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Chapter

Cytological Diagnosis of Infectious Diseases: Identification of Pathogens and Recognition of Cellular Reactions

Yutaka Tsutsumi

Abstract

Cytological diagnosis of infectious diseases is as important as the cytodiagnosis of malignancies, because the detection of pathogens in cytological specimens is crucially valuable for prompt and appropriate patients’ treatment. When compared with histological diagnosis, cytology is strong at detecting microbes under Papanicolaou and Giemsa stains. Host response against the infectious agent can be estimated by the type of background inflammatory cells. Patterns of the inflammatory cellular responses against extracellular and intracellular pathogens should be recognized. Immunocytochemical and molecular approaches can be applied, even when we have only one cytology specimen in hand. The cell transfer technique is useful to create plural material from one glass slide for immunocytochemistry and other techniques. In case of transmissible disorders including sexually transmitted diseases, the prompt and appropriate diagnosis will avoid avoidable transmission of infectious agents among people, and eventually contribute to the safety of the human society.

Keywords: cell transfer technique, cytodiagnosis, defense mechanism, host response, immunocytochemistry, infectious diseases, inflammatory cells, pathogens

1. Introduction

In the daily practice of cytological diagnosis, cytopathologists tend to focus on the diagnosis of premalignant and malignant diseases. Generally speaking, the cytology practice functions as screening for malignancy. However, the cytodiagnosis of infectious diseases and the identification of pathogens in cytological preparations must not be undervalued. The correct cytodiagnosis of infectious diseases leads patients to prompt and appropriate treatment. Histopathological diagnosis is strong at recognizing host responses against pathogens, while pathogens are more easily identified in the cytology specimen than the histology specimen. When infectious diseases are clinically suspected, it is better for us to perform Giemsa staining in addition to routine Papanicolaou staining.

In the present review article, the author presents varied aspects of cytomorphology of infectious diseases, in addition to general remarks for the defense mechanisms against infectious microorganisms. Immunocytochemistry
significantly contributes to the definite and final cytodiagnosis. Often times, only one cytology specimen is available in the daily practice, so that the special techniques “how we can detect pathogens in only one cytology preparation” are needed for evaluating with additional staining. Please refer to the previous articles, textbooks and web sites of the author, describing the cytological diagnosis of infectious diseases [1–7]. It is most regrettable that some of them were written in Japanese.

2. Defense mechanisms against infection

Defence mechanisms against infection are categorized into two types: nonspecific and specific. Both types cooperatively function as an effective anti-infection system. Varied inflammatory cells are involved in the processes [8–10].

2.1 Types of inflammatory cells

Types of inflammatory cells and their properties are briefly summarized in Table 1. Function of the cells and their proliferative and migratory activity are shown.

Representative light microscopic and electron microscopic features of the inflammatory cells are illustrated in Figures 1 and 2. Of note is that cytokines mediate intercellular communication with which the immune cells talk to each other [11]. Cytokines include interferons, interleukins, chemokines, lymphokines and tumor necrosis factors.

2.2 Nonspecific defense mechanisms against infection

2.2.1 Physical barriers

The epidermis of the skin and the surface mucosal layer on the mucosal membrane play an effective physical barrier against invasion of the pathogen. The cilia on the pseudostratified mucosa of the airway effectively excrete the pathogen.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Function</th>
<th>Proliferative activity</th>
<th>Migratory potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocyte</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophil</td>
<td>Phagocytosis</td>
<td>None</td>
<td>Migratory</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>Allergy, anti-helminth function</td>
<td>None</td>
<td>Migratory</td>
</tr>
<tr>
<td>Basophil</td>
<td>Histamine production</td>
<td>None</td>
<td>Migratory</td>
</tr>
<tr>
<td>Mast cell</td>
<td>Histamine production</td>
<td>Proliferative</td>
<td>Migratory</td>
</tr>
<tr>
<td>Monocyte</td>
<td>Phagocytosis</td>
<td>Proliferative</td>
<td>Migratory</td>
</tr>
<tr>
<td>Macrophage</td>
<td>Phagocytosis, granuloma reaction</td>
<td>Proliferative</td>
<td>Migratory</td>
</tr>
<tr>
<td>B-lymphocyte</td>
<td>Humoral immunity</td>
<td>Proliferative</td>
<td>Migratory</td>
</tr>
<tr>
<td>T-lymphocyte</td>
<td>Cellular immunity/helper activity</td>
<td>Proliferative</td>
<td>Migratory</td>
</tr>
<tr>
<td>NK cell</td>
<td>Innate immunity</td>
<td>Proliferative</td>
<td>Migratory</td>
</tr>
<tr>
<td>Plasma cell</td>
<td>Antibody production</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Dendritic cell</td>
<td>Antigen presentation</td>
<td>Proliferative</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 1. Inflammatory cells and their properties.
2.2.2 Antibacterial secretory proteins

The secretory juice secreted from secretory glands contains varied antibacterial proteins such as lactoferrin, lysozyme (muramidase) and defensins [12].

**Figure 1.** Types of inflammatory cells (may-Giemsa). a: Three kinds of granulocytes (from left to right: Basophil, neutrophil and eosinophil) seen in the bone marrow smear. b: Small lymphocyte. c: Plasma cell. d: Hemophagocytic (activated) macrophage. Compare the cytoplasmic granules in the granulocytes. The cytoplasm of the small lymphocyte is scanty, and the plasma cell contains basophilic cytoplasm with a prominent Golgi area. The macrophage actively phagocytizes red cells and platelets.

**Figure 2.** Electron microscopic appearance of inflammatory cells. a: Neutrophil (left) and lymphocyte (right), b: Eosinophil, c: Basophil, d: Two plasma cells in the small bowel mucosa, e: Activated macrophage in soft tissue. The lymphocyte has an indented nucleus, while the granulocytes possess segmented nuclei. The cytoplasmic granules feature the respective granulocytes: Small-sized granules in the neutrophil, large crystalline granules in the eosinophil, and large rounded granules often with a fingerprint image in the basophil. The plasma cells contain a round nucleus with peripherally condensed heterochromatin and the cytoplasm rich in rough endoplasmic reticulum. The large-sized macrophage possesses an ameboid cytoplasmic process and numbers of electron-dense lysosomal granules. Bars indicate 1 μm.

**2.2.2 Antibacterial secretory proteins**

The secretory juice secreted from secretory glands contains varied antibacterial proteins such as lactoferrin, lysozyme (muramidase) and defensins [12].
Lactoferrin shows a bacteriostatic function by combining and competing trivalent ferric ions mandatory for the growth of bacteria and fungi. Lactoferrin is secreted from the lactating breast, serous salivary glands, lacrimal glands, eccrine sweat glands, gastric glands and prostatic glands. Of particular note is that protease digestion of lactoferrin yields lactoferricin and lactoferrampin, potent antimicrobial peptides derived from the lactoferrin molecule [13]. Lysozyme operates as a bactericidal or bacteriolytic molecule by cutting the joint sequence of N-acetylglucosamine and N-acetylmuramic acid in the peptide glycan network on the cell wall of Gram-positive bacteria [14]. Defensins belong to bactericidal proteins strongly binding to phospholipids [12]. Numbers of serous secretory glands secrete both lysozyme and defensins, together with lactoferrin. In the small bowel mucosa, lysozyme and defensins are actively secreted from Paneth cells (Figure 3). Representative microscopic features of production of lactoferrin and lysozyme in varied secretory epithelial cells are displayed in Figure 4.

2.2.3 Phagocytes and natural killer (NK) cells

Bacteria are nonspecifically phagocytized by phagocytes such as neutrophils and macrophages [15]. Figure 5 exhibits bacteria phagocytized by neutrophils. Because of the lack of proliferative activity, neutrophils are predominantly seen in acute inflammation. Macrophages are proliferative, so that they mainly appear in chronic inflammation. Myeloperoxidase, lysozyme and defensins show bactericidal activities in the phagocytic vacuole (primary granule) of the neutrophil [16]. In the secondary (specific) granule of neutrophils, lactoferrin is contained. The main bactericidal enzyme functioning in the macrophage is lysozyme (see Figure 3b). NK cells correspond to CD56-positive large granular lymphocytes [17]. The cytoplasmic granules of the NK cell contain bactericidal, antiviral and apoptosis-inducing proteins common with the CD8-positive killer (cytotoxic) T-lymphocyte, such as perforin, granzymes (A and B) and T-cell intracellular antigen-1 (TIA-1).
These cells play significant roles in the host defense against pathogens for the initial two weeks after infection, until the establishment of the “specific” (humoral and/or cellular) immune reaction.

It should be noted that neutrophils form neutrophil extracellular traps (NETs), a filamentous spiderweb-like network entrapping bacteria, after cell death called NETosis [18]. NETs are composed of DNA stretches and anti-bacterial proteins, including lactoferrin and myeloperoxidase [19]. NETs are richly formed in the abscess lesion, as shown in Figure 6.
2.2.4 Innate immunity and Toll-like receptors

Acute viral infection usually calms down in one week. The strong anti-viral mediators are type I interferons (IFN-alpha and IFN-beta). The IFNs are produced by the keratinocyte of the epidermis and squamous mucosa, the columnar cells of the intestinal and airway mucosa and Langerhans (dendritic) cells distributed among the epithelial cells [20, 21]. Toll-like receptors (TLRs) expressed on these cells specifically recognize microbe-derived components such as lipoproteins, lipopolysaccharide, viral double-stranded RNA, non-methylated CpG islands of DNA and flagellin to induce IFN secretion. Toll means great and curious in German. In the human being, there are 10 kinds of TLRs. The TLR-mediated innate immunity, as well as phagocytosis by neutrophils and macrophages and the NK cell-mediated defense, comprise major functions of the vertebrate intrinsic system for the exclusion of the pathogen.

2.3 Specific defense mechanisms against infection

The specific acquired immunity consists of humoral immunity and cell-mediated (cellular) immunity [8, 9]. Production of specific antibodies by B-lymphocytes is the key mechanism of the humoral immunity. Serum complements secreted from the liver activate neutralizing activity of specific antibodies. The key players of the cell-mediated immunity are cytotoxic (killer) T-lymphocytes and activated macrophages. It takes a certain period (usually two weeks to one month) until establishing the specific acquired immunity.

The specific defense mechanisms against infection should be divided into two categories: the systemic immunity versus local (mucosal) immunity. The pathogen invading the inside of the body are specifically protected by IgG-mediated humoral immunity and also by CD8-positive cytotoxic T-lymphocyte-mediated cellular immunity.

The mucosal immunity provides a defense mechanism protecting invasion of the pathogen across the mucosa [22]. Dimeric secretory IgA (slgA) functions as a mediator of the mucosal humoral immunity, but it hardly shows a neutralizing (killing) activity. The lamina propria mucosae contains numbers of IgA-producing plasma cells. slgA is secreted onto the mucosal surface after coupling with “secretory component (SC)” produced by the columnar epithelial cells. Figure 7 schematically displays the process of formation of slgA. Microscopic features of IgA secretion in
intestinal metaplasia of the stomach are demonstrated in Figure 8. sIgA is uniquely resistant to protease digestion. It is of note that IgG can also be secreted onto the mucosal surface after coupling with IgG Fc-binding protein, a unique IgG Fc receptor of secretory type, produced by mucin-secreting cells in the mucosa [23, 24]. Extrathymic T-lymphocytes are distributed among the mucosal columnar cells as “intraepithelial lymphocytes” predominantly expressing CD8 and T-cell receptor gamma/delta on the cell surface [25] (Figure 9). Intraepithelial lymphocytes significantly increase in certain mucosal infections such as *Giardia lamblia* infection (giardiasis). The extrathymic T-lymphocytes locally recruit in the “crypt patch” located in the lamina propria mucosae. Because of the lack of education in the thymus, the extrathymic T-lymphocytes, self-reactive to provoke apoptosis of the

Figure 7.
Schematic presentation of the process of formation of secretory IgA (sIgA). IgA is secreted onto the mucosa after binding with secretory component (SC), a product of mucosa-lining columnar epithelial cells. Dimeric IgA consists of two molecules of monomeric IgA and J-chain. IgA-producing plasma cells are richly distributed in the normal bowel mucosa.

Intestinal metaplasia of the gastric mucosa showing secretory IgA transportation. a: H&E, b: IgA, c: Secretory component (SC). Goblet cells and absorptive-type cells with brush borders are observed in the metaplastic gland. The columnar cells of the intestinal type produce SC, which traps dimeric IgA secreted from IgA plasma cells (arrowheads) in the lamina propria mucosae. The apical cytoplasm of the columnar cells is immunoreactive for IgA, representing the intracellular transportation of sIgA.

Figure 8.
columnar epithelial cells, may control the number and function of indigenous bacterial flora living in the lumen.

The mucosa-associated lymphoid tissue (MALT) is distributed in the intestinal and airway mucosa. The largest MALT is called as Peyer’s patch in the ileal mucosa (Figure 10). The B-lymphocyte-rich lymphoid follicles with activated germinal centers are covered with dome-shaped columnar epithelial cells without villous structures. In contrast to the other part of the gut mucosa, B-lymphocytes and microfold (M) cells are distributed among the dome columnar cells. The M cells are special epithelial cells suited for efficient endocytosis and transcytosis, and function as gateways to the mucosal immune system [26]. The MALT is known to play a central role in the mucosal homing of B-lymphocytes destined to secrete IgA.
2.4 Three major patterns of host responses against infection

From the pathological point of view, there are three major mechanisms of host responses against infection, depending on the type of pathogens and the mode of infection (either extracellular or intracellular infection).

1. Neutrophilic reaction against extracellular pathogens

2. Cellular immune reaction against intracellular pathogens

3. Humoral immunity via neutralizing antibody reaction

Table 2 summarizes the features of the defense mechanisms and host responses against pathogens. Patterns of the host response against pathogens are listed up in Table 3.

2.4.1 Neutrophilic reaction against extracellular pathogens

The extracellular pathogens growing outside the host cell, such as suppurative bacteria (*Staphylococcus*, *Streptococcus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Actinomyces*, etc.) and hypha-forming fungi (*Candida*, *Aspergillus* and *Mucorales*),
are principally phagocytized by neutrophils [27]. Infection of the extracellular pathogen thus results in abscess and phlegmonous inflammation, solely composed of neutrophils. In case of infection by non-invasive (extracellular) protozoa like *Trichomonas vaginalis*, neutrophilic exudation is activated to characteristically form so-called “cannon balls” (clusters of neutrophils). When the specific antibodies against the pathogen and complements are present in the body fluid, the phagocytic activity of neutrophils is significantly enhanced through an opsonin effect. Capsule-forming microbes frequently escape phagocytosis by neutrophils. In case of infection by anaerobic bacteria, massive ischemic necrosis is commonly associated.

Representative cytological features of neutrophil-mediated inflammation are illustrated in Figure 11. Accumulated neutrophils grossly correspond to pus (pyogenic exudates).

2.4.2 Cellular immune reaction against intracellular pathogens

Neutrophils and antibodies are ineffective against intracellular (cytozoic) pathogens. Instead, cell-mediated immunity functions as the major defense mechanism [28]: The infected host cells themselves are eliminated. The intracellular pathogen represents viruses, chlamydia, rickettsia, protozoa, yeast-form fungi (*Cryptococcus, Histoplasma, Coccidioides*, etc.) and certain types of bacteria such as *Mycobacterium, Legionella* and *Salmonella*. Microscopically, lymphocytic infiltration (CD8-positive T-cell reaction) or granulomatous reaction is seen.

When macrophages are activated by the T-lymphocytes, epithelioid granulomas are formed. The epithelioid cells derive from activated macrophages. Multinucleated giant cells of Langhans type are often formed by plasma membrane fusion of the macrophages. Typical examples include tuberculosis, cryptococcosis and stage III syphilis. Necrosis is often associated with the granulomatous reaction, and in case of tuberculosis, caseous necrosis is characteristic. Typically, epithelioid granuloma with central necrosis is surrounded by small lymphocytes. Examples of cytology appearance of the granulomatous reaction in tuberculosis are shown in Figure 12.

![Figure 11. Neutrophilic response against extracellular pathogens.](image)

Neutrophilic response against extracellular pathogens. a: MRSA-induced microabscess in the heart muscle in septicemia (H&E), b: Enterococcus faecium-induced acute cystitis (urine sediment, Papanicolaou), c: Streptococcus milleri-induced pyothorax (Gram), d: Actinomyces israelii-induced endometritis (Papanicolaou), e: Hypha-forming Candida albicans-induced vaginitis (Papanicolaou), f: *Trichomonas vaginalis*-induced vaginitis (Papanicolaou). Extracellular pathogens, including *T. vaginalis* (arrowheads), are attacked by neutrophils. Neutrophils can fight against microbes larger than themselves (e and f).
In contrast, viral and rickettsial infection provokes infiltration of small T-lymphocytes without granulomatous reaction (Figure 13). In case of viral meningitis, lymphocytes are the major component in the cerebrospinal fluid. When lymphocytes are predominantly seen in the urine, the possibility of follicular cystitis caused by persistent infection of beta-hemolytic streptococci should be considered [29]. Similarly, the cervical smear preparation may contain lymphocytic reactions to be diagnosed as follicular cervicitis, and the possibility of Chlamydia trachomatis infection should be considered [30].
In case of *Legionella* pneumonia, macrophages comprise the main cellular reagent scarcely with lymphocytic infiltration. In cutaneous and visceral leishmaniasis, activated macrophages actively phagocytize the protozoan bodies. In visceral leishmaniasis (kala azar), the microbes are seen in activated Kupffer cells. In stages I and II syphilis, dense infiltration of plasma cells is characteristic. The gastrointestinal mucosa with chronic active gastritis and inflammatory bowel disease, as well as the nasal mucosa with chronic rhinitis and the gingival tissue with periodontitis or radicular dentigerous cyst, are also densely distributed by plasma cells. Plasma cells are often clustered in inflammatory foci of subacute inflammation or a subacute phase of inflammation [31]. Cytological features are represented in Figure 14. The cellular immunity-mediated removal of the infected parenchymal cells may cause functional insufficiency of the organs and tissues: Examples include hepatitis and encephalitis.

### 2.4.3 Humoral immunity via neutralizing antibody reaction

Neutralizing antibodies in the serum effectively eliminate pathogens that are distributed extracellularly. Typically, the neutralizing antibodies are produced against bacterial exotoxins, bacterial capsules and viral virions. Anti-viral antibodies are effective against the viral particles in the blood (during viremia) and body fluid. These features are applied to vaccination practice, and permanent immunity can be expected [32]. Vaccines injected subcutaneously induce IgG-type neutralizing antibodies in the serum. Oral vaccines such as Sabin vaccine against poliovirus and Rotavirus vaccine induce secretory IgA in the gut lumen. It is of note that IgG-type neutralizing antibodies in the serum can be transported with mucin by the IgG Fc-binding protein secreting from mucin-producing cells [23, 24]. Individuals with inherited complement deficiency, particularly the deficiency of C3 (the major opsonin), are vulnerable to recurrent pyogenic infections especially with...

![Figure 14. Macrophage activation and plasma cell infiltration.](image-url)
encapsulated bacteria, including *Streptococcus pneumoniae* and *Neisseria meningitidis*. It is of note that sporadic meningococcal meningitis in adults may accompany inherited complement deficiency [33].

The activated humoral immunity is microscopically represented by follicular hyperplasia with enlarged germinal center formation in the lymph node and a variety of organs and tissues [34]. In fact, follicular hyperplasia is a microscopic feature of autoimmune disorders [35]. It has been clarified that a variety of cytokines are secreted from immunocytes to communicate each other and to secrete immunoglobulins. In particular, tumor necrosis factor (TNF) receptor-1 signaling is required for the differentiation of follicular dendritic cells, germinal center formation and antibody responses [36]. The germinal center also contains stimulated lymphocytes secreting interleukin-2 (IL-2) and interferon-gamma (IFN-γ) [37]. Representative examples of activated humoral immunity are demonstrated in Figure 15.

### 2.5 Host reactions against pathogens of other types

Host reactions against pathogens of other types are commented below.

1. **Suppurative granuloma**: An intermediate form of abscess and granuloma, that is called "suppurative granuloma" (abscess surrounded by granuloma), is seen in cat scratch disease (Bartonella infection), tularemia, listeriosis, yersiniosis, melioidosis and cutaneous mycosis (sporotrichosis and chromomycosis) [38]. Microscopic features of cat scratch disease involving the spleen are demonstrated in Figure 16.

2. **Xanthogranuloma and malakoplakia**: In certain situations, low-virulent extracellular pathogens, particularly *E. coli*, may grow intracellularly in the cytoplasm of macrophages, resulting in formation of xanthogranuloma (yellow-colored granuloma) [39]. Malakoplakia, a special form of the
xanthogranuloma, is microscopically featured by Michaelis-Gutmann bodies (round-shaped calcified and basophilic cytoplasmic inclusions). These lesions may be seen in the kidney, urinary bladder, epididymis, colon and gallbladder. Examples of malakoplakia and xanthogranulomatous inflammation are illustrated in Figure 17.

Figure 16.
Suppurative granuloma seen in cat scratch disease (Bartonella henselae infection). Left: H&E (spleen), right: Stamp preparation (Papanicolaou). The surgically resected spleen grossly contains plural splenic abscesses. Histologically, suppurative granulomas, consisting of central abscess and surrounding epithelioid granuloma, are noted. Stamp cytological preparation demonstrates a cluster of epithelioid cells (arrowhead) in mildly necrotic background admixed with neutrophils and lymphocytes.

Figure 17.
Malakoplakia of the rectal mucosa (a, b) and xanthogranulomatous epididymitis (c, d). a and c: H&E, b and d: Immunostaining for E. coli antigen (pre-embedding immunoelectron microscopy using a paraffin section in d). Malakoplakia is microscopically featured by Michaelis-Gutmann bodies, rounded basophilic cytoplasmic inclusions immunoreactive for E. coli antigens (arrowheads). Xanthogranuloma consists of accumulated foamy macrophages. Both lesions are caused by E. coli infection under an immunocompromised condition. Rod-shaped bacteria with cell wall labeling are proven in the cytoplasm of the foamy macrophage (d). Bar = 1 μm.
3. Eosinophilic infiltration: Infestation of parasites, particularly round worm (nematode) and fluke (trematode), provokes infiltration of eosinophils and IgE-type immune response, common with the type 1 allergic reaction [40] (Figure 18). Cestode (tapeworm) infestation usually lacks the eosinophilic reaction. In case of allergic lung reaction against Aspergillus (allergic bronchopulmonary aspergillosis), eosinophilic granuloma (granuloma with eosinophilic infiltration) is observed [41]. Occasionally, foreign body reactions against worm bodies and ova are observed. Formation of multinucleated foreign body giant cells is characteristic (Figure 19). Parasitic ova induce foreign body granulomas to form so-called “worm egg tubercles”. When eosinophilic infiltration is associated, immune-mediated eosinophilic granulomas are formed [42]. In case of anisakiasis, foreign body reactions without eosinophilic infiltration are seen against the Anisakis larva if the infestation occurs for the first time in a non-immunized patient after eating raw sea fish [43]. The mechanisms may be similar to the nonspecific phagocytic action of macrophages against genuine foreign bodies such as asbestos bodies and injected paraffin by augmentation mammoplasty.

4. Acellular hemorrhagic necrosis: In opportunistic infection associated with neutropenia and cellular immunodeficiency, the inflammatory cellular reactions are poorly provoked, resulting in hemorrhagic necrosis of the tissue [44].

3. Use of immunocytochemistry for cytology specimens

You must not give up additional staining even when you have one and only cytology specimen in hand. The resources for applying immunocytochemistry to the one and only cytology specimen are presented below. In case of liquid-based cytology (LBC), additional plural specimens are easily available. In Japan, however, the LBC procedure is still underdeveloped because of the low cost-performance. Therefore, these techniques are practically valid and helpful [45].

3.1 Cell block preparation

The sediments of liquid specimens such as pleural and pericardial effusions, ascites, the content of cystic lesions and urine can be kept for a long period of time as cell blocks after formalin fixation and paraffin embedding [46]. Immunostaining and in situ hybridization (ISH) method can be performed by preparing multiple paraffin sections from the cell block. So far, several technical inventions have been reported how to prepare cell blocks [47].

In Figure 20, chronic active Epstein–Barr virus (EBV) infection seen in a male case aged at his 20’s is presented [48]. The patient manifested collagen disease-like signs and symptoms, such as fever, skin rash, muscle weakness, liver dysfunction and eosinophilia. In the ascites cytology specimen, a number of large granular lymphocytes (a form of atypical lymphocytes) were detected in the background with red cells, eosinophils and hemophagocytic macrophages. A cell block was prepared to know the nature of the lymphoid cells. The large-sized lymphocytes expressed natural killer cell markers such as CD45 and CD56, and EBV-encoded small nuclear RNA (EBER) was demonstrated in the nuclei by the ISH technique. The final diagnosis was chronic active EBV infection with virus-associated hemophagocytic syndrome. The prognosis of this disease is poor. In fact, the patient died of duodenal ulcer perforation seven months later. Of note is that EBV does not
Figure 18.
Eosinophilic infiltration. a: Bile cytology in clonorchiasis (Papanicolaou), b: Eosinophilia in pleural effusion in tuberculosis (Giemsa), c and d: Allergic bronchopulmonary aspergillosis (sputum cytology, c: Papanicolaou and d: Grocott). A small-sized ovum of Clonorchis sinensis with miracidium formation is seen in the bile and surrounded by eosinophils with bilobed nuclei (a). Eosinophils densely seen in the hemorrhagic pleural effusion in a case of tuberculosis may represent an allergic reaction against acid-fast bacilli (b). In allergic bronchopulmonary aspergillosis, rhomboid-shaped and red-colored Charcot-Leiden’s crystals (arrowheads) deriving from eosinophilic granules are seen. Degraded eosinophils are observed in the background (c). Grocott stain identifies a few distorted Aspergillus hyphae phagocytized by a multinucleated giant cell (d).

Figure 19.
Foreign body granulomatous reactions. a: A worm egg tubercle formed in the colonic submucosa in Schistosoma haematobium infestation (H&E), b: An omental nodule by Anisakis larva migration (H&E), c: Asbestos body in the sputum (Papanicolaou), d: Fine needle aspiration from a nodular lesion post augmentation mammoplasty (Papanicolaou). Foreign body reactions with multinucleated giant cells are noted around schistosoma eggs with miracidium formation (a) and a dead nematode larva (b). Eosinophilic reactions are scarcely seen. For the comparison, two examples of genuine foreign bodies are shown. Asbestos bodies (c) and paraffin oil droplets (d) injected by augmentation mammoplasty are phagocytized by macrophages. A long, brown-colored asbestos fibril is engulfed by two macrophages in c. vacuolated cytoplasm filled with lipid-soluble substances is characteristic, and multinucleated giant cells are dispersed in d. Arrowheads indicate multinucleated giant cells.
produce viral particles in the infected cell, so that no intranuclear inclusions are formed and thus the EBER technique is needed.

3.2 Re-staining method

A re-staining method is applicable to the single (one and only) cytology specimen [49, 50]. At first, the cells or areas of target should be marked on the back side of the glass slide with a diamond-tip pen and then photomicrographed. After removal of the coverslip in xylene, stained dyes can be removed by dipping in acid alcohol solution (50% ethanol containing 0.5% hydrochloric acid) for hours or simply dipping specimens in tap water overnight. The immunostained cells or areas of target are re-photomicrographed for comparison.

In case of Giemsa-stained glass slides or immunostained preparations on trimethoxy[3-(phenylamino)propyl]silane-coated glass slides, the re-staining method is especially valuable, since the cell transfer technique described below is not applicable due to tight attachment of the cells.

In Figure 21 showing scraping cytology of herpes simplex virus (HSV) infection on the vulva, the re-staining method visualizes intranuclear and intracytoplasmic viral antigens. A commercially available polyclonal antibody was used for immunostaining. Vulvar HSV infection belongs to sexually transmitted disease (STD).

Figure 22 demonstrates chlamydial cytoplasmic inclusions, so-called “nebular inclusion bodies”, in the scraping cytology from the uterine cervix. Chlamydiosis also represents STD. The inclusions are clearly re-stained with a monoclonal antibody B104.1 against Chlamydia trachomatis. Tiny cytoplasmic inclusions, visualized with immunostaining, are scarcely recognizable in the Papanicolaou-stained preparation.

3.3 Cell transfer technique

If you have one and only glass slide of Papanicolaou-stained cytology specimen or hematoxylin and eosin (H&E)-stained histology specimen and you want to
evaluate the expression of immunocyto/histochemical markers, the cell transfer technique [51] is quite useful and valuable (Figure 23). Firstly, cover slips must be removed by dipping in warmed xylene. Secondly, the specimen is covered with a mounting medium/resin at 2–3 mm thickness, in order to form a coating film of the solidified mounting medium in a warm incubator overnight. Then, the film of the solidified resin should be peeled off the glass slide by dipping in warm water for one hour to get the cells or tissues transferred onto the film side. The solidified resin film is placed in water on the silane-coated glass slide to be dried in a warm incubator. Finally, the resin component can be removed by dipping in xylene to get the cells and tissues transferred to a new glass slide. You can obtain plural glass slides if the solidified resin is cut by scissors into several pieces.

Cells smeared outside the cover slip can be transferred to another glass slide without removing the cover slip (Figure 23). This is particularly useful in case of gynecological cytology specimens.
By a conventional technique of cell transfer, it takes time to have the cells transferred. Itoh et al. [52] invented a time-saving method to get the cells transferred in one hour (Table 4). In brief, the mounting medium should be diluted two-fold by xylene, and the rein film should be solidified on a hot plate at 70–80°C.

When the target cells in the specimen are few in number, it is recommended to have the cells marked with a diamond-tip pen on the back side of the glass slide before removal of the cover slip. When an archival long-kept and fully dried specimen is used, the cover slip removal is not easily achieved. In order to accelerate the removal, xylene solution should be warmed up to 70–80°C and/or the cover slip should be cracked with a diamond-tip pen.

Harada et al. [53] reported that detachment of the cover slip is accelerated by using a packaging duct tape, as summarized in Table 5. Figure 24 illustrates the step of Harada’s method for rapid removal of the cover slip. The method is applicable to archival glass slides long kept at room temperature for 20 years. It takes only one hour to remove the cover slip. By combining Harada’s method with the above-mentioned Itoh’s quick method for the cell transfer, old cytology glass slides become ready for immunocytochemical analysis within a few hours.

1. Removal of cover slip
   The cover slip can be removed by dipping in warm xylene
2. Application of mounting medium
   The mounting medium (e.g. Malinol®, Muto Chemicals, Tokyo) should be two-fold diluted by xylene and one mL of the diluted resin is placed onto the smeared cells on the glass slide.
3. Solidification
   The resin should be solidified for 30 minutes on a hot plate at 70–80°C.
4. Softening
   The glass slide with an adherent resin film should be soaked for 15 minutes in warm water at 50–60°C.
5. Detachment
   The softened resin film can be peeled off the glass slide with forceps. The detached film can be divided into several pieces with scissors.
6. Pasting
   The resin film is soaked in water and pasted onto a new silane-coated glass slide. Caution is needed for the recognition of the right side of the film. After removal of excessive water, the cell-transferred glass slide should be dried on a hot plate at 70–80°C. You can prepare plural glass slides when the resin film is cut into several pieces by scissors.
7. Removal of solidified resin
   The solidified resin can be removed by dipping in xylene. Hydration is then achieved through placing the glass slide in ethanol. The specimen is ready for immunostaining.

Table 4.
Itoh’s time-saving cell transfer technique.
Figure 25 displays identification of human papillomavirus (HPV) type 16 genome in the nuclei of severely dysplastic cervical squamous cells. The cells in the routine cervical smear were transferred onto the silane-coated glass slide, and the ISH technique was applied to localizing the viral genome in the nuclei of the dysplastic cells. This cell transfer step is essentially required to avoid detaching the cells during the staining step, because heating pretreatment of the specimen is inevitable for the ISH technique. In this way, the Papanicolaou-stained cytomorphology and the state of HPV infection can directly be compared in the same cervical cells.

The cervical smear was prepared from a postmenopausal lady, and we had one and only glass slide in hand. The cells on the glass slide were transferred to another silane-coated glass slide. The clustered atypical parabasal keratinocytes in the background of senile colpitis were positively stained for p16-INK4a, indicating the
carcinogenic HPV infection (HPV-infected genuine dysplasia or high-squamous intraepithelial lesion) [45], as illustrated in Figure 26. In this way, genuine HPV-related dysplasia was distinguished from reactive (benign) atypia of the parabasal keratinocytes secondary to senile atrophy. Heating pretreatment is an essential step for immunolocalizing p16-INK4a that is a tumor suppressor gene product for modulating the cell cycle. Carcinogenic HPV infection inactivates retinoblastoma (RB) gene leading to the overexpression of p16-INK4a. In other words, the p16-INK4a is a marker of HPV-infected cells in the uterine cervix [54].

It should be noted that the cell transfer technique is applicable to paraffin sections, as well as Papanicolaou-stained cytology specimens in the gynecologic and respiratory fields, but Giemsa-stained cytology preparations employing dry fixation

Figure 25.
Severe dysplasia of uterine cervix (left: Papanicolaou, right: ISH for HPV, type 16 genome). The cells were transferred onto a silane-coated glass slide to localize HPV, type 16 genome by ISH technique, requiring heating pretreatment. Dotted signals are seen in the dysplastic nuclei. The microscopic features of HPV infected cells can directly be compared with those of pap staining.

carcinogenic HPV infection (HPV-infected genuine dysplasia or high-squamous intraepithelial lesion) [45], as illustrated in Figure 26. In this way, genuine HPV-related dysplasia was distinguished from reactive (benign) atypia of the parabasal keratinocytes secondary to senile atrophy. Heating pretreatment is an essential step for immunolocalizing p16-INK4a that is a tumor suppressor gene product for modulating the cell cycle. Carcinogenic HPV infection inactivates retinoblastoma (RB) gene leading to the overexpression of p16-INK4a. In other words, the p16-INK4a is a marker of HPV-infected cells in the uterine cervix [54].

It should be noted that the cell transfer technique is applicable to paraffin sections, as well as Papanicolaou-stained cytology specimens in the gynecologic and respiratory fields, but Giemsa-stained cytology preparations employing dry fixation

Figure 26.
Moderate dysplasia of uterine cervix after menopause (left: Papanicolaou, right: Immunostaining for p16-INK4a). Dysplastic change and reactive atypia secondary to senile colpitis should be distinguished. The expression of p16-INK4a confirmed the precancerous state of the cervix in this postmenopausal female patient. After the cell transfer onto the silane-coated glass slide, p16-INK4a was immunostained by employing heat-induced antigen retrieval.
and cytology specimens of liquid form smeared on silane-coated glass slides are not suitable for the cell transfer technique.

The cell transfer technique can be applied to the transfer of the cohesive cells cultured on the plastic slide (Nunc Lab-Tek® chamber) to the silane-coated glass slide. Xylene is not applicable to the plastic slide and the cover slip cannot be placed on the plastic slide. The broken glass slides can be repaired by employing the cell transfer technique (Figure 27) [55].

3.4 Application of cytology specimens to ultrastructural study and polymerase chain reaction analysis

The ethanol-fixed cytology specimens can be applied to electron microscopic and immunoelectron microscopic study. Fine structures of viral particles and chlamydial bodies are preserved even after ethanol fixation. Figure 28 demonstrates immunoelectron microscopic observation of chlamydial antigen in a uterine cervical columnar cell. In the nebular inclusion body in the ethanol-fixed cell, both elementary bodies and reticulate bodies of the chlamydial microbe are clearly observed. The plasma membrane of the particles is positively labeled with the monoclonal antibody [56]. Figure 29 schematically illustrates the cell cycle of C. trachomatis in the infected cell. Smaller-sized elementary bodies represent the infectious particles, while larger-sized reticulate bodies belong to the proliferative form. ISH technique can also be applied to cytology preparations [57].

Pathogens are often localized in a limited part in the specimen. It is practical and convenient for us to focus target on the infected cell for (immuno)electron microscopic study. At first, immunostaining with diaminobenzidine color reaction should be performed at the light microscopic level. After taking photomicrographs, the cover slip is removed, the specimen is re-fixed in 1% osmium tetroxide solution, and the cells are targeted for epoxy resin (Epon) block preparation by employing the inverted beam capsule method. Ethanol fixation accelerates penetration of antibody molecules into the cell, so that routine method for the light microscopy gives us an excellent result also at the ultrastructural level. Fine morphology of particulated microbes is well preserved even after ethanol fixation.

The cytology specimen can be analyzed with polymerase chain reaction (PCR) analysis by employing the cell transfer technique. The solidified resin film prepared

Figure 27. Repair of a broken glass slide employing the cell transfer technique. a: The glass slide was broken; b: The broken slide is supported by another glass slide underneath adhered with epoxy glue; c: Cover slip is removed in warm xylene; d: Mounting medium (resin) is covered on the glass slide; e: Solidified resin film is peeled off after dipping in warm water; f: The resin film is pasted onto a new glass slide. After enough drying, the resin is removed in xylene and then a cover slip is set.
from a Papanicolaou-stained smear should be cut by scissors. Parts of the specimen are kept as Papanicolaou-stained slides, while DNA or RNA can be extracted from the other parts after xylene treatment [45].

**Figure 29.**
Schematic presentation of the growth cycle of *Chlamydia trachomatis*. Smaller-sized elementary bodies (red) infect the cell, and larger-sized reticulate bodies (green) proliferate to form intracytoplasmic nevular inclusions. Intermediate bodies (yellow) indicate a transitional form between the reticulate and elementary bodies. N = nucleus.

**Figure 30.**
Illustrates *Entamoeba gingivalis* colonization in inflamed exudate seen around an intrauterine contraceptive device (IUD). Microscopically, amebic trophozoites are scattered around actinomycotic grains [58]. The patient aged in her 50’s complained of white-colored fluor genitalis. After removal of the IUD, cytological specimens were sampled from the surface of the device. The postmenopausal lady totally forgot the artificial material inserted in her uterus. In order to confirm the diagnosis, PCR analysis was performed by utilizing the cell transfer technique.
The DNA sequence of *E. gingivalis* was identified from the 221 based band on the gel. It was evident that oral sex had caused the infection of anaerobic residents of the oral cavity (both *Actinomyces* and *E. gingivalis*) [59] around the artificial material. The growth of *Actinomyces*, an obligate anaerobic microbe, allowed the survival of *E. gingivalis*, an obligate anaerobic protozoan, in the uterine cavity.

4. Cytodiagnosis of bacterial infection

The cytology service has a significant role in the detection and presumptive identification of microorganisms [60]. Generally speaking, the cytodiagnosis of bacterial infection can be reached more easily for extracellular pathogens than for intracellular pathogens. It should also be noted that bacteria are more steadily observed in Giemsa-stained preparations than in Papanicolaou-stained preparations. For immunocytochemical confirmation, the techniques mentioned above (the usage of cell block and re-staining or cell transfer technique) are valuable. Representative examples of the cytodiagnosis of bacterial infection are described below.

4.1 Bacterial vaginosis

Large-sized Gram-positive rods, *Lactobacillus* or so-called Döderlein bacilli, are the normal flora (indigenous microbiota) of the vagina and maintain the local acidity at pH 3.8–4.5 by producing lactic acid. Their length ranges from 2 to 9 μm, with the width of 0.5 to 0.8 μm. The lactic flora produces hydrogen peroxide and antimicrobial peptides (bacteriocins) to inhibit growth of other microbes [61]. The number of the non-mobile bacilli is increased around the period of ovulation through the secretory phase of the menstrual cycle. Döderlein bacilli are seen in healthy mature (premenopausal) women, but after menopause without hormonal activity, they are no longer observed in the cytology preparation. In Papanicolaou-stained preparations, they look like homogeneously basophilic large rods without...
spore formation. The background generally shows paucity of inflammation, but they are occasionally phagocytized by neutrophils [62].

In case of bacterial vaginosis (vaginitis), abnormal bacteria grow and Döderlein bacilli are markedly decreased or totally disappear [63, 64]. Representative example is Gardnerella vaginitis caused by infection of *Gardnerella vaginalis*, a small (1 to 1.5 μm-sized) Gram-negative non-mobile coccobacillus. The small bacteria, *G. vaginalis*, often cluster on the squamous cells of the superficial type to form so-called “clue cells”. *Gardnerella* infection is often evident in a proliferative phase of the menstrual cycle. The infection is commonly associated with neutrophilic reaction, but poor neutrophilic response may be seen in some cases, hence the term “bacterial vaginosis”, instead of bacterial vaginitis.

Another microbe causing bacterial vaginosis is *Mobiluncus*, spp., a V-shaped or crescentic, mobile, obligate anaerobic bacillus with unstable Gram reactivity. The size is intermediate between Döderlein bacillus and *G. vaginalis*. *Atopobium (Fannyhessea) vaginae*, a small-sized (less than 1 μm), obligate anaerobic Gram-positive elliptical coccobacillus often forming a short chain (somewhat resembling streptococcus), is a recently reported member causing bacterial vaginosis [65]. The growth of filamentous long-shaped bacillus, *Leptothrix*, may be associated with bacterial vaginosis. After menopause, these bacteria may often be replaced by enterobacteria such as *Escherichia coli* and *Klebsiella pneumoniae*. *Pseudomonas aeruginosa* may colonize the vaginal mucosa, accompanying biofilm formation (refer to Figure 37). All these microbes provoke neutrophilic exudation.

Figure 31 displays representative cytomorphology of vaginal bacteria in Papanicolaou-stained preparations.

### 4.2 Chlamydial infection

Chlamydiosis is a representative and common STD. Symptomatic non-gonococcal urethritis is seen in male patients, while symptoms are mild in females. Columnar epithelial cells infected with *Chlamydia trachomatis* contain round-shaped cytoplasmic inclusion bodies named as nevular inclusion bodies [66].
life cycle of Chlamydia is shown in Figure 29. Immunocytochemistry using the re-staining method or cell transfer technique is quite effective for making a diagnosis of chlamydiosis (refer to Figures 22 and 28). In most cases of chlamydiosis, bacterial vaginosis is associated, so that a variable number of neutrophils are seen in the background. Chlamydial infection commonly causes lymphoid follicle formation in the mucosa: Lymphocytic background may be seen in the cervical smear preparation, as C. trachomatis-associated follicular cervicitis [67].

Chlamydial inclusions are also seen in cytology specimens scraped from male urethra. C. trachomatis causes epididymitis and salpingitis. Extragenital chlamydiosis should be of notice [68]. Chlamydial pharyngitis and proctitis are mediated by oral sex and anal sex, respectively. Acute chlamydial conjunctivitis occurs in sexually active young men, and the cytoplasmic inclusions are demonstrated by quick Giemsa (Diff-Quik) staining. Representative features are shown in Figure 32.

4.3 Gonococcal infection

Gonorrhea is a classic example of STD. Neisseria gonorrhoeae, a Gram-negative paired coccus (diplococcus), causes acute urethritis in male, and it shows high affinity to urethral epithelial cells [69]. Figure 33 demonstrates urine cytology from a 28-year-old single Japanese male patient. It should be of note that paired cocci are specifically attached onto the large-sized squamous cells of urethral origin. Typically, diplococci are phagocytized by neutrophils. Cytological diagnosis of gonorrhea can be made immediately.

4.4 Bacteria growing in liquid specimens and effusions

Acute cystitis is common in women, most often caused by Escherichia coli infection. Pyuria is an important sign of bacterial cystitis. In case of acute cystitis in

Figure 32.
Chlamydial infection (left: Immunostaining for chlamydial antigen in scraped male urethra with methyl green counterstain, right: Giemsa-stained scraping cytology of conjunctiva). Numbers of urethral and conjunctival epithelial cells possess chlamydial cytoplasmic inclusion bodies. Note extragenital infection of C. trachomatis on the eye (arrows).
aged men, the association of prostatic gland hyperplasia causing urethral stenosis should be considered. Urinary bladder cancer may often associate bacterial growth in the urine. Rods mostly represent *E. coli*, while chained cocci usually belong to *Enterococci* (Figure 34). Refer also to Figure 11b, where cocci (*Enterococcus faecalis*) are actively phagocytized by neutrophils in urine. Particularly when neutrophilic reaction is evident, the diagnosis of bacterial cystitis should be added to that of urothelial carcinoma.

Similarly, bile cytology specimens may reveal bacillary growth around adenocarcinoma cells. The possibility of ascending purulent cholangitis due to malignant...
bile duct obstruction should be excluded. It may indicate an emergency state requiring prompt antibiotics therapy. Therefore, the cytodiagnosis must be adenocarcinoma plus bacillary colonization. Giemsa staining is superior to Papanicolaou staining for identifying infection of the extracellular bacteria.

When you find bacilli in specimens of ascites or pleural effusion, you should check how the specimen was kept until the centrifugation procedure to get the sediment [70]. If the specimen was kept overnight at room temperature, bacterial grew after the specimen sampling. Neutrophilic response is absent. In the urine sample left for a prolonged time, urease activity of the bacteria, yielding ammonium irons, provokes urine alkalization that leads to deposition of ammonium-magnesium phosphate crystals and non-crystalline phosphate. Representative cytological features are shown in Figure 35. Compare them with the specimen of genuine bacterial pleuritis caused by *Streptococcus milleri* as shown in Figure 11c.

### 4.5 Morphological change of Gram-negative rods

Administration of wide-spectrum penicillin and cefem antibiotics may provoke considerable morphological changes of the Gram-negative *Enterobacteirae* in the bile and urine. These include filamentous deformation and spheroplast change. The beta-lactam antibiotics bound to the penicillin-binding proteins on the bacterial cell membrane hamper the bacterial growth, leading to the shape changes [71, 72]. In the bile shown in Figure 36, *Klebsiella pneumoniae* accompanied marked elongation and spheroid change. Microbial culture of the bile was positive for *K. pneumoniae*. The morphologically altered bacteria somewhat resemble fungi. The filaments and spheroplasts are negative with Gram and Grocott stains. *Pseudomonas aeruginosa* in the urine may also show marked filamentous change. Because of the effect of antibiotics treatment, neutrophilic response may be suppressed.
Under an immunocompromised condition, *Enterobacteria* such as *E. coli* and *K. pneumoniae* may proliferate within the cytoplasm of macrophages in the digestive and urinary organs to manifest xanthogranulomatous inflammation and malakoplakia, as mentioned above (Figure 17).

4.6 Biofilm infection

*Pseudomonas aeruginosa* of mucoid form commonly accompanies biofilm infection. Biofilm-forming bacteria stick to each other and also to the surface of material or injured mucosa. The adherent bacteria become embedded in a slimy (mucoid) extracellular matrix or secretory capsule. The biofilm protects the microbe from the attack by neutrophils, antibodies, complements and antibiotics: biofilm infection represents a state of resistance of the bacteria to antibiotics therapy [73, 74]. The biofilm-forming *P. aeruginosa* may thus cause persistent and intractable infection particularly in the airway. The neutrophilic host response is thus often minor in degree. Representative examples of biofilm infection are displayed in Figure 37. Refer also to Figure 44f. Gallbladder adenocarcinoma was cytologically associated with biofilm infection of rods, *P. aeruginosa*, embedded in mucoid matrix. In the vagina of the aged after hysterectomy, infection of *P. aeruginosa* of mucoid-type is proven cytologically.

Biofilm may also be formed by capsule-forming bacteria such as streptococci, staphylococci and enterococci.

4.7 Scraping/touch smear cytology of autopsied lung

We pathologists commonly encounter pneumonia in autopsy cases. Nosocomial (hospital-acquired) pneumonia is often caused by Methicillin-resistant *Staphylococcus aureus* (MRSA) or *Enterobacteria*, while community-acquired pneumonia may result from infection of *Streptococcus pneumoniae*, *Haemophilus influenzae*,

Figure 36. Antibiotics-induced shape changes of Klebsiella pneumoniae in the bile: Formation of filaments and spheroplasts (a/b: Papanicolaou, c: Giemsa). The rounded form is called as spheroplast (arrows). Beta-lactam antibiotics bound to penicillin-binding protein on the bacterial cell membrane provokes shape changes of the gram-negative rods. Microbial culture confirmed infection of *K. pneumoniae* in this case. Distinction from fungal colonization is requested.
Moraxella (Branhamella) catarrhalis, etc. In case of lethal lobar pneumonia, candidate causative microbes include *S. pneumoniae* and *Legionella pneumophila* [75]. Giemsa-stained scraping or touch smear cytology sampled from the pneumonia lesion is practical in determining the causative microorganism during autopsy services. It is important for pathologists to avoid biohazard. *S. pneumoniae* is transmissible by droplet transmission, while *L. pneumophila* does not show human-to-human transmission. Giménez staining is also useful for demonstrating the microbe. Figure 38 illustrates scraping cytology sampled from lethal lobar pneumonia in the aged patient. See also Figure 14a. Rods were phagocytized by macrophages, so that the causative microbe was identified as *L. pneumophila*, an intracellular microorganism. It is of note that the main cellular reactants against *L. pneumophila* are macrophages. Because of the paucity of lymphocytic response, granulomas are not formed. The importance of *L. pneumophila* as a cause of community-acquired lobar pneumonia of the aged should be emphasized [76].

### 4.8 Nocardiosis

Nocardiosis is usually encountered in immunocompromised patients [77, 78]. *Nocardia asteroides*, a Gram-positive filamentous aerobic bacterium, can be demonstrated in bronchial brushing cytology specimens. A young male suffering from ulcerative colitis under steroid treatment complained of fever, coughing and phlegm. Cavitation was radiologically identified in his left upper lobe of the lung, and mycotic infection was clinically suspected. A quick-witted cytotechnologist performed Grocott staining in the cytology preparation. Grocott-stained filamentous bacteria were identified in the background of neutrophilic response, and the diagnosis of nocardiosis was made. The filamentous bacteria were not easily recognized in Papanicolaou-stained preparation, because they do not form aggregated grains. They were additionally positive with Gram and Ziehl-Neelsen’s stains. Gram positivity and weak acid-fastness characterize *Nocardia*. Figure 39 illustrates
cytopathologic appearance of lung nocardiosis. Microbial culture identified *N. asteroides*, and administration of sulfonamides was clinically quite effective.

4.9 Actinomycosis

Actinomycosis, infection of *Actinomyces israelii*, happens in immunocompetent individuals [79, 80], in sharp contrast to nocardiosis. Formation of sulfur granules,
reaching 1–2 mm in size, is characteristic of actinomycosis (Figure 40). Refer also to Figure 11d. The sulfur granule is a dense cluster of obligately anaerobic filamentous bacteria embedded in the homogeneous, periodic acid-Schiff (PAS)-reactive matrix called Splendore-Hoeppli material. The filaments are visualized with Gram, PAS and Grocott stains. Active neutrophilic response against the grains can be observed. In the lung, the sulfur granules are commonly seen within the destroyed airway, and inflammatory pseudotumor may be formed as a result of severe inflammatory fibrosis. Actinomycosis is also encountered in the oral cavity, liver and pelvic organs, including the endometrium (see also Figure 30). Actinomycotic grains are often seen in the pit of the enlarged pharyngeal tonsil as a non-pathogenic resident microbe.

4.10 Tuberculosis and non-tuberculous mycobacteriosis

When epithelioid cell granuloma is seen in bronchial scraping cytology, the possibility of lung tuberculosis should be considered (Figure 41). See also Figure 12. Often times, necrotic background is associated [81, 82]. Infrequently, tuberculous pleuritis may induce eosinophilic exudation (Figure 18b).

It is an important mission of the cytopathologist to have the hospital staff noticed for the biohazard [83]. Under an immunosuppressed condition, numerous acid-fast bacilli are phagocytized by macrophages, and Giemsa staining discloses negatively stained long rods in their cytoplasm [84] (Figure 42). The outer membrane of the cell wall of mycobacteria contains large amounts of glycolipids, especially mycolic acids [85]. This unique cell wall structure not only gives acid-fastness but also inhibits the penetration of dyes in the Giemsa solution. Mycobacterium tuberculosis, a representative acid-fast bacillus, shows airborne transmission. Bronchial sampling is performed in the isolated room equipped for bronchofiberscopy, so that check-ups for the close contact persons are requested. Cytology laboratory may be contaminated with the transmissible dryness-resistant pathogen inside the droplet nucleus. The bacterial morphology is indistinguishable between tuberculosis

Figure 40.
Actinomycosis of the endometrium (scraping cytology, Papanicolaou, left: Low-power, right: High-power). Formation of sulfur granules is characteristic of Actinomyces israelii infection. The granule is surrounded by neutrophils, and it consists of filamentous bacteria embedded in the hyaline matrix called Splendore-Hoeppli material. In contrast to nocardiosis, the diagnosis of actinomycosis can be reached with Papanicolaou staining. Endometrial actinomycosis may be provoked by the insertion of intrauterine contraceptive device.
and non-tuberculous mycobacteriosis [82]. The distinction of the two is important since non-tuberculous mycobacteria do not show human-to-human transmission. Identification of \textit{M. tuberculosis} by polymerase chain reaction, as well as the interferon gamma-releasing assay (QuantiFeron or T-Spot) [86] using the blood of the patient and close contact persons, are essentially important to avoid occupation-related infection. In case of tuberculosis, not only correct cytodiagnosis but also prompt warning against intrahospital biohazard are thus strongly requested.

Figure 41. Pulmonary tuberculosis (bronchial brushing cytology, Papanicolaou, left: Low-power, right: High-power). Clusters of epithelioid cells represent a granulomatous reaction. The association of necrotic background (left) strongly suggests mycobacterial infection. It is difficult to distinguish tuberculosis (\textit{Mycobacterium tuberculosis} infection) from non-tuberculous mycobacteriosis. Not only correct cytological diagnosis but also prompt warning against intrahospital biohazard are requested. Note also that non-tuberculous mycobacteria accompany no biohazard.

Figure 42. Negative staining of mycobacteria phagocytized by macrophages (bronchial brushing cytology, left: Papanicolaou, right: Giemsa). Epithelioid granuloma is seen in Papanicolaou-stained preparation. Giemsa stain is useful to identify acid-fast bacilli, since the mycobacteria are resistant to be stained. Therefore, unstained bacillary images are clearly discernible in the cytoplasm of macrophages. No bacilli are visible in the pap smear. In this case, \textit{Mycobacterium avium} (a representative non-tuberculous mycobacterium) was cultured. By courtesy of Mr. Tomohiro Watanabe, Chuken Kumamoto, Japan.
When epithelioid granuloma and neutrophilic reaction are seen in the same specimen, the possibility of suppurative granuloma should be suspected. The typical example is cat scratch disease (bartonellosis) (Figure 16) caused by *Bartonella henselae* infection [87]. This tick-associated infection is commonly seen in the cervical or axillary lymph node and infrequently involving the spleen.

### 4.11 Bacteria seen in the blood

Some bacteria may be observed in the peripheral blood (Figure 43). Spiral microbes of *Borrelia recurrentis* are seen in the peripheral blood in an early stage of relapsing fever. The febrile disease is endemic in the African continent [88]. In case of bacterial septicemia, bacteria phagocytized by phagocytes (neutrophils and monocytes) are occasionally identified in peripheral blood preparations. In *Capnocytophaga canimorsus* septicemia caused by dog bite, a few bacilli are phagocytized by neutrophils [89]. *Streptococcus suis*, an important pathogen of pigs, may cause meningitis and lethal septicemia in the human who farms pigs or handles pork. The disease is endemic in southeastern Asia [90]. *In situ* hybridization (ISH) study of theuffy coat of the peripheral blood in septicemia infected with *Escherichia coli/Klebsiella pneumoniae, Staphylococcus aureus* or *Pseudomonas aeruginosa* exhibits bacterial DNA signals in the cytoplasm of neutrophils, even after chemotherapy [91].

### 4.12 Bacteria seen in sputum preparations: importance of Gram staining

Gram staining is cheap and quick technique to identify pathogens on smear preparations of the sputum, exudates, liquid materials and effusions. The importance of Gram staining in the diagnosis of pneumonia should be emphasized [92, 93]. It takes minutes to get results.
Typical microscopic appearance of Gram-stained sputum preparations is illustrated in Figure 44. These include Streptococcus pneumoniae, Methicillin-resistant Staphylococcus aureus (MRSA), Moraxella (Branhamella) catarrhalis, Haemophilus influenzae, Klebsiella pneumoniae, and Pseudomonas aeruginosa. Gram staining gives us prompt identification of pathogens causing pneumonia. Cocci are seen in a–c, and rods in d–f. Gram stain is positive in a & b, but negative in c–f. The bacteria are phagocytized by neutrophils in b & c, while the capsule-forming pathogens are escaped from phagocytosis in a, d–f. Mucoid form is observed in f. The rods in e & f are much larger than those in d.

Figure 44.
Pathogens causing pneumonia in the sputum (Gram, a: Streptococcus pneumoniae, b: Methicillin-resistant Staphylococcus aureus (MRSA), c: Moraxella (Branhamella) catarrhalis, d: Haemophilus influenzae, e: Klebsiella pneumoniae, f: Pseudomonas aeruginosa). Gram-stained preparations give us prompt identification of pathogens causing pneumonia. Cocci are seen in a–c, and rods in d–f. Gram stain is positive in a & b, but negative in c–f. The bacteria are phagocytized by neutrophils in b & c, while the capsule-forming pathogens are escaped from phagocytosis in a, d–f. Mucoid form is observed in f. The rods in e & f are much larger than those in d.

5. Cytodiagnosis of fungal infection

Invasive fungal infection is treatment-resistant and often lethal [94]. Fungi are commonly visualized with PAS reaction and Grocott (methenamine silver) staining. Gram staining may be positive. Fungi infectious to the human being are divided into two forms: yeast and hyphal-forming types. Hypha-forming fungi belong to extracellular pathogens, and provoke neutrophilic reaction. Yeasts, round in shape and not forming hyphae, infect intracellularly and protected by cellular immunity provoking granulomatous cellular reaction. Candida accompanying both yeast and hypha-forming (myceliform) morphology is placed in an intermediate form [95].

5.1 Candidosis

Superficial candidosis (moniliasis) represents the most common mycosis. Candida albicans, the major species of Candida, is characterized by dimorphic
appearance: ovoid yeast cells (germ spores) and filamentous pseudohyphae. *C. albicans* is a normal indigenous flora of the oral cavity, intestinal lumen and skin, residing as a form of yeasts.

*Candida vaginitis* is the most frequently encountered candidosis in cytology specimens [96]. Hypha-forming colonies are surrounded by neutrophils, and Döderlein bacilli, the normal flora, are no longer observed. Yeast form fungi are also intermingled (Figure 45). Refer also to Figure 11e. Vaginal candidosis is often associated with pregnancy, diabetes mellitus and acquired immunodeficiency syndrome (AIDS), and it is also experienced as a form of STD. *Candida (Torulopsis) glabrata* can be identified in the Papanicolaou-stained cervical smear preparation as a form of paired and orange-colored yeasts. No hyphae are formed, Döderlein bacilli are preserved, and neutrophilic reaction is mild [97]. *C. glabrata* thus represents a normal vaginal flora and must not be misinterpreted as candida vaginitis. The simple and thoughtless comments such as “Candida-positive” may mislead the clinician to inappropriate and unnecessary treatment against candida vaginitis.

*Candida* is often seen in sputum and urine cytology (Figure 46). The appearance of yeast-form Candida without hypha formation in the sputum may represents the increased non-pathogenic flora. In fact, the neutrophilic response is minor in degree. In the pathogenic state, Candida consistently forms pseudohyphae. It should be recognized that mucosal candidosis is common in the oral cavity and upper airway, but candida scarcely provokes pneumonia. In case of candida cystitis, the urine cytology reveals both yeasts and pseudohyphae in the inflammatory (neutrophil-rich) background [95]. *Trichosporon cutaneum (beigelii)*, showing a microscopic resemblance with *Candida albicans*, may also cause mycotic cystitis [98]. Uneven PAS reactivity of *T. cutaneum* gives a hint for differentiation from *C. albicans*, but microbial culture is essential for confirming the causative fungus.

Figure 45.
Pathogenic and non-pathogenic Candida in cervical smear preparations (Papanicolaou, left: Candida vaginitis, right: Candida (Torulopsis) glabrata as normal vaginal flora). Pathogenic *Candida albicans* forms pseudohyphae in the vagina and provokes neutrophilic reaction, causing candida vaginitis. *C. glabrata* forms paired and orange-colored yeasts without forming hyphae (arrows). The preservation of Döderlein bacilli (arrowheads) in the background is the proof for the lack of pathogenicity.
5.2 Cryptococcosis

Figure 46. Candidosis and trichosporonosis (Papanicolaou, a: Yeast form Candida in sputum, b: Candidosis in sputum, c: Candida cystitis, d: Trichosporon cystitis, inset; PAS). Candida yeasts often stain yellowish with Papanicolaou staining. When only yeast form is observed in the sputum, we can judge the microbe as non-pathogenic (a). Since Candida pneumonia is rare, hypha-forming Candida growth may occur in the oral cavity or pharynx (b). In the urine, typical orange-colored microscopic features of Candida infection, accompanying both yeasts and pseudohyphae, are noted (c). Trichosporon cutaneum, microscopically resembling Candida, occasionally causes fungal cystitis (d). Uneven PAS reactivity is a feature of the Trichosporon species (inset).

5.2.2 Cryptococcosis

Figure 47 demonstrates bronchial brushing cytology of pulmonary cryptococcal granuloma. Multinucleated giant cells of macrophage origin phagocytize small
transparent rounded yeasts in the cytoplasm. *Cryptococcus neoformans* grows in the cytoplasm of macrophages to provoke a granulomatous cellular reaction. It should be noted that cryptococcal infection is accelerated by impaired cellular immunity, e.g. after steroid therapy and in AIDS [99].

*C. neoformans* may infect the meningeal space [100]. Indian ink method clearly demonstrates capsule-forming yeasts in the cerebrospinal fluid (CSF). Usually, yeasts in the CSF are few in number (Figure 47c). In case of disseminated cryptococcosis seen in patients with suppressed cellular immunity, a large number of yeasts are observed, and they are often not phagocytized by macrophages. Urine cytology in disseminated cryptococcosis is displayed in Figure 47d.

### 5.3 Pneumocystosis

*Pneumocystis jirovecii*-induced acute interstitial pneumonia is seen in patients with severe suppression of cellular immunity after administration of steroid or in AIDS [101, 102]. *P. jirovecii* (previously called as *P. carinii*) is now categorized in the fungus, but unculturable *in vitro*. Grocott staining is essential for making the diagnosis of pneumocystosis in bronchial/alveolar lavage specimens (Figure 48). Pneumocystis pneumonia often manifests dry cough without formation of phlegm. Grocott-positive small cysts are clearly observed. PAS reactivity is negative. Lymphocytic reaction may be seen in the cytology specimen. In heavily infected specimens in AIDS patients, pathogens (cysts) look like clustered hemolytic red cells in Papanicolaou-stained preparations. With Giemsa staining, the nuclei of smaller-sized ameboid trophozoites are stained purple. Response of small lymphocytes may be provoked, as illustrated in Figure 13d.

![Figure 48](image)

*Figure 48.* *Pneumocystis pneumonia* (bronchial lavage, *a*: Papanicolaou, *b*: Grocott, *c*: Giemsa). Under suppressed cellular immunity, *Pneumocystis jirovecii* appears in the airway as clustered translucent cysts in the pap smear, somewhat resembling hemolytic red cells (*a*). Lymphocytes and macrophages surround the pathogens. The cysts are clearly detected with Grocott stain (*b*). The cyst wall and dot-like structure within the cyst are stained black. Giemsa stains nuclei of small-sized trophozoites in purple (*c*).
5.4 Aspergillosis

Aspergillus is a representative example of hypha-forming (myceliform) filamentous fungi, growing extracellularly [103]. Basophilic hyphae, typically accompanying Y-shaped bifurcation and septum formation, are arranged in the same direction (Figure 49). Non-viable hyphae show less basophilia. Neutrophils accumulate around the hyphae. The most common species is *Aspergillus fumigatus*. *A. flavus* occasionally secretes orange/red-colored pigment [104]. Melanin pigment is seen in *A. niger* that also produces calcium oxalate crystals [105].

In aspergilloma containing a fungus ball within the cavitated bronchus, conidial heads, globose, radiate or broom-shaped, are formed in the aerobic (air-filled) cavity, and Grocott-positive conidia (conidiospores) may be seen in the bronchial lavage specimens. It should be noted that the round-shaped conidia closely resemble *Cryptococcus neoformans*. An important point of distinction is that the conidia floating in the air are not phagocytized by macrophages.

Aspergillus infrequently provokes an allergic reaction with eosinophilic granuloma formation. The status is called as allergic bronchopulmonary aspergillosis. A number of eosinophils and eosinophilic Charcot-Leiden crystals appear in the sputum, in association with a few injured fungal hyphae (see Figure 18c and d).

5.5 Mucormycosis

Mucormycosis (zygomycosis) is the infection by *Zygomycetes*, including *Mucor ramosissimus*, *Rhizomucor pusillus*, *Rhizopus oryzae*, etc. *Zygomycetes* is an opportunistic microbe mainly affecting premature babies and patients with neutropenia or severe diabetes mellitus. When compared with Aspergillus, the hyphae are less basophilic and thick and lack septum formation. The lamified angle of the hypha tends to be wide. The infection provokes neutrophilic responses. The main sites of
involvement include the paranasal cavity and lung. Calcium oxalate crystal deposition and yellow pigment secretion may be associated with mucormycosis [106]. Lethal systemic dissemination may occur in neutropenic patients and in premature neonates [107]. Figure 50 illustrates scraping cytology of the invasive brain lesion seen in a young boy. Thick branching hyphae without septum formation are noted.

6. Cytodiagnosis of viral infection

Intranuclear clusters of viral particles are recognized as intranuclear inclusion bodies. Intranuclear inclusion bodies characterize DNA virus infection, while most of the RNA viruses do not form inclusion bodies. Exceptionally, measles virus, an RNA virus, forms intranuclear inclusions. Some DNA viruses may cause cytoplasmic viral inclusions: hepatitis B virus to form ground-glassed hepatocytes and molluscum contagiosum virus (a family of pox viruses) to form molluscum bodies in keratinocytes. There are two types of intranuclear inclusion bodies, smudge (homogeneous or full) type and Cowdry A (haloed) type [108]. The viral infection principally provokes lymphocytic host response, when the patient is immunocompetent (see Figure 13b).

6.1 Viral inclusions

Representative examples of intranuclear viral inclusion bodies seen in cytology specimens are shown in Figure 51.

Human papillomavirus (HPV), a wart-forming DNA virus, provokes skin and mucosal lesions. Intranuclear inclusions are seen in the skin lesion (wart), but not observed in the mucosal lesions (refer to Figures 25 and 26). Both types associate koilocytosis, namely perinuclear halo formation in the superficial keratinocytes. In gynecologic cytology specimens, koilocytosis is seen in condyloma acuminatum.
Cytological Diagnoses of Infectious Diseases: Identification of Pathogens and Recognition...

Epidemic keratoconjunctivitis is a highly contagious eye disease caused by infection of adenovirus, types 8, 19 or 37. Quick Giemsa-stained epithelial cells scraped from the conjunctiva reveal intranuclear inclusion bodies of smudge type [110].

Infection of herpes simplex virus (HSV; human herpesvirus-1 or -2) typically accompanies intranuclear inclusion bodies of both smudge and Cowdry A types [111]. Scraping cytological features of herpetic keratitis and HSV infection of the nipple are illustrated. See also Figure 21, where vulvar keratinocytes are infected by HSV as a form of STD. Epstein-Barr virus (EBV, human herpesvirus 4) may cause neoplastic growth of lymphocytes and gastric epithelial cells, but intranuclear inclusions are not formed [112]. In case of chronic active EBV infection, the induction of large granular lymphocytes of NK cell lineage is characteristic, as demonstrated in Figure 20.

6.2 Opportunistic viral infections

Cytomegalovirus (CMV; human herpesvirus-5) is a representative example of the opportunistic virus activated in case of impaired cellular immunity. CMV provokes enlargement of the infected cells with formation of large basophilic (owl-eyed) intranuclear inclusion bodies. Granular intracytoplasmic inclusion bodies are also noted [113]. Hemorrhagic varicella (lethal infection of varicella-zoster virus [VZV; human herpesvirus-3) occurred in a child case of acute lymphoblastic leukemia after bone marrow transplantation. The cytology specimen aspirated from a hemorrhagic vesicle shows intranuclear inclusion bodies [114].
BK virus-infected cells in the urine sediment are called “decoy cells,” somewhat resembling urothelial cancer cells. Intranuclear inclusion bodies of smudge type are observed in the infected urothelial cells under suppressed cellular immunity. BK virus antigen or SV40 antigen can be demonstrated in the nuclei. Electron microscopy identified numerous round and small-sized viral particles in the nuclei [115]. Representative cytological features of opportunistic viral infection are displayed in Figure 52. The cellular (lymphocytic) response is scarcely seen in these immunocompromised cases.

7. Cytodiagnosis of protozoan infection

Protozoa, unicellular microbes usually measuring 5–20 μm, may accompany pseudopodia, flagellae or cilia. Most protozoa infect not only human but also animals, categorized in zoonotic infection [116]. Some protozoa such as Entamoeba histolytica, Giardia lamblia and Trichomonas vaginalis lack mitochondria [117].

7.1 Protozoa in cytology specimens

Representative features are demonstrated in Figure 53.

The most common protozoan experienced in routine cytology services is Trichomonas vaginalis in cervical smear preparations. This STD pathogen is seen adjacent to glycogen-rich superficial keratinocytes. Döderlein bacilli are no longer observed in the background. Neutrophils are often clustered to form so-called cannon (pus) balls [118]. T. vaginalis is a non-invasive protozoan and grows extracellularly, so that neutrophilic response is induced (Figure 11f). Cannon ball formation (clustering of neutrophils as cannon balls) is a microscopic hallmark of trichomoniasis. Giardia lamblia commonly colonizes the duodenal and gallbladder mucosae. Bile cytology preparations may contain flagellated, symmetrical pear-shaped protozoan cells, characteristically having paired nuclei [119]. G. lamblia, non-invasive protozoan, commonly induces lymphoid follicle formation with marked increase intraepithelial lymphocytes in the duodenal mucosa [120], as displayed in Figure 9.
Cellular reaction in the bile is usually poor, but lymphocytic background may be associated. Regarding the enteric co-infection of *G. lamblia* and *Entamoeba histolytica* in an AIDS case, refer to Figure 55. Acanthamoeba is a free-living protozoan widely seen in environmental water. When one wears contaminated contact lenses, painful keratitis may happen. Scraping cytology from the turbid and eroded cornea identified cysts and trophozoites of *Acanthamoeba*, spp. [121]. Touch smear preparation or fine needle aspiration sampled from a biopsied skin tissue of cutaneous leishmaniasis demonstrates amastigotes of *Leishmania tropica* growing in the cytoplasm of macrophages [122]. Both round basophilic nuclei and small-sized kinetoplasts are observed in the non-flagellated protozoan cells. Indistinguishable cytological features are seen in visceral (systemic) leishmaniasis (kala azar), as shown in Figure 14b.

### 7.2 Protozoa in blood preparations

Blood smear preparations may contain protozoa. Malaria, Babesia and *Trypanosoma* should be of notice (Figure 54). Regarding the methods for detecting blood parasites (protozoa and nematode larvae), refer to review articles [123, 124].

Falciparum malaria and tertian malaria, mosquito-mediated febrile diseases, are caused by infection of *Plasmodium falciparum* and *P. vivax*, respectively. In falciparum malaria, ring forms are seen in normal-sized red cells, and often times two or more ring forms infect one red cell. Black-colored malaria pigment is associated. Neither ameboid forms nor schizonts appear in the peripheral blood. The red cells infected by the ameboid form of *P. falciparum* strongly express cell adhesion molecules on the surface, so that they adhere to the capillary endothelial cells expressing CD36 and intercellular cell adhesion molecule-1 (ICAM-1). This process of capillary obstruction is the direct cause of cerebral (malignant) malaria [125]. In tertian malaria, ring forms are fewer in number, and the ring forms are associated
with cytoplasmic granules (Schüffner spots) in enlarged red cells. Ameboid forms and schizonts are scattered [126].

Infection of *Babesia microti* is mediated by tick bite. Babesiosis is mainly seen in animal blood, and human cases are rarely encountered, particularly after splenectomy. Ring forms are seen in red cells, and formation of cruciform bodies (tetrads), resembling a maltese cross, is pathognomonic [127].

*Trypanosoma cruzi*, mediated by Triatoma bite, is identified in the peripheral blood in an acute stage of infection. C-shaped trypomastigotes with a distinct kinetoplast at the end, 20 μm in length, are seen outside the red cells. The clinical course is benign. In chronic stage, *T. cruzi* infects the cardiomyocytes causing chronic heart failure by Chagas disease. In African sleeping disease, lethal meningencephalitis occurs. The flagellated trypomastigotes of *T. gambiense* are larger (20–30 μm in length) than those of *T. cruzi* [128].

7.3 Opportunistic protozoan infection

Opportunistic protozoan infection is commonly complicated by impaired cellular immunity (particularly in AIDS). These include amebic dysentery, giardiasis, cryptosporidiosis, toxoplasmosis and microsporidiosis. The inflammatory cellular response is poor in immunocompromised patients.

Trophozoites of *Entamoeba histolytica* are identified in cytology preparations aspirated from amebic liver abscess. The environment allowing the growth of obligate anaerobic trophozoites lacking mitochondria results in karyorrhexis of
neutrophils and loss of PAS reactivity in the background fluid due to advanced anoxia. Karyosomes (aggregated chromatin centrally located in the nucleus) are pathognomonic of protozoan cells. The plump cytoplasm of the trophozoite consists of thick perinuclear endoplasm and thin peripheral ectoplasm [129]. They often phagocytize red cells. A monoclonal antibody EHK153 detects the ameba in cell block preparations. No cyst form is discerned in the lesion. Rectal lavage from an AIDS patient complaining of severe diarrhea demonstrates opportunistic co-infection of G. lamblia and E. histolytica. Figure 55 illustrates cytological features of amebiasis.

As reference, poorly pathogenic Entamoeba gingivalis, a normal and anaerobic resident in the oral cavity, may appear in the sputum (see Figure 62c), and neutrophils are typically phagocytized by the trophozoites [59]. E. gingivalis may colonize the endometrium around an intrauterine contraceptive device (IUD) in healthy women, and co-infection with Actinomyces israelii is needed, as illustrated in Figure 30.

Cysts of Cryptosporidium parvum in diarrheal discharge in an AIDS patient show acid-fastness, red-colored with Ziehl-Neelsen's staining. The acid-fast cysts are small-sized, measuring 3–5 μm. Cryptosporidiosis in AIDS is lethal due to the lack of effective therapeutic drugs [130].

A patient with acute myeloid leukemia post bone marrow transplantation complained myalgia the leg. Fine needle aspiration was performed from the painful muscle. Clustered tachyzoites (pseudocysts) are seen in the cytoplasm of striated muscle cells. The diagnosis of Toxoplasma gondii-induced myositis was made [131].

Microsporidiosis caused by Encephalitozoon cuniculi is seen in ascites fluid of an immunosuppressed mouse. Giemsa staining clarifies the nuclei of small cysts clustered in the cytoplasm of macrophages. Microsporidiosis may be encountered in the

Figure 55.
Entamoeba histolytica infection (Papanicolaou [a&c], Giemsa [d], PAS for cell block preparation [b]). The aspirate from liver abscess contains trophozoites of E. histolytica. They are characterized by a karyosome, (a centrally located chromatin aggregate) unique in protozoan nuclei, and plump cytoplasm consisting of thick perinuclear endoplasm and thin peripheral ectoplasm (a). Trophozoites in the cell block preparation reveal PAS reactivity. Neutrophils are devoid of glycogen because of anaerobic environment (b). Rectal lavage from an AIDS patient complaining of severe diarrhea demonstrates opportunistic co-infection of E. histolytica and Giardia lamblia (c&d). Yellow arrows indicate trophozoites of G. lamblia, and its cyst form is shown by the yellow arrowhead. The large-sized trophozoite of E. histolytica (red arrows) phagocytizes neutrophils.
intestine, striated muscle and brain as an opportunistic complication in AIDS patients [132]. Recent studies indicate that the genus microsporidium belongs to the specialized fungus, instead of the protozoan. Representative microscopic appearance of the latter three infections is demonstrated in Figure 56.

8. Cytodiagnosis of helminthic infection

Larval parasites (nematodes) and parasitic ova are occasionally experienced in cytology specimens. It should be noted that manifesting helminthic parasitosis is mostly caused by visceral larva migrans in the human body.

8.1 Larval nematodes in cytology specimens

Typical example includes disseminated strongyloidiasis, opportunistically happening in immunocompromised patients suffering from AIDS or adult T-cell leukemia/lymphoma [133, 134]. Strongyloides stercoralis shows percutaneous infestation of the larva via normal skin in tropical and subtropical areas. In Japan, the disease is endemic in southern Okinawa and Amami districts. Adult worms (nematodes), 2–3 mm in length, infest the small bowel mucosa, and persistent autoinfestation occurs through direct intraluminal hatching to infective larva, up to 600 μm in length. In disseminated strongyloidiasis, larval nematodes migrate to a variety of organs and tissues, and they may be seen in cytology specimens of the sputum, urine and body fluids (Figure 57). The cellular response against the worm is poor.

8.2 Larval nematodes in blood preparations

In human filariasis encompassing several types [135], microfilariae, 200–400 μm in length, are observed in the peripheral blood smears (Figure 58). Bancroftian (lymphatic) filariasis caused by Wuchereria bancrofti is seen worldwide, and scrotal
swelling and elephantiasis of the lower extremities are clinically featured. Brugian filariasis caused by *Brugia malayi* is endemic in subtropical Asia. The microfilariae are sheathed in both forms. Conjunctival infestation of *Loa loa*, an African eye worm, provokes sheathed microfilariae in the peripheral blood. A transparent, 2–7 mm-long adult worm is seen beneath the conjunctival mucosa. In onchocerciasis causing river blindness in the highland of central America and tropical Africa and mediated by blackfly bite, unsheathed microfilariae of *Onchocerca volvulus* appear in the peripheral blood and preferably invade the eye ball. In canine filariasis,
unsheathed microfilariae of *Dirofilaria immitis* appear in the peripheral blood. See the review articles [123, 124].

8.3 Parasitic ova in cytology specimens

Parasitic ova may appear in cytology specimens. Based on their unique morphology, parasitosis of adult helminthic worms can be indicated (Figure 59). Small ova (30 μm in length) of *Clonorchis sinensis* [136] and large ova (130 μm in length) of *Fasciola hepatica* [137] may be seen in the bile. Large ova (around 100 μm in length) of *Paragonimus westermani* [138] and *Schistosoma haematobium* [139] may appear in the hemosputum and hemorrhagic urine, respectively. Regarding ova of *C. sinensis* in the bile, refer also to Figure 18a. Eosinophilic background is often associated. Foreign body granulomatous reaction (so-called egg tubercle) is provoked against ova of *S. haematobium* as illustrated in Figure 19a. The ova of *C. sinensis* is the smallest one, while the ova of *F. hepatica* is the largest. Large-sized asymmetric ova of *P. westermani* are yellow/golden-colored. The large-sized ova of *S. haematobium* are spiked at one end. The ova of *C. sinensis* and *S. haematobium* contain multinucleated and ciliated miracidium. The ova of *F. hepatica* and *P. westermani* contain unembryonated yolk cells without miracidium formation.

9. Structures confusing with infectious agents

Certain microscopic structures seen in cytology specimens are occasionally confusing with infectious agents [140]. Representative examples are shown below.
9.1 Incidental airborne contamination during specimen sampling

Incidental contaminants during the process of specimen preparation should be noticeable (Figure 60). A variety of living bodies floating in the air may attach onto cytology specimens rich in sticky mucinous material. These include pollen [141], non-pathogenic fungi (conidia of Alternaria alternata [142] and hyphae of Helicosporium [143]) and mites [144] in house dust. Hairs of carpet beetle larvae may be contaminated from cotton swabs or wooden spatulas [145]. Star-shaped algae commonly found in fresh water marsh may be contaminated in cytology specimens via laboratory water supply [146]. They are positive with PAS and Grocott stains. Ointment matrix may be contaminated in gynecologic cytology sampled from patients suffering from vaginal candidosis. The important notice is the absence of cellular response against the substances.

9.2 Larval nematodes incidentally contaminated in cytology specimens

Sputum cytology preparations may contain a larval nematode [147]. The larva is microscopically indistinguishable from pathogenic S. stercoralis, but the patient is asymptomatic with negativity of human immunodeficiency virus antibody. Only one larva is observed in the specimen, and repeated examination fails to show the nematode any longer. In such a case, the patient inhaled an egg of the free-living nematode in the soil, and the ovum hatched to larva in the airway. Nematode larvae may be directly contaminated from the soil in pediatric urine preparations and scraping cytology specimens sampled from severe-degree eroded athlete foot. Representative pictures are displayed in Figure 61.

9.3 Structures confusing with pathogenic microbes

Certain microscopic structures may resemble pathogenic microbes [140], as shown in Figure 62. Sharp-margined vacuoles formed in the cytoplasm of uterine

![Figure 60](http://dx.doi.org/10.5772/intechopen.95578)

**Figure 60.** Incidental airborne contamination during specimen sampling (Papanicolaou [a,b,c,d], Giemsa [c], a: Pollen, b: Alternaria alternata, c: Helicosporium, d: Mite). A variety of living bodies floating in the air may attach onto cytology specimens rich in sticky mucinous material. A pollen is seen in the cervical smear (a). The shape, color and size of pollen depend upon the kind of flowers and blossoms. The brown-colored conidia of A. alternata in the cervical smear show short breaks (b). Hyphae of Helicosporium in the blood sample should not be confused with microfilaria (c). Mites living in house dust have four pairs of short leg (d).
cervical columnar/metaplastic cells should be distinguished from chlamydial inclusions. Calcium urate crystals in the sediment of acidic urine may be confusing with *S. haematobium* ova. Note the size variation, thinness and the lack of miracidium to avoid confusion. Starch granules in sputum cytology may resemble *Paragonimus* eggs [148].

Aspirated *Entamoeba gingivalis* may be observed in sputum cytology specimens [149]. The non-pathogenic protozoa are especially plentiful in the mouth of patients with periodontitis and bad breath. Characteristically, they phagocytize neutrophils, as shown in Figure 30.
Airway aspiration of food residue may contain pieces of mushroom. Mushrooms in sputum cytology microscopically consist of parallel-arranged lamified hyphae similar to pathogenic *Aspergillus*. The presence of clamp connection at the site of septum is characteristic of mushroom cells [150]. Co-aspirated food-derived plant cells are often seen in the background (Figure 63).

9.4 Non-pathogenic fungi in sputum

Aspiration of air-floating non-pathogenic fungal conidia (spores) may induce growth of hypha-forming fungi in the sputum. Little cellular response is seen. Four different kinds of such fungi are presented in Figure 64: *Penicillium* spp., *Ductylaria (Ochroconis) gallopava*, *Petriellidium (Allescheria) boydii* and unknown fungus with a beaded appearance. From a clinical point of view, the appropriate recognition of non-pathogenic microorganisms in cytology specimens is requested. In other words, pathogenic hypha-forming fungi belong to either *Aspergillus*, *Mucor* or *Candida*. It should be noted, however, that these fungi may cause pneumonia in immunocompromised patients [151–153].

9.5 Myospherulosis

In myospherulosis (or spherulocytosis), macrophages contain clustered small globular material (endoboby) in the cytoplasm, suggesting infection of yeast-form fungi such as cryptococcosis and coccidioidomycosis. They accumulate in aspirated fluid of cystic lesions in the paranasal cavity or in the breast [154, 155]. PAS and Grocott stains are negative, and they may represent hemolytic red cells or fat droplets phagocytized by the macrophages (Figure 65). The term myospherulosis comes from small globular structures seen in a cystic lesion formed in the striated muscle of the neck [156].

Figure 63.
Aspirated mushroom in the sputum (Papanicolaou, a: Aspirated food debris, b-d: Clamp connection). Airway aspiration of food residues may contain pieces of mushroom. Co-aspirated food-derived plant cells (red arrow) are often seen in the background (a). Mushroom cells in sputum cytology microscopically consist of parallel-arranged, lamified hyphae similar to pathogenic *Aspergillus*. The presence of clamp connection (blue arrows) at the site of septum is characteristic of the mushroom cells (b-d). By courtesy of Mr. Tomohiro Watanabe, Chuken Kumamoto, Japan.
9.6 Grocott stain-positive structures confusing with cryptococcal yeasts or Pneumocystis jirovecii

It should be noted that Grocott methenamine silver staining stains not only fungi but also some microorganisms. Grocott may stain *Strongyloides stercoralis*, CMV and *Mycobacterium tuberculosis* [157]. Neutrophils and mucin granules are also
When bronchial brushing (scraping) cytology specimens are stained with Grocott method, mucin granules released from destroyed goblet cells show black granularity. The black-stained mucin granules resemble cryptococcal yeasts or cysts of *P. jirovecii* (Figure 66). For the diagnosis of pneumocystosis, bronchial/alveolar lavage solution should be evaluated, instead of the bronchial brushing cytology specimen.

Starch grains powdered on the surface of rubber gloves may be contaminated in sputum/bronchial cytology specimens [159]. Starch grains are also Grocott-reactive, and may be confused with yeast-type fungi. It is requested to use gloves without starch powders for preparing cytology specimens. Size variation and Papanicolaou-stained appearance (navel-forming figure and birefringence) make hints for distinction.

10. Conclusive remarks

The present review described varied cytomorphologic features of infection. Inflammatory cellular responses against pathogens are emphasized. Changes of sexual behavior, globalization-based increase of imported infection and the growing application of immunosuppressive therapy accelerate the chance to encounter unexpected or little-known infection. A wide variety of pathogens may cause infectious diseases. It is not easy for cytopathologists to prove the causative pathogen in cytology specimens. We must realize that the exact and prompt pathogenic diagnosis, with the aid of clinical and epidemiological information, may lead the patient to appropriate treatment. Avoidance of avoidable microbial transmission
eventually contributes to the safety of the human society. The recognition of the type of background cellular responses helps us make an appropriate cytodiagnosis.

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Innate Immunity in Health and Disease


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