evidence of adenocarcinoma consistent with a colonic primary. *Comment:* CDX-2 is a useful marker for neoplasms with intestinal-type differentiation, including colorectal carcinomas (CDX-2, 100×).

- In general, other primary neoplasms of the female genital tract such as endometrial and cervical carcinomas less commonly, involve the serosal surfaces.

> Ovaries are frequently the site for metastatic carcinomas from the breast and the GI tract. In most cases, distinction between ovarian adenocarcinomas from those of metastatic origin cannot be made based on cytomorphology alone.

Immunomarkers

Estrogen Receptor

- The nuclear staining for ER is helpful in the diagnosis of endometrial adenocarcinomas and majority of tubo-ovarian carcinomas with the exception of the mucinous subtype.

- ER may be very useful in distinguishing between serous carcinomas (ER positive in 88% of metastatic serous carcinomas of the ovary and 86% of the primary peritoneal serous carcinomas) from malignant mesotheliomas. ER is negative in mesotheliomas.
- Progesterone receptor (PR) immunostaining is less sensitive than ER in effusions.
- Interpretation of ER and PR immunostaining in peritoneal fluids from female patients should be done with caution, since benign epithelial Mullerian inclusions are commonly ER and PR positive.

CK7

- It is expressed in all female genital-tract carcinomas. Its positivity is helpful in distinguishing ovarian from metastatic colonic carcinomas.

CK 20

- It may be positive in some female genital-tract carcinomas.

CA-125

- Although a nonspecific marker, it is expressed in endometrial and tubo-ovarian carcinomas (95% and 67% of ovarian and primary peritoneal carcinomas, respectively), may be helpful in the distinction of female genital-tract from other primary sites.

WT-1

- Nuclear WT-1 expression is present in normal ovarian surface epithelium and has been used in the diagnosis of ovarian carcinomas, especially in the serous subtype.

p16

- Diffuse staining of this marker is seen in HPV-induced cervical carcinomas. Most cervical carcinomas of squamous, glandular, and small-cell type are p16 positive.
- p16 has also been detected in many other noncervical malignancies such as ovarian, head and neck, lung, pancreas, and bladder. Therefore, p16 should not be used as the only marker to establish the diagnosis of metastatic cervical carcinoma.

p63

- This nuclear marker is expressed in squamous cell carcinomas of the cervix and in general in carcinomas with squamous differentiation.

Carcinomas of Urothelial-lined Organs

- These neoplasms uncommonly metastasize to serosal membranes. They should, however, be considered in the differential diagnosis when there is a history of bladder, ureteral, or renal–pelvis primary carcinomas.
- Interestingly, metastatic urothelial carcinoma cells in the effusion samples may resemble mesothelial cells and create diagnostic difficulties using cytomorphology alone. In such cases, immunocytochemistry is extremely helpful.

Immunomarkers

p63

- Positive nuclear staining differentiates urothelial carcinoma cells from mesothelial and adenocarcinoma cells (Figure 5.4A, B).

> p63 immunostain does not differentiate urothelial carcinomas from squamous-cell carcinomas.

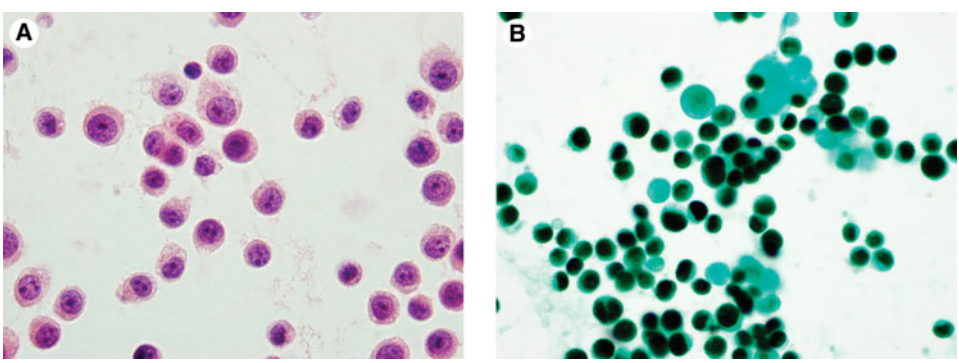


FIGURE 5.4 (A) Pleural fluid: Cellular evidence of carcinoma. *Comment:* This patient had deeply invasive urothelial carcinoma of the bladder, who presented with lung nodules and pleural effusion. These isolated cytologically abnormal cells may superficially resemble reactive mesothelial cells (PAP, 100×). (B) Pleural fluid: Cellular evidence of carcinoma consistent with metastatic urothelial carcinoma. *Comment:* We used p63 to confirm the urothelial origin of these abnormal cells. p63 is not expressed in cells of mesothelial origin (p63, 100×).

CK7

- It is expressed in majority of carcinomas, including urothelial carcinomas.

CK 20

- It is positive in majority of urothelial carcinomas, usually demonstrating a focal pattern of staining.

Carcinomas of Head and Neck Origin

- Squamous cell carcinoma is the most common carcinoma arising in the tongue, larynx, and hypopharynx.
- Squamous cell carcinomas rarely metastasize to the serosal surfaces. However, when a patient with a history of squamous cell carcinoma of head and neck origin presents with pleural effusion, the differential diagnosis should include a squamous cell carcinoma from lung origin and it should be clinically investigated.

Immunomarkers

p63

- It is a squamous cell marker; however, it cannot differentiate carcinomas from head and neck from those of other sites of origin (Figure 5.5A, B).

p16

- It is expressed in HPV-induced head and neck carcinomas, and therefore, may be helpful in differentiating head and neck squamous cell carcinomas from others.

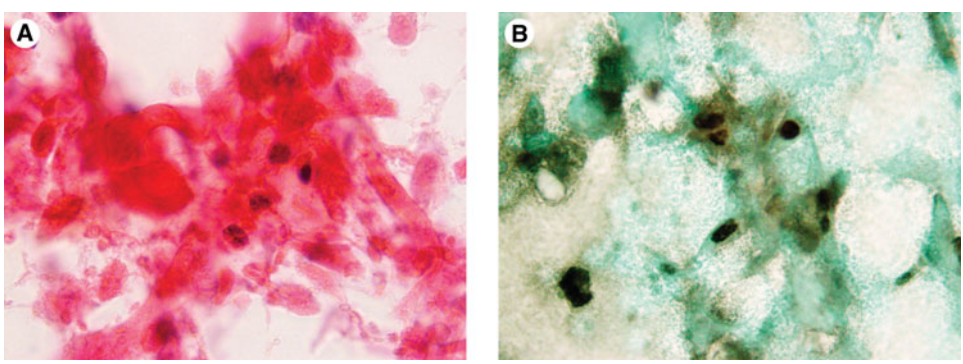


FIGURE 5.5 (A) Pleural fluid: Cellular findings suggestive of squamous cell carcinoma. *Comment:* This patient was treated for squamous cell carcinoma of tongue origin (PAP, 100×). (B) Pleural fluid: Cellular evidence of squamous cell carcinoma. *Comment:* p63 nuclear staining confirms the diagnosis of squamous cell carcinoma. However, this marker cannot determine the site of origin (p63, 100×).

Carcinomas of other Primary Sites

Carcinomas of Prostatic Origin:

- Rarely found in serous effusions and they are morphologically similar to those of other sources.
  - *PSA (prostatic specific antigen)*: It is a specific marker to establish the prostatic origin in the presence of clinical history (Figure 5.6A, B).

Carcinomas of Thyroid Origin:

- These neoplasms rarely metastasize to the serous membranes.
- Their distinction from other papillary carcinomas such as those of lung origin (bronchioloalveolar type) may be impossible on the basis of cytomorphology alone.
  - *TTF-1 (thyroid transcription factor-1):* is expressed in both lung and thyroid carcinomas (Figure 5.7A, B).
  - *Thyroglobulin:* is a specific marker for thyroid carcinomas that is used as a confirmatory marker, when lung and thyroid are both considered as potential sites of origin for carcinoma cells in effusions (Figure 5.7C).

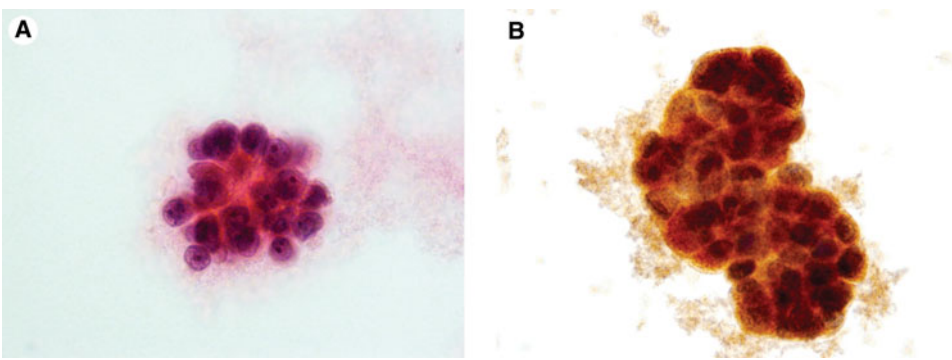


FIGURE 5.6 (A) Pleural fluid: Cellular evidence of adenocarcinoma. *Comment:* patient with metastatic hormone refractory prostatic carcinoma. Prostatic carcinomas are rarely found in effusion samples; therefore, clinical history is important in selection of immunomarkers (PAP, 100×). (B) Pleural fluid: Cellular evidence of adenocarcinoma consistent with metastatic prostatic carcinoma. *Comment:* Prostatic specific antigen is a specific marker to determine prostatic site of origin (PSA, 100×).

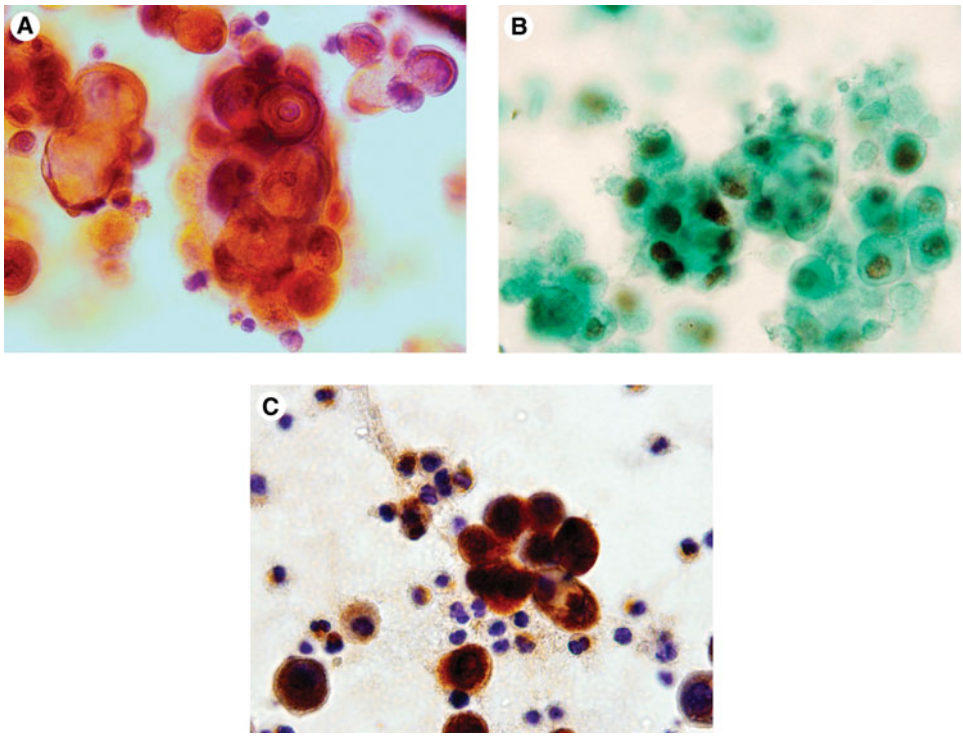


FIGURE 5.7 (A) Pleural fluid: Cellular evidence of carcinoma with psammoma bodies. *Comment:* patient with a known history of papillary thyroid carcinoma presenting with lung nodules. Thyroid papillary carcinomas rarely metastasize to serous membranes. Therefore, distinction between metastatic thyroid carcinoma and more commonly seen carcinomas such as those of lung origin should be investigated by ICC. It should also be noted that presence of psammoma bodies in this case does not necessarily indicate a carcinoma of thyroid origin. (B) Pleural fluid: Cellular evidence of carcinoma consistent with metastatic papillary thyroid carcinoma. *Comment:* We used a small panel of TTF-1 and thyroglobulin to confirm the thyroid origin of this tumor. Nuclear positivity for TTF-1 is observed in both, thyroid and lung carcinomas; therefore, use of a second confirmatory marker is essential in patients with history of thyroid carcinoma and lung masses (TTF-1, 100×). (C) Pleural fluid: Cellular evidence of carcinoma consistent with metastatic papillary thyroid carcinoma. *Comment:* Since TTF-1 is a marker that is expressed in both lung and thyroid carcinomas, thyroglobulin should be used to confirm the thyroid origin (Thyroglobulin, 100×).

- Thyroid medullary carcinomas rarely metastasize to the serous membranes. Cytologically they resemble other endocrine neoplasms such as those from pancreas (islet cell tumors), gastrointestinal tract, and bronchial carcinoids. They are positive for TTF-1, calcitonin, and CEA.

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6

Cerebrospinal Fluid Cytology

Introduction

- Microscopic examination of cerebrospinal fluid (CSF) is an important diagnostic task in both symptomatic and asymptomatic patients with the involvement of the central nervous system (CNS) by malignant or infectious processes.
- Cancer patients with CNS involvement diagnosed by cytology require further CSF monitoring to optimally adjust their treatment.
- Cytologic diagnosis of CSF should be based not only on the appearance of the cells, but the patient's clinical history, location of the lesion, method of collection (spinal tap or through a shunt), and the knowledge of differential diagnoses.

Specimen Handling and Preparation

- Adequate specimen handling and preparation is important for an accurate diagnosis in CSF specimens.
- The best CSF technical preparation is "centrifugation." The cytocentrifuged–cytospin method is simple and rapid with optimal cell recovery and good cytologic details. Further, the slides are suitable for ancillary studies.
- In our laboratory, we prepare four cytospin slides: three alcohol-fixed slides stained with the Papanicolaou method, and one air-dried slide with Wright–Giemsa stain.
- Should additional histochemical, immunocytochemical, or *in situ* hybridization analysis be required, one or two of the Papanicolaou-stained slides are used. We usually perform immunocytochemistry (ICC) on a previously Papanicolaou-stained cytospin slide without destaining (see Chapter 7).

Preparation of CSF should be done as rapid as possible after the lumbar puncture is performed. The specimen should be refrigerated if immediate cytopreparation is not possible. We only recommend the use of fixatives when immediate transportation to the cytology laboratory is not possible.

Diagnostic Categories

- Benign conditions
- Malignant conditions

Benign Conditions

- Normal CSF
- Contaminants in CSF
- Inflammatory conditions
- Infectious diseases

Normal CSF	
Lymphocytes (most common) Monocytes	Pia-arachnoid/leptomeningeal/ mesothelial cells (PAM cells) Ependymal cells (rare)

A normal CSF is a paucicellular or an absolutely acellular specimen; a few mature lymphocytes are the most commonly found cells.

- Although cytologic evaluation of CSF is not usually a quantitative procedure, cellular specimens are easily noticed on low-power magnification.
- Typically, a "normal CSF" contains very few cells that are usually lymphocytes (less than 5 cells/mm^3 in adults and less than 10 cells/mm^3 in neonates) (Figure 6.1).
- Pia-arachnoid mesothelial/monocytoid (PAM) cells are the arachnoid lining cells and the counterpart of mesothelial cells in other body cavities. These cells are not true mesothelial cells and they resemble monocytes (Figure 6.2). The cytoplasm is abundant with defined cell borders. Their eccentric nuclei have bean-shaped and fine chromatin. Nucleoli are rare. The PAM cells may show features of macrophages including cytoplasmic pigments.
- In occasions, rare neutrophils are present in "normal CSF."

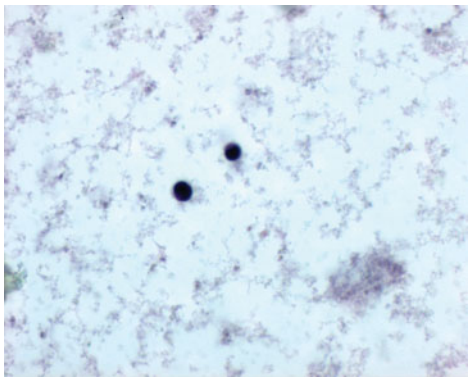

FIGURE 6.1 *CSF: Negative for malignancy. Comment:* Presence of few mature lymphocytes is normal in CSF. CSF is a naturally paucicellular specimen (PAP, 25×).

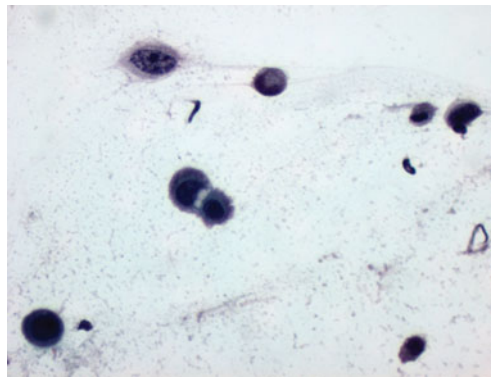

FIGURE 6.2 *CSF: Negative for malignancy. Comment:* Presence of a few monocytes is normal in CSF (PAP, 60×).

Contaminants in CSF	
Peripheral blood	Corpora amylacea
Bone marrow	Fungal elements
Cartilage	

- *Peripheral blood*: Contamination by peripheral blood can be a source of diagnostic error. Red blood cells may indicate pathologic bleeding or a traumatic tap. Since white blood cells accompany red blood cells, an erroneous diagnosis of infectious process should be avoided in the presence of a bloody tap.
- *Bone marrow*: Bone marrow cells may be present in CSF and, especially the megakaryocytic cell line might be confused by atypical cells of unknown origin.
- *Cartilage*: Cartilage cells may be present in CSF. The cells are characterized by eccentric nuclei with dense amphophylic cytoplasm that can be confused with adenocarcinoma or chordoma cells (Figure 6.3).
- *Corpora amylacea*: Corpora amylacea can be present in CSF of elderly patients, and should not be confused with yeasts or with psammoma bodies seen in meningiomas.
- *Talc powder*: Talc particles are due to contamination from surgical gloves. They are characterized by polarization (Figure 6.4).

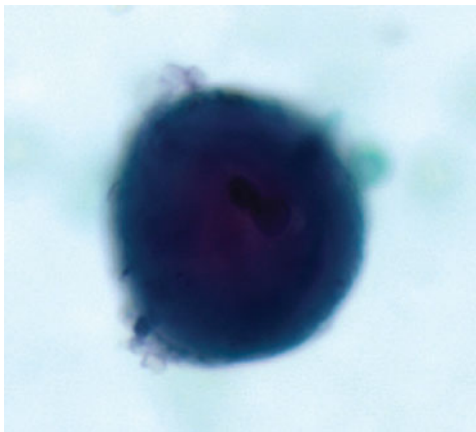

FIGURE 6.3 *CSF: Negative for malignancy. Comment:* Cells of cartilaginous origin may be a contaminant in CSF and should not be confused with a malignant cell (PAP, 100×).

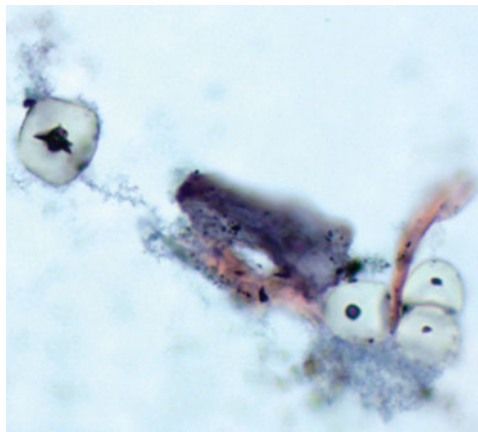

FIGURE 6.4 *CSF: Negative for malignancy. Comment:* Presence of talc powder crystals is a contaminant in CSF (PAP, 40×).

- *Fungal elements*: Fungal elements can be found in CSF as a result of contamination.

> In pathogenic conditions, fungal elements are seen in association with a profuse inflammatory reaction. The one exception to this rule is in cases of *cryptococcal meningitis*.

- In inflammatory conditions, CSF predominantly contains lymphocytes, monocytes, and neutrophils.

Inflammatory Conditions
Acute inflammation
Chronic inflammation

Acute inflammation

- Neutrophils are the most common type of inflammatory cells frequently associated with bacterial meningitis (Figure 6.5).
- If only a few neutrophils are seen in CSF, their source maybe a bloody tap or an impending reaction to an early bacterial infection.
- Eosinophils are noted occasionally. When they are found in large numbers, a parasitic infection such as *cysticercosis* should be suggested.

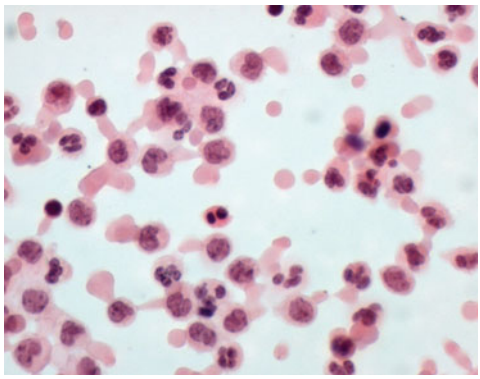

FIGURE 6.5 *CSF: Negative for malignancy. Cellular evidence of acute inflammation. Comment:* Presence of many polymorphonuclear cells may represent acute bacterial meningitis and microbacterial cultures should be suggested (PAP, 60×).

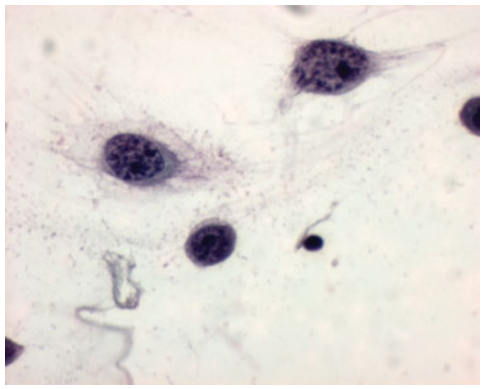

FIGURE 6.6 *CSF: Specimen consists of a few atypical lymphocytes. Clinical correlation is recommended. Comment:* Presence of atypical lymphocytes may be seen in viral infections such as the one caused by Epstein–Barr virus. However, flow cytometric analysis should be performed if malignancy is clinically considered (PAP, 100×).

Chronic inflammation (lymphocytosis)

- A mature lymphocytic population often indicates a chronic and indolent inflammatory state.
- A mixed population of immature lymphocytes and plasma cells usually represents an immune response.
- T-lymphocytes are the predominant mononuclear cells found in many conditions including tuberculosis, multiple sclerosis, and viral meningitis.
- Plasma cells are rarely seen as the predominant cell type. If present in large numbers, neurosyphilis, or plasmacytoma/multiple myeloma should be considered in the differential diagnosis.
- The presence of atypical lymphocytes in acute Epstein–Barr virus infection may be a source of diagnostic pitfall (Figure 6.6).
- When lymphocytes show cytologic atypia, differential diagnosis should include a lymphoproliferative disorder (lymphoma/leukemia).

When a lymphoreticular process is suspected cytologically, a consultation with the hematopathology service is recommended and flow cytometry should be the next test of choice.

Benign and Malignant Lymphocytes

	Benign	Malignant
Cytoplasm	Less	More
Nuclear membrane	Smooth	Irregular
Chromatin	Finer	Coarser
Nucleoli	Rare	Often multiple
Nuclear Fragmentation	Absent	Present

- "Mollaret disease" is a rare type of benign recurrent aseptic meningitis that is characterized by a hypercellular CSF within the first 24 hours of its clinical manifestation. The cellular component includes large monocytes with eccentric and irregularly shaped nuclei (Mollaret's cells), neutrophils, and lymphocytes. Later on, during the course of the disease, the cytologic pattern changes into a pure lymphocytosis.
- "Multiple sclerosis" is characterized by the presence of numerous enlarged monocytoid cells with bean-shaped nuclei and enlarged lymphocytes.
- In reactive inflammatory conditions due to meningeal irritation, an increased number of PAM cells may be seen in the CSF samples that may represent a source of false-positive results.
- The following table highlights the most useful cytologic findings differentiating PAM cells from carcinoma cells.

	PAM Cells	Carcinoma Cells
Size	Small	Large
Nucleus	Kidney bean	Round/Oval
Chromatin	Bland	Irregular
Nucleoli	Indistinct	Prominent

Infectious Diseases

- *Cryptococcosis* is the most common fungal infection seen in CSF. It is characterized by lack of inflammatory reaction and geometrical distribution of budding yeasts with thick walls that allow its identification (Figures 6.7 and 6.8). The diagnosis of *Cryptococcosis* can be confirmed by mucicarmine stain (Figure 6.9).

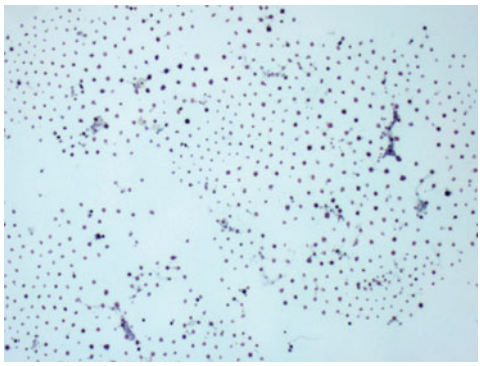

FIGURE 6.7 *CSF: Negative for malignancy. Cellular evidence of cryptococcosis. Comment:* Note the geometrical distribution of the cryptococcal yeasts (PAP, 25×).

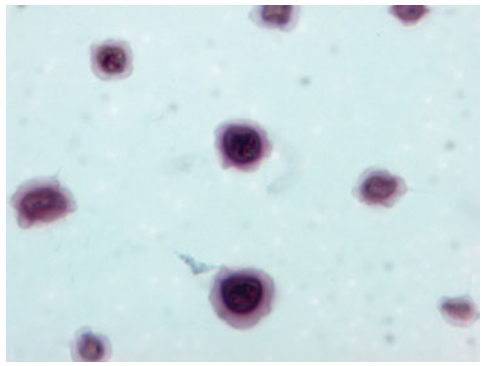

FIGURE 6.8 *CSF: Negative for malignancy. Cellular evidence of cryptococcosis. Comment:* Note the presence of thick capsule (PAP, 100×).

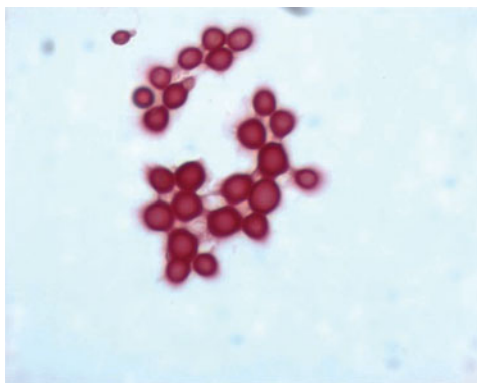

FIGURE 6.9 CSF: Negative for malignancy. Cellular evidence of cryptococcosis. *Comment:* The glycoprotein capsular material is positive with mucicarmine (Mucicarmine, 60×).

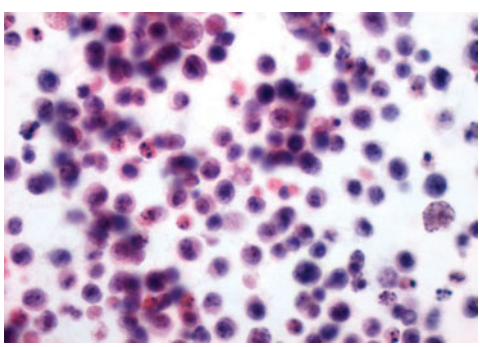

FIGURE 6.10 *CSF: Cellular evidence of malignant lymphoreticular process (lymphoma/leukemia). Flow cytometric analysis is required for a specific classification. Comment:* Apoptotic bodies are abundant (PAP, 100×).

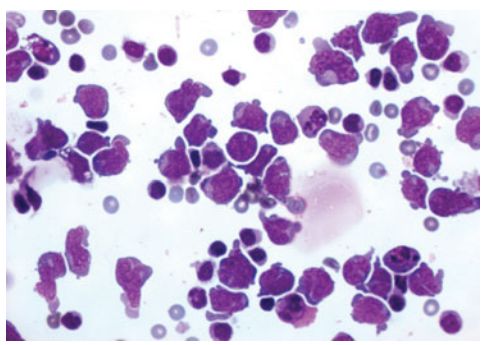

FIGURE 6.11 *CSF: Cellular evidence of malignant lymphoreticular process (lymphoma/leukemia). Flow cytometric analysis is required for a specific classification. Comment:* Prominent abnormal nuclei are identified. Flow cytometry result: Large B-cell lymphoma (Wright–Giemsa stain, 100×).

- In clinically suspected cases, ancillary techniques such as cultures and PCR for the identification of the microorganisms are recommended.

Malignant Conditions

- Lymphoreticular processes such as lymphomas/leukemias are the most frequently diagnosed malignancies in CSF.

CSF Sensitivity of Cytologic Examination in Malignancies

Lymphoreticular processes	66%
Metastatic neoplasms	50%
Primary CNS neoplasms	33%

Lymphoreticular Processes: Lymphomas/Leukemias

- Diagnosis of malignant lymphoma in CSF (lymphomatous meningitis) may be the result of a primary CNS lymphoma or secondary involvement by an extracerebral lymphoma. The cytologic findings are similar in both conditions (Figures 6.10 and 6.11).
- In adults, the majority of lymphoreticular processes involving CNS are B-cell lymphomas.
- The neoplastic cells are numerous and monomorphic. The cells have high nucleo-cytoplasmic ratio. The nuclei are infolded or lobulated. Multiple nucleoli may be present.

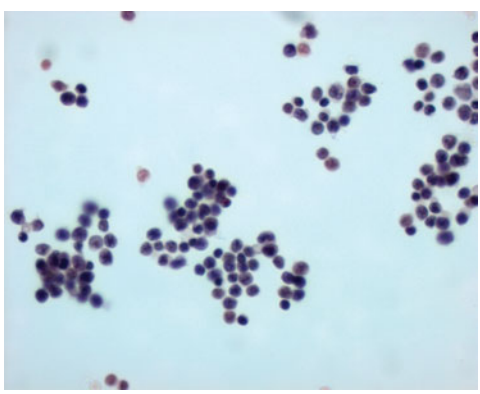

FIGURE 6.12 *CSF: Cellular evidence of malignant lymphoreticular process (lymphoma/leukemia). Flow cytometric analysis is required for a specific classification. Comment:* Flow cytometry result: Acute lymphoid leukemia (Wright-Giemsa stain, 60×).

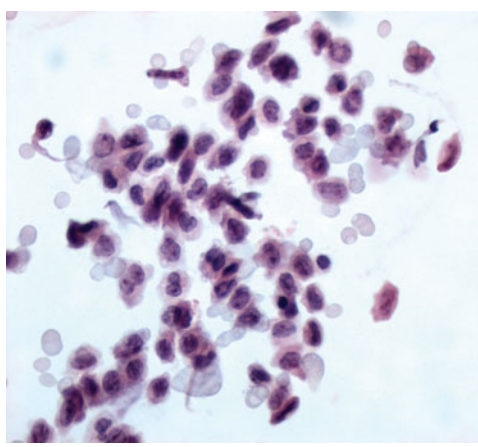

FIGURE 6.13 *CSF: Cellular evidence of malignant lymphoreticular process (lymphoma/leukemia). Flow cytometric analysis is required for a specific classification. Comment:* Flow cytometry result: Acute myeloid leukemia (Wright–Giemsa stain, 100×).

Cytomorphology alone does not allow distinction between different subtypes of malignant lymphomas in CSF. Ancillary studies such as flow cytometry, are extremely helpful in the characterization of lymphoreticular processes involving the CSF.

- In cases of acute lymphoid and myeloid leukemias, the cell of origin may be determined by using periodic acid-Schiff (PAS) stain and myeloperoxidase immunostain, respectively (Figures 6.12 and 6.13).

In majority of cases, the initial diagnosis of CSF involment by a malignant lymphoreticular process is easily achieved by routine diagnostic cytology, whereas subclassification of the neoplasm is usually done by ancillary techniques such as ICC, flow cytometry, and gene rearrangement studies.

Leptomeningeal Carcinomatosis

- Leptomeningeal carcinomatosis is an important neurologic complication seen in approximately 10% of patients with a known solid malignancy.
- Favorable clinical outcome in patients with leptomeningeal carcinomatosis largely depends on early diagnosis. Therefore, cytologic evaluation of CSF should be rapid and accurate.

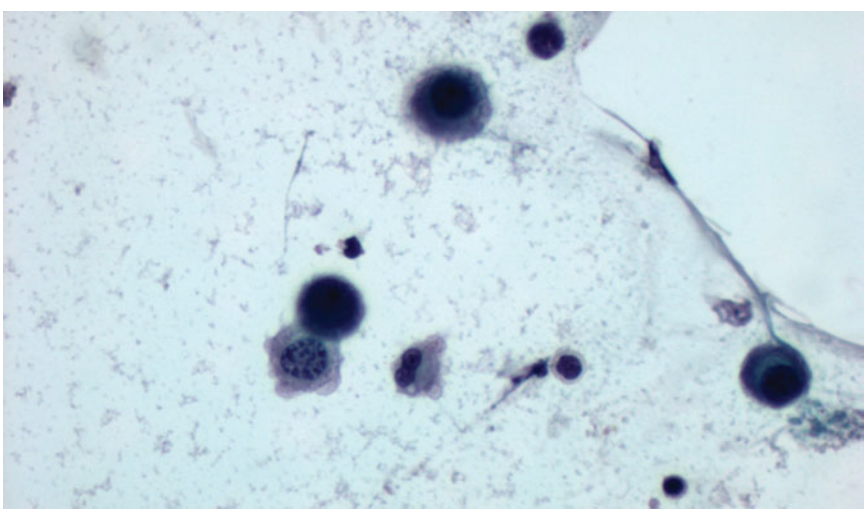

FIGURE 6.14 *CSF: Cellular evidence of malignant neoplasm. Immunostains are pending. Comment:* Few but very abnormal cells are present. Immunostains such as EMA and CEA should be used to confirm a malignant epithelial process involving CSF. Final diagnosis: Metastatic breast carcinoma (PAP, 100×).

Cytologic examination of CSF in cases of suspected leptomeningeal carcinomatosis is considered to be the best diagnostic test.

- Breast and lung carcinomas, followed by malignant melanoma, are the most frequent sources of metastasis in CSF (Figures 6.14–6.16).
- *Sensitivity*: Routine CSF cytology for the detection of carcinoma cells has a sensitivity ranging from 50% to 80%.

The false-negative results in CSF cytology are largely due to the limited number of isolated carcinoma cells with minimal cytologic abnormalities. This is in contrast to other body cavity fluids such as pleural and peritoneal, where the carcinoma cells are commonly grouped and easily detectable in low-power magnification.

- *Specificity*: Routine CSF cytology for the detection of carcinoma cells has a specificity ranging from 90% to 100%.

The most frequent source of false-positive result in CSF cytology is the misinterpretation of reactive PAM cells as carcinoma cells. We use immunocytochemical markers such epithelial membrane antigen (EMA) and carcinoembryonic antigen (CEA) in this differential diagnosis.

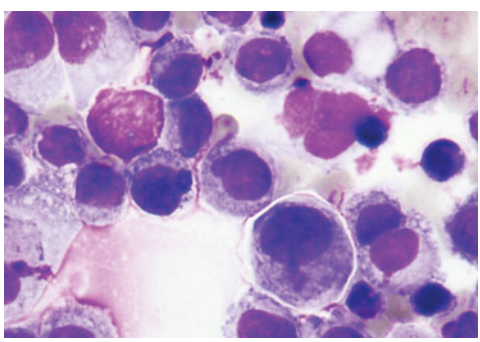

FIGURE 6.15 *CSF: Cellular evidence of carcinoma. Comment:* Numerous isolated malignant cells represent leptomeningeal carcinomatosis. Final diagnosis: Metastatic breast carcinoma (Wright–Giemsa stain, 100×).

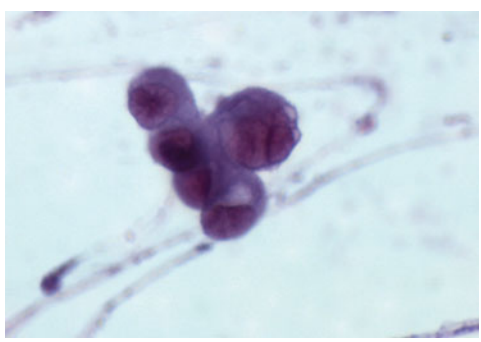

FIGURE 6.16 *CSF: Cellular evidence of carcinoma. Comment:* Presence of malignant cells in tight clusters is an uncommon finding in CSF. Final diagnosis: Metastatic lung carcinoma (PAP, 100×).

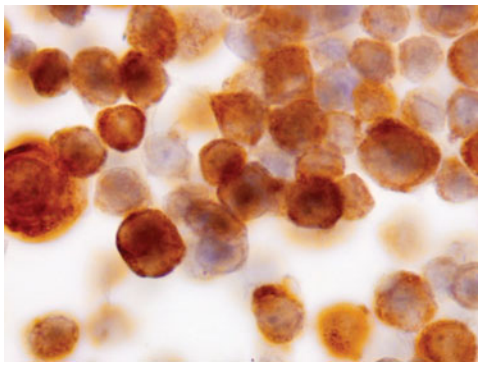

FIGURE 6.17 *CSF: Cellular evidence of carcinoma confirmed by EMA immunostain. Comment:* Note strong cytoplasmic positivity for EMA. Final diagnosis: Metastatic breast carcinoma (EMA, 100×).

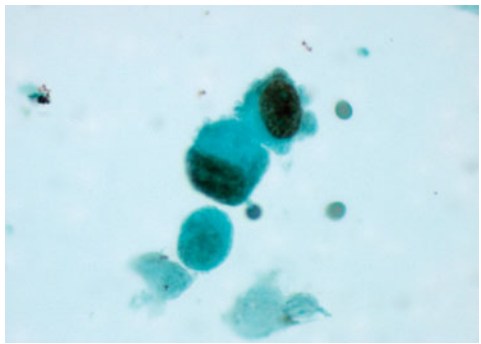

FIGURE 6.18 *CSF: Cellular evidence of carcinoma consistent with a neoplasm of lung origin. Thyroid transcriptor factor-1 immunostain is positive. Comment:* Note easily detectable nuclear positivity (TTF-1, 100×).

- EMA and CEA are proven to be of diagnostic value in cases of leptomeningeal carcinomatosis. Strong intracytoplasmic staining with either marker indicates the presence of carcinoma cells in CSF (Figure 6.17).
- Other tumor markers, such as thyroid transcription factor-1 (TTF-1) and estrogen receptor (ER-1D5), are used to confirm the cell of origin (Figures 6.18 and 6.19) in cases of metastatic lung and breast carcinomas, respectively.
- If malignant melanoma is in the differential diagnosis, we recommend using S100 protein and HMB-45 immunostaining for confirmation of the

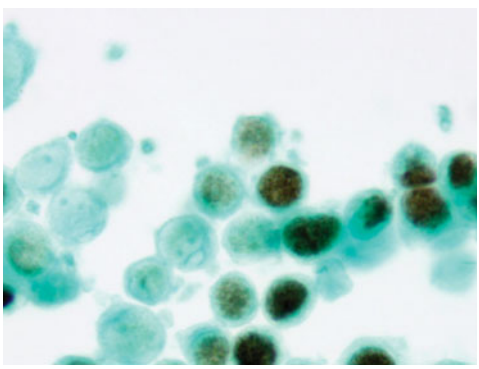

FIGURE 6.19 *CSF: Cellular evidence of carcinoma consistent with a neoplasm of mammary origin. Estrogen receptor immunostain is positive. Comment:* Note nuclear positivity (ER clone 1D5, 100×).

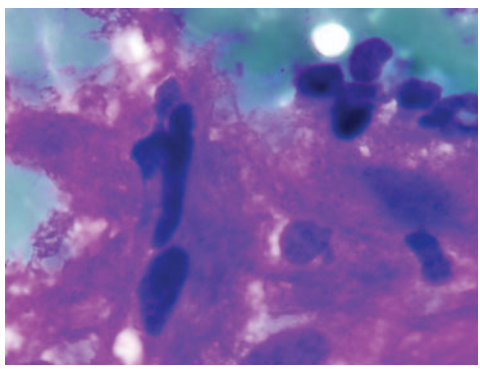

FIGURE 6.20 *CSF: Cellular evidence of a malignant neoplasm. Comment:* Atypical elongated cells are identified; this neoplasm proved to be an astrocytoma on resected specimen (Wright-Giemsa stain, 100×).

diagnosis. Likewise, when other malignancies are clinically suspected, the use of a variety of available ICC tumor markers is indicated.

- New flow cytometric assays to assign lineage to nonhematopoietic neoplasms using fluorescent dye-conjugated antibodies to several proteins have been developed. Potential implications of these assays include samples with limited cellular content such as CSF.

Primary CNS Neoplasms

In adult patients, primary CNS neoplasms are the least common malignancies diagnosed in CSF.

- Astrocytoma is the most frequent primary CNS neoplasm diagnosed in CSFs of adult patients (Figure 6.20). The neoplastic cells should be differentiated from those of more commonly found leptomeningeal carcinomatosis. Immunocytochemical stain for glial fibrillary acid protein (GFAP) may be helpful in this distinction.

Suggested Readings

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7

Laboratory Techniques

- Accurate cytologic interpretation of body-cavity fluids is dependent upon properly prepared cytologic slides.
- Many technical problems in the cytopreparation of body-cavity fluids arise in samples containing a large amount of blood, protein, or inflammatory cells.

> It is desirable to cytoprepare the body fluids in a fresh state that will allow reaching an optimal cytopreparation.

- In this chapter, the authors discuss the methodology used at their cytology laboratory.

Routine Cytopreparatory Techniques

Fixation and Slide Preparation

- All specimens should be submitted fresh, without added fixatives.
- If there is a delay in transportation of the fluid, the specimen must be refrigerated.
- With appropriate refrigeration (2–8 °C), cellular integrity is well preserved for at least 72 h and up to one week.
- In occasions, when transportation of the specimen to the cytology laboratory is delayed, an addition of Saccomanno's preserving solution (Lerner Laboratories, Pittsburgh, PA) with equal volume of the effusion sample provides a better cellular preservation.
- In addition when the effusion sample is mainly composed of blood, Saccomanno's fixative is used as an equal volume of fluid sample to accomplish lyses of the red blood cells.

Cytospin Preparation

- Record the following information upon receipt of the specimen:
  - Amount of fluid received (mL)
  - Gross appearance of the specimen (e.g., bloody, cloudy, clear, xanthochromic, chylous, mucinous, watery, etc.)
- Resuspend the cells gently prior to processing, as the cells may have settled to the bottom of the container.
- Place the specimen in 15 mL centrifuge tubes (two tubes for cytospin or six tubes for cell block preparations), and centrifuge at 2400 rpm for 1.5 min. *Note:* Specimens that are 2 mL or less can be put directly in the cytospin sample chambers.
- Prepare glass slides while the specimen is spinning.
- Two to four slides with the patient's name, access number, and specimen type are prepared. Extra slides may be made if special stains are requested.
- Slides are coated with albumin to promote better cellular adhesion.
- Assemble cytocentrifuge sample chambers and place it in the cytocentrifuge.
- The supernatant is decanted and the specimen is resuspended.
- The size of the cell button determines how much material is placed in the sample chamber. For small cell buttons, 3–6 drops can be used in each

sample chamber. If the cell button is large, usually only a drop or two is placed in each sample chamber.

- Spin the specimen at 1000 rpm for 1 min.
- After the centrifuge stops, remove the sample chamber as quickly as possible as one unit.
- Place the slides immediately in 95% isopropyl alcohol for fixation.
- Slides can be stained after 5 min of fixation using the Papanicolaou-staining technique.
- We routinely use the modified Papanicolaou-staining method (see table below).

Modified Papanicolaou Staining

Solution	Time (s)
80% Isopropyl alcohol	8
70% Isopropyl alcohol	8
50% Isopropyl alcohol	8
H_2O	8
Harris hematoxylin	35
H_2O	8
H_2O	8
H_2O	8
50% Isopropyl alcohol	8
NH_4OH	60
70% Isopropyl alcohol	9
80% Isopropyl alcohol	8
95% Isopropyl alcohol	8
OG-6	15
95% Isopropyl alcohol	8
95% Isopropyl alcohol	8
EA-65	75
95% Isopropyl alcohol	8
95% Isopropyl alcohol	8
95% Isopropyl alcohol	8
100% Isopropyl alcohol	8
100% Isopropyl alcohol	8
100% Isopropyl alcohol and xylene	8
Xylene	10

All solutions are changed weekly. Water and heavily discolored alcohols are changed daily. Alcohols (50% and 70%) and (NH_4OH) are filtered daily after each run.

Cell Block Preparation

- On selected cases, we prepare cell blocks when we anticipate the use of ICC.
- We do not encourage the use of cell blocks when specimens are paucicellular.
  - Centrifuge 20–60 mL of material for 2 min at 1500 rpm and obtain a cell button. Decant supernatant.
  - Add 95% isopropyl alcohol and resuspend the specimen.
  - Centrifuge for 10–20 min at 1500 rpm. Prepare a cell block cassette with the patient's name and case number.
  - Decant supernatant.
  - Remove the cell button from the centrifuge tube.
  - A second centrifugation with 95% alcohol may be needed if the cell button is not hard.
  - When the cell button is free, lift the bottom from the tube and place it on tissue paper. Fold the paper and place it in cassette.
  - Fix the cell block in formalin and send it to the histology laboratory for processing.

Immunocytochemistry Technique

- ICC is the most frequently used ancillary technique to aid in cytologic interpretation of serous effusions when cytomorphology alone is not sufficient for diagnosis.
- Immunohistochemistry is an important diagnostic tool in tumor pathology and traditionally used on histologic material and cytologic cell blocks.
- The technique applied to cytologic material for diagnostic purposes is less widely used due to:
  - Limited cytologic material
  - Problems in interpretation
  - Lack of specific markers to differentiate benign from malignant cells
- If the cell sample is adequate, one should prepare cell blocks as they represent the ideal specimens for ICC.
- All too often, however, the cell sample is limited to few smears or cytocentrifuge preparations. In fact, not uncommonly, the necessity for performance of ICC may only arise after reviewing the Papanicolaou-stained slides. In such cases, the slide coverslip is removed and the ICC is performed on the previously Papanicolaou-stained slides without destaining.

> ICC is simply performed on previously Papanicolaou-stained slides without the need for destaining. We have experienced similar sensitivity rates for immunocytochemical analysis on cytospin material including previously Papanicolaou-stained slides and those of the cell block material.

- Although removal of the glass coverslip from previously stained slides is relatively simple, some plastic and liquid-film coverslips are not easily removable and may interfere with the performance of ICC.
- Filter preparations are not suitable for ICC, since filters absorb immunologic reagents and chromogens, causing an unacceptable background staining.
- Cytocentrifugation of serous fluids with high protein content may cause precipitation of a protein film over cellular material and prevent adequate

penetration of reagents. In such cases, a brief washing of the cells by isotonic-saline solution before centrifugation will remove the excess protein.

Immunocytochemistry Is not Recommended	
Filter preparation	Air-dried cytospins
Specimens with excess blood and proteins	Diff-Quick-stained slides
Slides with plastic coverslip	Destained slides (cellular antigens may be removed)

- We have performed ICC on a liquid-based material with optimal results. However, in our laboratory, we use cytospin as the preparation method of choice for effusion samples.

Fixation and Slide Preparation

- Fixation using alcohol (95% isopropyl) is recommended.
- Other fixatives suitable for ICC analysis:
  - Buffered formalin
  - Formol-acetone
  - Mixture of ethanol and formalin
- A brief fixation in any of the above fixatives is quite adequate for most cytologic preparations.
- With the exception of a few cell-membrane antigens, air-dried specimens are not suitable for ICC. Furthermore, most air-dried samples are stained with Diff Quick or similar Romanowski methods. These stains interfere with subsequent ICC procedures. Attempts for destaining such preparations may in fact remove the antigenic content of the cells.
- Prolonged fixation (weeks/months) in *formalin* may result in antigenic loss.

Prolonged fixation in *alcohol-based* fixatives does not interfere with immunocytochemical analysis.

Alcohol-fixed, Pap-stained archival slides can be used for immunocytochemical analysis. No destaining is necessary.

- Cytospin slides are prepared using charged or silanated microscopic slides (Fisher Scientific, Pittsburgh, PA).

Immunoperoxidase Procedure for Cytospin and Archival Slides

Steps 5 through 9 are carried out in an automated instrument (Autostainer Plus, DakoCytomation).

- There is no need for modification of the immunohistochemical technique, using cytologic material. We use the same immunostaining methods for cytology specimens as used for histologic material.
- It is not unusual that in a cytologic sample, the target cells for ICC are too few and too far in between. To facilitate quick identification of those cells after immunostaining, circle them with ink over the coverslip, and then use a diamond pen to etch the inked area from the back of the slide.
- The following is the stepwise immunocytochemical procedure as performed at the authors' laboratory.

1. Remove the glass coverslips by heating the slides on a heating block for 2 s, and immediately immerse them in xylene (2 min).
2. Removal of the plastic coverslip is more time consuming, and can be done by immersing the slides for 2–3 days in xylene.
3. Rehydrate slides in decreasing ethanol grades (100%, 95%, 90%, and 85%).
4. Block endogenous peroxides by using a 6% solution of hydrogen peroxide in water (3 min, room temperature).
5. Place slides in target retrieval solution (S1699, DakoCytomation, Carpinteria, CA) and heat at 90 °C in a pressure cooker for 10 min.
6. Block endogenous biotin by the biotin-blocking reagent (X0590, DakoCytomation).
7. Incubate with the primary antibody, 22 min at room temperature.
8. Add the linking solution; biotinylated antimouse immunoglobulin; incubate for 22 min (K0690, DakoCytomation).
9. Add streptavidin-peroxidase conjugate and incubate for 22 min (K0690, DakoCytomation).
10. Place slides in diaminobenzidine (DAB) solution for 10 min (K33468, DakoCytomation).

11. Counterstain with Harris hematoxylin for 15 s.
12. For nuclear antigens, replace step 10, and apply 1% cupric sulfate (1 min, room temperature) to intensify the signal; counterstain with 0.2% fast green for 2 s.
13. Dehydrate in increasing grades of ethanol, clear in xylene, and mount.

- All washes and dilutions are made with tris-buffered saline (DakoCytomation, S1968).

> Vegetable steamers or pressure cookers induce uniform and gentle heat and is used as the heat-induced antigen retrieval method for ICC. Microwave methods may lead to inconsistent results.

- If the retrieval of an antigen requires predigestion by a protease, one should reduce the digestion time to 1/4th or 1/5th of what ordinarily is used for histologic sections.

Immunoperoxidase Procedure for Cell Blocks

- We use the same immunostaining methods for cell blocks as used for histologic material (see above).

Immunocytochemical Controls

- Because the sensitivity and specificity of cell-marker identification is similar in histologic and cytologic preparations, it is not necessary to use separate positive and negative cytology controls with every run of ICC.
- The most valuable controls in immunochemistry are internal controls. But unlike histologic sections that are usually composed of several cellular components, cytology samples seldom contain more than two or three cell types. This reduces the possibility of having an internal control in cytology and hence, negative ICC results in cytologic specimens are not as meaningful as positive reactions.

> Preparation and storage of various cytologic samples to be used exclusively for ICC controls is not practical and may even be impossible. We use the same controls as in histology when we perform ICC.

Selection of Markers

- A reasonable differential diagnosis usually is based on the cytomorphology characteristics, clinical information, and the probability of certain neoplasms occurring in specific age groups and in specific anatomic locations.
- Another important factor is the availability of markers for the entities within the differential diagnosis.
- The experience of the observer is an essential factor determining the clinical value of ICC. This factor has a major impact from when the moment markers are selected to when the results are evaluated and a final diagnosis is rendered.
- Since most diagnostic problems in effusion cytology can be narrowed down to two or three possibilities, the choice of antibodies can also be restricted to two or three.
- This "tailor-made" approach requires the pathologist's input and necessitates one's active participation by formulating a working diagnosis.
- The authors have used this nonalgorithmic, differential diagnosis-driven limited antibody approach in all cases discussed and illustrated in this book. This is a reflection of our constant preoccupation for maintaining practicality in ICC.

Evaluation of Results

- The hallmark of a true positive ICC reaction is the heterogeneous distribution of crisp granular staining within single cells or among a group of cells.
    - Only nuclear or intracytoplasmic reactions are acceptable as truly positive, because any cell that floats in effusion specimens may show a nonspecific surface membrane staining.
    - Depending on the antigen, the DAB reaction may be on the cell membrane, within the cytoplasm, perinuclear, or intranuclear.

False-positive Reactions

With rare exceptions, diffuse monotonous pale brown staining of cells is, in all likelihood, nonspecific.

- Similar to histopathology, common sources of false-positive reaction in cytologic material includes the nonspecific staining of crushed, degenerated, and necrotic cells.
- Histiocytes, macrophages, cells in mitosis, and tumor giant cells may also show a false-positive reaction.
- Large, three-dimensional cellular clusters in cytocentrifuge specimens may entrap immunologic reagents and lead to nonspecific positive results. In such cases, the evaluation of positive reaction should be limited to single cells or two-dimensional groups.

False-negative Results

- In the absence of internal controls, the true nature of a negative reaction is difficult to verify.

> Negative reactions in Immunocytochemistry are not as meaningful as positive reactions.

- When samples contain cells with delicate and fragile cell membranes, intracytoplasmic antigens may be released by the act of smearing and appear as nonspecific background staining. Therefore, background staining in cytologic material is usually not a major problem. In fact, most of what appears to be a nonspecific background staining in reality represents true staining.
- In effusion samples, the cells may show a nonspecific membrane staining for an antigen that is present in the fluid but is not elaborated by those cells; for example, nonspecific cytoplasmic membrane reaction of mesothelial cells for an immunoglobulin. Therefore, only nuclear or intracytoplasmic reactions are acceptable as truly positive results.

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8

Flow Cytometry, Immunoassays, and Molecular Techniques

Flow Cytometry

Basics of Flow Cytometry

- In laser flow cytometry (FCM), excitation from a laser source is used for rapid detection and quantitation of light scatter and fluorescent emission from individual cells in a suspension.
- FCM is a valuable tool for rapid quantitation of cellular components (such as DNA in nuclei stained with a fluorescent dye) or from cytoplasmic or cell surface markers labeled with a fluorescent antibody.
- Although laser FCM has become the main tool for phenotype analysis of hematological malignancies, its use for the study of cells in body-cavity fluids has been limited.
- Most of the earlier studies in body-cavity fluids have focused on flow cytometric analysis of DNA content for the detection of aneuploid malignant cells. Some of the earlier studies used DNA binding dye DAPI (4′-6-diamidino-2-phenylindole) for the generation of DNA distribution histograms using excitation from a high-pressure mercury light source. In more recent studies, excitation from a 488-argon laser has been used to analyze cells stained with propidium iodide.

DNA Aneuploidy

- Several earlier workers explored the possible utility of FCM for rapid detection of tumor cells with aneuploid DNA content.
- Flow cytometric studies in body-cavity fluid samples are controversial.
- Several studies conclude that by combining flow cytometric analysis of DNA aneuploidy and conventional diagnostic cytology, sensitivity, predictive value of a negative result, and specificity increased to more than 90%.

> Flow cytometric analysis of DNA aneuploidy and conventional diagnostic cytology increases sensitivity, specificity, and negative predictive value in the diagnosis of fluid samples.

- Some suggested that approximately one-quarter of the body-cavity samples containing cells with aneuploid DNA content (as detected by laser FCM) were cytologically benign; upon further review some indeed contained malignant cells.
- Flow cytometric detection of the aneuploid cells may significantly reduce the false-negative rate of effusion cytology for the diagnosis of malignancy.
- Flow cytometric analysis has been suggested to be a helpful technique to resolve cytologically equivocal results.
- One of the major problems in DNA FCM of body-cavity fluids has been the presence of an unacceptable number of false-positive results. As the resolution of a DNA histogram is measured as a coefficient of variation of the cells in the G0/G1 peak, in samples with broad CVs, it is often difficult to differentiate between normal cells with diploid and tumor cells with near-diploid DNA content. Thus, in studies, where the instrument resolution is not optimal and the CVs are large, markers such as cytokeratins or nuclear volume can be used to differentiate between the normal and the tumor cells.
- Although DNA FCM can provide quantitative information that complements the morphologic data from cytology, the optimal use of FCM requires that more than one parameter besides that of the DNA content alone should be used to refine and generate useful data.

> DNA FCM has been complemented by the examination of cell volume, protein content, or marker expression for the detection of tumor cells in body-cavity fluids.

DNA, Protein Content, and Nuclear Volume

- FCM can be used for the detection of tumor cells with near-diploid tumor cells that may have increased nuclear volume or protein content as compared to that of the normal cells.
- Staining of cells from body-cavity fluids with propidium iodide or DAPI for nuclear DNA content can be combined by staining with fluorescein isothiocyanate for the protein content.
- By comparing DNA content versus protein content, one can often distinguish between the normal cells with diploid DNA content and smaller protein content and the larger tumor cells with near-diploid DNA content but larger nuclear volume and protein content.
- Tumor cells with near-diploid DNA content but larger protein content and volume can be easily differentiated from the smaller normal cells (Figure 8.1).

DNA Content and Diagnostic Marker Expression

- Several well-known markers are used in diagnostic cytology to determine the possible site of origin of malignant cells in the body-cavity fluids. It should

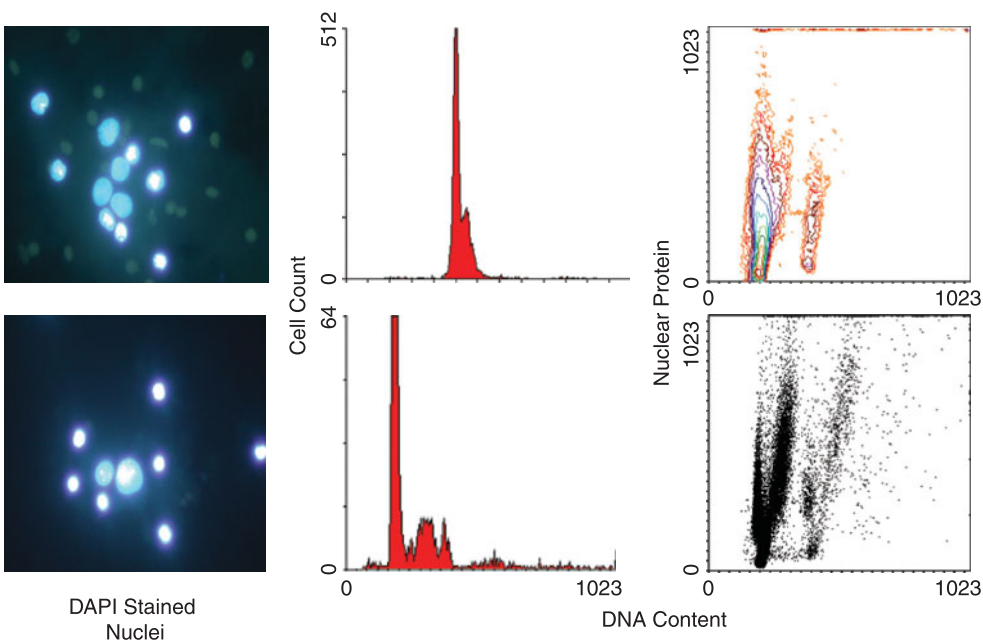

FIGURE 8.1 Pleural fluid: DNA versus nuclear volume and protein content of cells with aneuploid DNA content. *Comment:* This FCM analysis was performed on a cytologically negative specimen. Patient proved to have carcinoma cells in subsequent conventional cytologic analysis.

be noted that most of the studies on the use of immunocytochemical markers have relied on the classical immunocytochemical methods for the processing of sections or cells on a slide. There have hardly been any studies on the use of FCM for the evaluation of diagnostic markers in cells from body-cavity fluids.

- We have modified and refined the classical immunohistochemical procedures and developed protocols for rapid sample processing and marker analysis of body-cavity fluids by multiparametric FCM.
- We have used flow cytometric protocols which involve the incubation of cells with Ber-EP4-FITC (DAKO cat. # F0860) for 30 min at room temperature followed by fixation and staining with propidium iodide.
- Some of the well-known markers and their tissue-specific expression are described in the following sections.

Ber-EP4

- It is an epithelial antigen used for the detection of malignancy in cells from body-cavity fluids. The predictive value of positive Ber-EP4 staining in distinguishing adenocarcinomas from malignant mesotheliomas is more than 95%.

Using FCM, Ber-EP4 is one of the best markers to increase sensitivity and specificity for the detection of malignant cells in body-cavity fluids (Figure 8.2)

- In serous carcinomas of the ovary and when combined with estrogen receptor (ER), its sensitivity for the identification of carcinoma cells reaches 95%.

Cytokeratins

- Several workers have used cytokeratin subtypes to determine the site of origin (e.g., colon vs. other sites) in body-cavity fluids. The expression of cytokeratins can be readily monitored by laser FCM.
- CK7−/CK20+ immunophenotype is seen in majority of colonic adenocarcinomas but not in the lungs, ovary, stomach, or breast adenocarcinomas. All primary and almost 90% of metastatic colorectal adenocarcinomas are CK7−/CK20+.

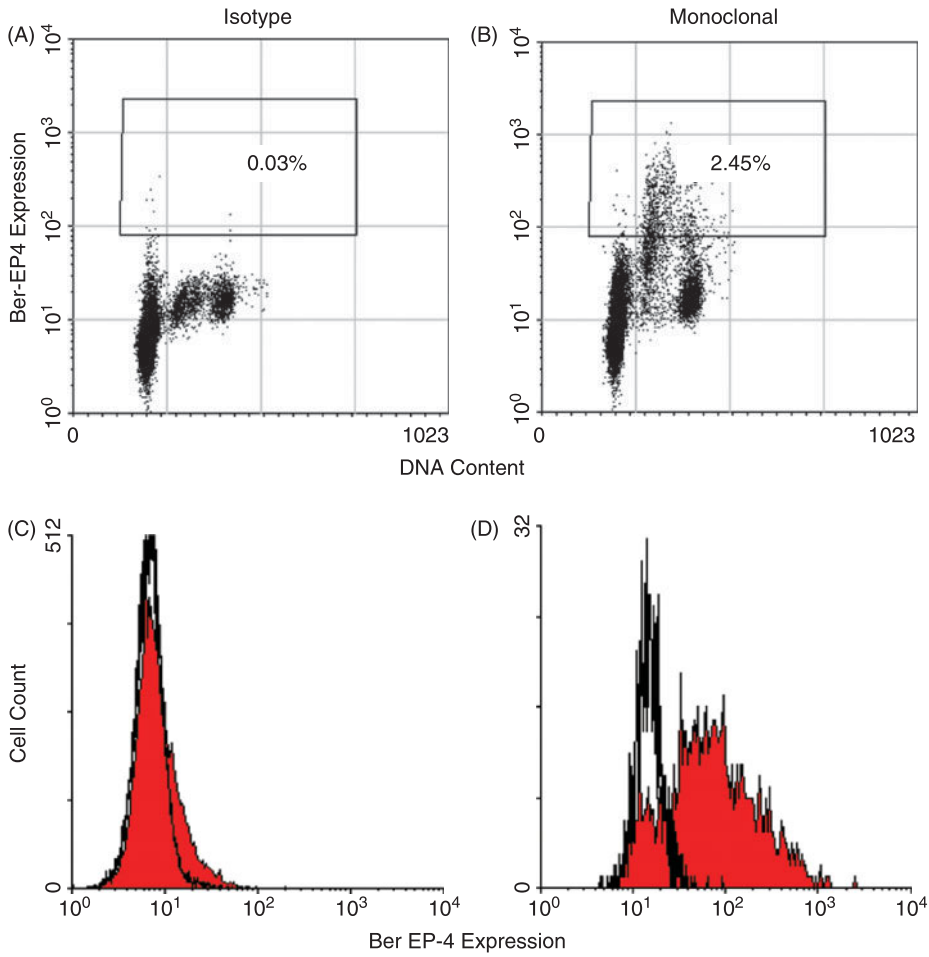

FIGURE 8.2 Peritoneal fluid: Cells with triploid DNA content and high Ber-EP4 expression. *Comment:* Expression of Ber-EP4 versus DNA content of the isotype (A, C) and the antibody treated sample (B, D). In contrast to the cells with the diploid DNA content, the aneuploid cells had high Ber-EP4 reactivity. This finding supported the cytomorphologic diagnosis of adenocarcinoma.

- The CK7+/CK20− immunophenotype is seen 100% in lung, 88% in breast, and 87% in ovarian cases. CK7+/CK20− pattern characterized a majority of primary and metastatic lung adenocarcinomas.

Estrogen Receptor/Progesterone Receptor

- Several studies have reported the expression of hormone receptors ER and progesterone receptor (PR) in tumor cells from metastatic sites such as the pleural cavity.

- Most of the studies have immunocytochemical techniques.
- Approximately three-quarters and half of mammary carcinomas are positive for ER and PR, respectively. Mullerian tumors are also frequently positive for ER and PR.

> The detection of ER and PR in metastatic adenocarcinoma from pleural or pericardial effusions can distinguish breast and Mullerian origin carcinomas from lung primary sites.

- ER and PR immunostaining can also be helpful in distinguishing between peritoneal mesotheliomas and serous carcinomas.

> None of the mesotheliomas expressed ER or PR or Ber-EP4 or TTF-1.

- Although methods for the detection of nuclear ER/PR expression by FCM have been well established, their use for the study of cells in body-cavity fluids has been limited.
- We have developed methods based on the analysis of both formalin-fixed/paraffin-embedded tumors and isolated nuclei for the detection of ER/PR receptors in cells from body-cavity fluids.

Thyroid Transcription Factor-1 (TTF-1)

- Thyroid transcription factor-1 (TTF-1) combined with CK7 and CK20 has been used for identifying the primary site of metastatic adenocarcinoma in serous effusions.
- All nonpulmonary adenocarcinomas lacked TTF-1 staining.
- Majority of lung adenocarcinomas have positive TTF-1 reactivity; in contrast, none of the metastatic adenocarcinomas of the ovary, stomach, colon, and breast are positive for TTF-1. Majority of lung squamous cell carcinomas are also negative for this marker.

> Combination of cytokeratins and TTF-1 correctly identifies the tumor site of origin in more than 80% of patients with malignant body-cavity fluids of unknown origin.

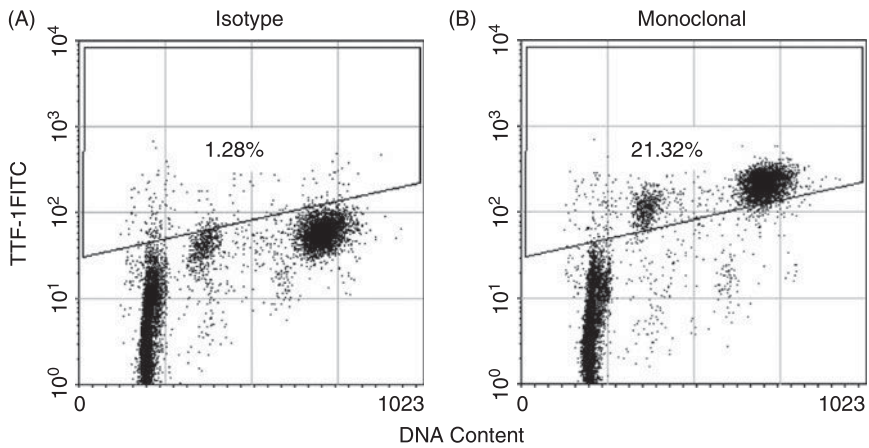

FIGURE 8.3 Pleural fluid: Expression of TTF-1 versus DNA content. The aneuploid cells have high TTF-1 expression. *Comment:* More than 20% of the cells expressed TTF-1. This FCM result confirmed the cytomorphologic impression of adenocarcinoma from lung origin.

- Some reports have suggested that although TTF-1 is generally considered to be a specific marker in lung and thyroid tumors, its occasional expression in other carcinomas should be kept in mind.
- As TTF-1 is a nuclear marker, its identification by laser FCM is relatively easy and one can use the propidium iodide/hypotonic citrate-staining method and incubation with an anti-TTF-1 antibody for monitoring DNA content versus TTF-1 expression (Figure 8.3).
- In some of the samples, TTF-1-RPE was used in combination with Ber-EP4-FITC for dual parametric analysis of marker expression in body-cavity fluids.

Preparation Method for FCM Evaluation of TTF-1

The cells were fixed for 10 min in 70% ethanol.

The cells were permeabilized with 0.1% Triton X100 in PBS for 30 min at room temperature.

The cells were then incubated with TTF-1 (DAKO, Inc., Carpinteria, CA, Clone AG7G3/1, cat. # M 3575) antibody overnight at 4 °C.

The cells were washed once and labeled with GAM-FITC (Sigma, St. Louis, MO, and catalog. # F9006) or GAM-RPE (Sigma cat. # P9670) for 2 h.

The cells were then washed twice and resuspended in PBS for analysis.

p63 Expression

- TTF-1 and p63 has been used in combination for distinguishing between small-cell lung carcinoma and poorly differentiated pulmonary squamous cell carcinoma.
- Since most of lung small-cell carcinomas express TTF-1 and do not express p63, this combination has been useful for distinguishing between the two histological types of lung tumors.
- Due to the availability of anti-p63 antibodies, it is relatively easy to stain cells from body-cavity fluids for the presence of these two markers for flow cytometric differentiation of small-cell versus squamous cell carcinomas.

Stem Cell Markers

- The expression of specific markers which identify stem cells has been studied in body-cavity fluids.
- In our laboratory, we have studied the expression of ALDH1, CD44, CD24, and CD133 in cells from body-cavity fluids.
- *ALDH1*: Aldehyde dehydrogenase 1 is a stem cell marker. It has been identified as a non-small cell lung cancer (Figure 8.4) and mammary carcinoma stem cell marker and it correlates with prognosis. Its expression is increased in cells with CD44+/CD24– and CD133+ phenotype.

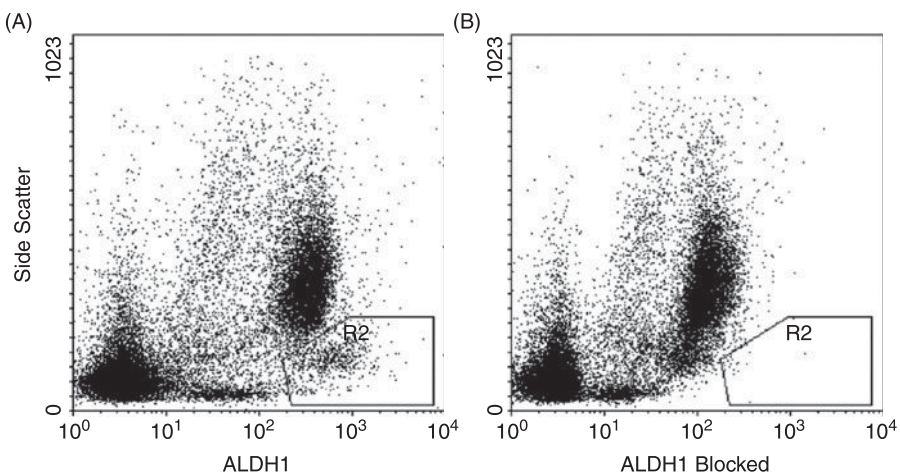

FIGURE 8.4 Pleural fluid: A comparison of dot plot A versus B shows the presence of ALDH1 positive cells in region R2. *Comment*: this stem cell marker has been identified in non-small cell lung cancer and mammary carcinomas with prognostic value.

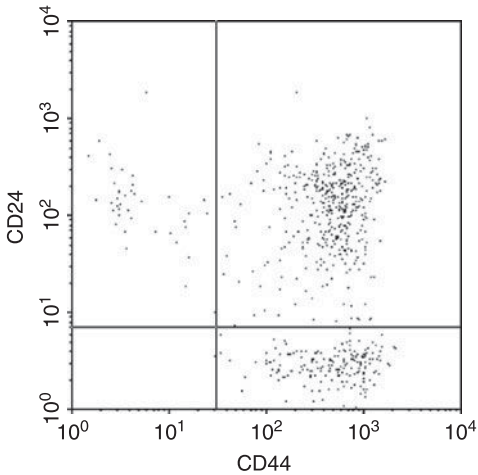

FIGURE 8.5 Pleural fluid: Expression of CD44 versus CD24. *Comment*: CD44+/CD24− cells are believed to be the tumor stem cells.

- *CD44+/CD24−*: CD44+ and CD24− are cell surface glycoproteins. CD44− is also a stem cell marker. Cells with CD44+/CD24− expression are able to form tumors in thymic mice. They can be successfully detected by FCM (Figure 8.5).
- In a recent study, we reported that although the frequency of $ALDH^{bright}$/CD44+/CD24− cells was threefold higher in malignant samples, cells with this phenotype are also seen in a small group of benign samples. Their presence indicates either the false-negative result or that this phenotype is also expressed by some nonmalignant cells.
- In one of our recent studies, Ber-$EP4^{pos}$ cells from body-cavity effusions, 9/20 benign and 6/9 malignant samples had CD44+/CD24− cells.

Immunoassay and Molecular Techniques

Introduction

- In the majority of patients with malignant disease, the occurrence of effusions in the pleural, pericardial, or peritoneal cavity indicates metastatic disease. Thus, sensitive and reliable detection of tumor cells in effusions is desirable.
- The detection of malignancy in effusions by conventional cytology is hampered by the problem of differentiating malignant cells from reactive cells. Thus, new methods complementing cytology for the diagnostic work-up of effusions need to be evaluated.
- Exfoliated sampling methods as well as fluid samples represent an ideal way to screen for malignancy.
- Epigenic analysis of pleural fluid can detect malignant DNA from a variety of neoplasms, providing complementary information to conventional cytology of effusions.
- Several immunoassay and molecular techniques have been investigated in order to improve sensitivity of diagnostic cytology in effusion samples:
  - Enzyme immunoassay,
  - Electrochemiluminescence immunoassay,
  - Microparticle immunoassay,
  - Interphase cytogenetics by fluorescence *in situ* hybridization (FISH) to detect aneuploidy,
  - Reverse-transcriptase polymerase chain reaction (RT-PCR).
- These techniques proved to be a sensitive and specific diagnostic approach to identify malignancy in effusions in combination with conventional cytology. They can test for biomarkers with high sensitivity and detect occult tumor providing important prognostic and predictive information.

Applications in Body-cavity Fluid Cytology

- Several markers are been studied in order to increase the sensitivity of conventional cytology in the diagnosis of effusions. Still, none has a role in daily pathology practice.

- Some of these markers are:
  - CEA, CA 15-3, CYFRA 21-1, and CA 125 levels may predict survival in patients with adenocarcinoma or squamous cell carcinoma involving pleural surfaces.
  - Heat-shock (HSP70) expression in effusions may be a prognostic marker of poor survival.
  - Human mammaglobin gene expression is a highly sensitive assay to detect occult tumor cells in effusions from patients with mammary and gynecologic malignancies.
  - CD44v8-10 mRNA expression could be an important adjunct to cytologic examination in cancer diagnosis, especially in detecting exfoliated cancer cells in pleural effusions.
  - The expression of mucin genes—MUC1 and MUC5AC, are significantly higher in malignant than in benign pleural fluids. In combined evaluation with both MUC1 and MUC5AC, the sensitivity for the diagnosis of malignant effusion was 86.1% and specificity was 91.5%.
  - In lung and breast cancer patients, high rates of sensitivity and specificity values were found for several metalloproteinases.
  - Micro RNA expression patterns can be used to differentiate malignant pleural mesothelioma from lung adenocarcinoma.
  - MN/CA9 is a new molecular marker for the detection of cancer cells in pleural effusions. The sensitivity and specificity of MN/CA9 gene expression has been reported to be 89.8% and 91.7%, respectively.
  - Microsatellite analyses of pleural-fluid supernatants may raise the sensitivity of cytologic diagnosis of malignancy.
  - Combination of CEA, mammaglobin, and Ep-CAM significantly improves the detection sensitivity of tumor cells in serous effusions.
  - Claudin-4 gene expression can improve the sensitivity of detection of malignant pleural and peritoneal effusions.
  - Telomerase activity can also be a good adjunct method in the diagnosis of malignant pleural effusions.
  - Folate receptor alpha (FOLR1) and gamma (FOLR3) levels differentiate ovarian carcinoma from breast carcinoma and malignant mesothelioma in serous effusions.
  - K-ras point mutations may be detected in malignant effusions which were overlooked by conventional cytologic methods.

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