THE INTERACTION IN VITRO OF PNEUMOCYSTIS CARINII WITH MACROPHAGES AND L-CELLS*

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Pneumocystis is an important cause of interstitial pneumonia in immunocompromised patients, yet little is known about the interaction of this organism with host humoral and cellular defense mechanisms (1–3). Investigations of Pneumocystis have been hampered in the past by difficulty in separating large numbers of viable organisms from host tissue, and by problems in documenting persistence or multiplication of organisms in vitro (4, 5). This report describes a model for studying in vitro Pneumocystis carinii with mammalian cells, and demonstrates features of the interaction of these organisms with alveolar and peritoneal macrophages and with fibroblasts.

Materials and Methods

Rat Alveolar Macrophage Cultures. 250 g male Sprague-Dawley rats (Taconic Farms, Inc., Germantown, N. Y.) were killed by intraperitoneal administration of 50 mg pentobarbitol (Abbott Laboratories, Diagnostics Div., South Pasadena, Calif.). The trachea was exposed, a polyethylene tube was inserted into the trachea by aseptic technique, and 50 cm³ of sterile saline was introduced in 5-cm³ aliquots. Material from one rat was used for each experiment. The lavage fluid was centrifuged at 30 g for 5 min, and the resulting sediment was resuspended in 4 cm³ of Eagle's minimum essential medium (MEM; Flow Laboratories, Inc., Rockville, Md.), with 20% heat-inactivated fetal calf serum (HIFCS; Grand Island Biological Co., Grand Island, N. Y.). 0.5 ml of this suspension (3 x 10⁷ cells/ml; approximately 70% macrophages) was placed on 22-mm square cover slips or 25-mm round cover slips in 35-mm plastic tissue culture dishes (Falcon Plastics, Div. BioQuest, Oxnard, Calif.). Dishes were incubated at 37°C in 5% CO₂-balanced air for 1 h to allow the macrophages to adhere firmly to the cover slip. The medium was then aspirated, the cover slips were washed once with MEM, then overlaid with 2.5 ml of fresh MEM containing 20% HIFCS, and incubated at 37°C in 5% CO₂ and balanced air.

Mouse peritoneal macrophage monolayers and L-cell monolayers were prepared by methods previously described by this laboratory (6).

Collection of Pneumocystis Carinii. Sprague-Dawley rats spontaneously developed Pneumocystis pneumonia 6–12 wk after treatment with Decadron (Merck Sharp & Dohme, West Point, Pa.), 0.01 mg/ml drinking water, and low (8%) protein diet (ICN Pharmaceuticals Inc., Life Sciences Group, Cleveland, Ohio). Tetracycline hydrochloride (Pfizer Inc., New York), 1 mg/ml drinking water, was added to decrease complications due to bacterial infections. Supernate resulting from centrifugation of bronchial lavage fluid at 30 g for 5 min was recentrifuged at

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Abbreviations used in this paper: HIFCS, heat-inactivated fetal calf serum; MEM, Eagle's minimum essential medium.
1,300 g for 30 min. The resulting sediment was resuspended in 0.5-2 cm³ of MEM/20% HIFCS, and a drop was placed under a cover slip and examined for the presence of *Pneumocystis* by phase contrast and light microscopy.

**Preparation of Antipneumocystis Serum.** Sediment from high speed centrifugation was lyophilized. Rabbits were inoculated at three injection sites, intrascapular and both thighs, with 10 mg total of lyophilized *Pneumocystis* suspended in distilled water and mixed with equal volumes of complete Freund’s adjuvant (Difco Laboratories, Detroit, Mich.). 14 days later, second inoculations of 10 mg of lyophilized *Pneumocystis* mixed with equal volumes of incomplete Freund’s adjuvant were given. The rabbits were bled 14 days after the second inoculations. The serum was separated and frozen at -35°C. Before use, serum was heat inactivated at 56°C for 30 min and adsorbed with normal rat lung for 12 h at 37°C. Control serum was obtained from rabbits that had not been immunized.

**Evaluation of Pneumocystis in Mouse Peritoneal Cavities.** High speed sediment from two heavily infected rats was resuspended in 2.0 cm³ of MEM and inoculated into the peritoneum of six CFW mice. Two mice were sacrificed at 1, 3, and 7 days each. The peritoneum was exposed and lavaged with 2 cm³ of phosphate-buffered saline. The lavage fluid was centrifuged at 1,300 g for 20 min, and the pellet resuspended in 0.2 cm³ of MEM. The resulting material was cytacentrifuged, stained with Giemsa, and examined by light microscopy.

**Methods for Evaluating Pneumocystis—Cell Interaction**

**Light and Phase Contrast Microscopy.** The culture medium was aspirated from *Pneumocystis*-infected macrophage monolayers. For light microscopy the cells were fixed in methanol for 3 min, and then stained with Giemsa for 30 min. Cover slips were then mounted on a glass slide and examined with a Zeiss photomicroscope with × 63 oil immersion objective (Carl Zeiss, Inc., New York). For phase contrast microscopy, the cells were fixed in 2.5% glutaraldehyde (Fisher Scientific Company, Pittsburgh, Pa.) in 0.1 M Na cacodylate buffer, pH 7.4, for 10 min at 4°C. Cover slips were then removed, inverted on a drop of distilled water, and rimmed with paraffin—vaseline. Cover slips were examined employing a Zeiss photomicroscope with × 63 Neofluor phase objective. Cultures were examined for general morphology of cells and organisms, and for degree of *Pneumocystis* infection. *Pneumocystis* cysts and trophozoites were readily identified by light or phase contrast microscopy, and were easily distinguished from round cellular structures or debris. The position of *Pneumocystis* in relation to the cells was evaluated, the percent of macrophages infected was determined, and the number of *Pneumocystis* adherent to each macrophage was counted.

For study of viable, unfixed infected monolayers, the macrophages were placed on round cover slips and infected with *Pneumocystis*. After incubation for 1 h, the cover slips were removed from plastic dishes and placed in a Sykes-Moore chamber (Bellco Glass, Inc., Vineland, N. J.), which was filled with fresh culture medium, and maintained at 37°C by an air curtain incubator (Sage Instruments, White Plains, N. Y.) and observed continuously by phase contrast microscopy. Polyethylene tubes (inside diameter 0.018) attached to 25-gauge needles provided inlet and outlet ports for introduction of fresh medium or antipneumocystis serum.

**Electron Microscopy.** Medium was aspirated from the cultures, and the cover slips were rinsed once with saline at room temperature. Cover slips were then flooded with 2.5% glutaraldehyde at room temperature and after 5 min, the cell sheet was scraped off with a plastic policeman. The suspended cells were transferred to a 3-ml conical tube and chilled in an ice bath. The cells were then fixed in a mixture of glutaraldehyde and osmium, exposed to uranyl acetate, and embedded in agar and then in epon as described in detail previously (7). Thin sections stained with lead and uranyl solutions were examined in a Siemens Elmiskop I (Siemens Corp., Iselin, N. J.) at 80 kV with a 50-μm objective aperture.

**Autoradiography.** Macrophage and L-cell monolayers were exposed for 1-20 h to fresh medium (MEM with 20% dialyzed HIFCS) containing 1-20 μCi/ml of tritiated uridine, tritiated thymidine, or tritiated leucine (New England Nuclear, Boston, Mass.) either before or after infection with *Pneumocystis*. Cover slips were then fixed in glutaraldehyde, washed, treated with 5% trichloroacetic acid for 1 h at 4°C, washed and dried, coated with L-4 emulsion (Ilford Ltd., Essex, Eng.), reacted for 6 days in the dark, developed for 2 min at 20°C, and stained with Giemsa.
Results

Preliminary Studies on Identifying Pneumocystis and Establishing Infection of Cell Cultures. Pneumocystis organisms were obtained in sufficient number from the bronchial lavage fluid of about 20% of the rats that had been treated with corticosteroids and low protein diet for 8–12 wk. It was found that a majority of Pneumocystis could be separated from alveolar macrophages and other cells by differential centrifugation. The sediment obtained from low speed centrifugation (30 g) contained $3 \times 10^7$ mononuclear cells and approximately 10% of the total Pneumocystis. The sediment from the high speed centrifugation (1,300 g) contained $1 \times 10^6$ mononuclear cells and 90% of the total Pneumocystis. The total number of Pneumocystis obtained from the bronchial washings was dependent on the severity of infection of the individual animal, but the number reached as high as $10^9$ organisms. Bronchial lavage fluid occasionally contained a few erythrocytes. Platelets were not identified. Lavage fluids heavily contaminated with blood cells were easily identified, and they were discarded.

Under phase contrast microscopy, both the trophozoite form and the cyst form of Pneumocystis could easily be recognized either with fresh or glutaraldehyde-fixed material. The vast majority of Pneumocystis were round or oval, 3 to 6 μm in diameter, and had a thin phase-dense limiting membrane, and a phase-dense nucleus (Fig. 1a). Less than 1% of the organisms were round, 4 to 8 μm in diameter, had a thick phase-dense limiting membrane, and contained four to eight round, phase-dense bodies, which were 1 μm in diameter (Fig. 1b). The former have been designated the trophozoite form; the latter have been designated the cyst form (3, 8, 9). Copious Pneumocystis were seen only in lavage fluid which also showed Pneumocystis by Giemsa and Gram Weigert staining of bronchial lavage fluids and lung sections. No trophozoite or cyst forms were seen in lavage fluid from control, noncorticosteroid-treated rats. Trophozoites adhered to glass cover slips but they did not appear to spread or change morphologically. Under electron microscopy, two forms of Pneumocystis which conformed to stages previously designated the trophozoite and the cyst could be recognized (3, 8, 9). The trophozoite was the predominant stage observed (Fig. 2a). Forms similar to the sporozoite were seen without a surrounding trilaminar membrane. A few thick-walled cysts were also seen (Fig. 2b). Numerous forms were observed that could not clearly be classified as cyst or trophozoite, suggesting transitional stages between these forms.

Rat alveolar macrophages from corticosteroid-treated animals were large glass-adherent cells (30–70 μm), which increased in size during cultivation in vitro. About 30% became flat and well spread. The rest remained round, with a small portion of flat ruffled membrane. The alveolar macrophages contained numerous phase-lucent vacuoles, many of which contained phase-dense debris. No difference in morphology could be observed among rat alveolar macrophages from control animals, from steroid-treated Pneumocystis-infected animals, and from steroid-treated uninfected animals.

When $10^6$–$10^7$ Pneumocystis trophozoites were added to rat alveolar or mouse peritoneal macrophage monolayers (Fig. 1a) or L-cell monolayers, the majority of organisms were observed by phase contrast microscopy to adhere to the
FIG. 1. Phase contrast microscopic appearance of *Pneumocystis*-infected alveolar macrophages from a steroid-treated rat, cultured for 4 h. The well-spread macrophage on the left (a) shows a central nucleus, multiple phase-lucent vacuoles, granules, and lipid bodies, and a large ruffled edge of clear cytoplasm. Multiple *Pneumocystis* trophozoites are adherent to the surface of the macrophage. The trophozoites appear as round bodies 3-6 μm in diameter with a phase-dense limiting membrane and a phase-dense nucleus. The alveolar macrophage on the right (b) is out of the plane of focus, but an adherent *Pneumocystis* cyst (arrow) is clearly seen. The cyst appears as a round organism 4-8 μm in diameter with a thick phase-dense limiting membrane which encloses round phase-dense bodies, the sporozoites, three of which are in the focal plane shown. (Glutaraldehyde fixation, × 1,200.)

Surface of the cells. The number of trophozoites adherent to an individual cell depended on the quantity of *Pneumocystis* added to the monolayer. Well-spread cells had up to 40 adherent *Pneumocystis* when heavy inocula were added. No change in macrophage or L-cell morphology was observed after the addition of the *Pneumocystis*. Some trophozoites adhered to glass and some floated free in the medium. Repeated washings of the monolayer did not cause significant detachment of *Pneumocystis* from the macrophages or L-cells. After 24 h in culture and two to three washings, however, only cell-adherent organisms were common; few *Pneumocystis* remained free in the medium or adherent to the glass cover slip.

FIG. 2. Electron microscopic appearance of *Pneumocystis carinii* after 4 h in vitro culture with rat alveolar macrophage. (a) A *Pneumocystis* trophozoite (t) is shown. The trophozoite has a trilaminar limiting membrane. The nucleus is visualized, and structures consistent with mitochondria are seen in the cytoplasm. (× 54,500). (b) A *Pneumocystis* cyst is shown. The limiting membrane is composed of multiple layers. Tubular structures exterior to the limiting membrane can be seen in cross section. Other sections not shown here demonstrate that these arise from the cyst's surface. The complex limiting membrane encloses electron-dense sporozoites (s), four of which can be seen here, each with its nucleus. × 44,500.
Most trophozoites were clearly adherent to the surface of well-spread macrophages and L-cells. Occasionally a phase-lucent vacuole was identified in an alveolar or peritoneal macrophage in which a well-defined trophozoite was identified. Trophozoites were not seen within fibroblasts. A few cysts were seen adherent to macrophages and fibroblasts, a few were seen free in the medium and, in rare instances, cysts were seen within vacuoles in macrophages.

Under electron microscopy, trophozoites were identified in close approximation to rat alveolar macrophage surface membranes. Some appeared to be adherent to villous projections of the macrophage. No special morphologic feature was evident at the attachment site. A clear space of approximately 100 Å separated the unit membrane of the macrophage and the attached *Pneumocystis*. By electron microscopy, trophozoites or cysts were not seen intracellularly in macrophages in the absence of antiserum.

**Persistence of Pneumocystis In Vitro.** Trophozoites remained adherent to the surface of rat alveolar macrophages, mouse peritoneal macrophages, or L-cells for at least 72 h without significant changes in their total number, morphology or relationship to cell surfaces (Fig. 3). About 20% of adherent trophozoites developed one or more phase-lucent vacuoles during this time. Trophozoites with phase-lucent vacuoles were present in small numbers in fresh bronchial lavage fluid, but they were abundant in monolayers incubated for as little as 12–24 h in nonsupplemented balanced minimum essential media. During the 72-h period, infected mouse peritoneal macrophages and L-cells remained well spread. Many rat alveolar macrophages became rounded and detached from the cover slip after 72 h, leaving a sparse monolayer of cells. The morphology of well-spread, heavily infected rat alveolar macrophages, mouse peritoneal macrophages, or L-cells did not change substantially during the 72-h period, although phase-dense amorphous material could be seen in vacuoles of some macrophages.

Addition of [*H]uridine (20 µg for 1–20 h), [*H]thymidine (20 µg for 1–20 h), or [*H]leucine (20 µg for 1–20 h) to *Pneumocystis*-infected rat alveolar macrophages or L-cells demonstrated grains of [*H]uridine and [*H]thymidine over 10–20% of trophozoites. No specific pattern of uptake over the individual trophozoites was seen. Uptake of [*H]leucine was not seen. Several cysts were observed with heavy uptake of [*H]uridine and [*H]thymidine. Addition of [*H]uridine, [*H]thymidine, or [*H]leucine to monolayers for 4–20 h before infection and then addition of trophozoites resulted in heavy labeling of the monolayer cells, but no labeling of the trophozoites.

Attempts to maintain *Pneumocystis* by inoculation of organisms into mouse peritoneal cavities were unsuccessful. *Pneumocystis* were not seen on Giemsa stain of peritoneal cells lavaged from mice 1, 3, and 7 days after intraperitoneal inoculation of 10⁷ trophozoites per mouse.

**The Interaction of Trophozoites and Macrophages and the Effect of Rabbit Anti-Pneumocystis Serum.** Continuous monitoring of heavily infected rat alveolar macrophages in a Sykes-Moore chamber for 120 min revealed that trophozoites remain adherent to the cell surface when MEM with 20% FCS is used, and the system maintained at 37°C. Observation of cells with 10–35 adherent trophozoites demonstrated no detachment of the trophozoites from the surface and no ingestion of the organisms. When MEM with 20% normal
rabbit serum was added to the system, no change in morphology of the cells or trophozoites was observed, nor was there a change in the relationship of the organisms and cells. When MEM with 20% rabbit antipneumocystis serum was added after 30 min of observation, dramatic changes occurred (Fig. 4 a and b). Within 20 min after the addition of the antiserum, each adherent trophozoite was directly observed to be engulfed by the surface membrane of the rat alveolar macrophage. The trophozoite could be recognized within the phase-lucent vacuole for only 2–4 min, after which it lost its characteristic morphology. Only amorphous phase-dense material within the vacuole could be seen, and the trophozoite could no longer be recognized. The rat alveolar macrophage became more conspicuously vacuolated, with phase-dense amorphous material in many vacuoles. The cell rounded slightly and exhibited extensive ruffled membranes. No morphologic changes were recognized in trophozoites adherent to the cover slip. Addition of rabbit antipneumocystis serum to rat alveolar macrophages from either control animals or from corticosteroid-treated but noninfected animals resulted in no morphological changes in the macrophages.

By electron microscopy, in preparations incubated for 1 h with MEM/20% rabbit antipneumocystis serum, macrophages were seen to contain more electron-lucent vacuoles than noninfected cells. Some vacuoles contained well-defined trophozoites (Fig. 4 c). Others contained trophozoites with poorly defined membranes, consistent with degenerating forms, whereas some vacuoles contained only amorphous electron-dense material. In no vacuole was there any evidence that trophozoite or cyst replication was taking place.

The opsonic titer of the antiserum was determined by morphologic examination of infected cells 1 h after addition of antiserum. The fraction of adherent trophozoites that were ingested by the macrophages was dependent on the degree of infection. When the rat alveolar macrophage had 1–10 adherent trophozoites, all well-spread macrophages ingested virtually all adherent organisms. When rat alveolar macrophages had 30–50 adherent trophozoites, most cells ingested less than 75% of adherent organisms. Fig. 5 shows a representative experiment assessing the effect of rabbit antipneumocystis serum on adherent trophozoites. In this experiment, a serum dilution of 1:256 or less caused 50% of infected cells to ingest all surface organisms.

**Fig. 3.** Persistence of *Pneumocystis* trophozoites in vitro adherent to alveolar macrophages.
Mouse peritoneal macrophages were also able to ingest adherent trophozoites. Incubation of heavily infected mouse peritoneal macrophages for 2 h with MEM/20% FCS or MEM/20% normal rabbit serum resulted in no decrease in the number of adherent trophozoites per cell, and no change in morphology of the cell macrophage or the organism. Addition of rabbit antipneumocystis serum resulted in ingestion of surface organisms, and more prominent vacuolization of the macrophages. The fraction of adherent trophozoites that were ingested was dependent on the heaviness of the initial infection.

Addition of rabbit antipneumocystis serum to heavily infected L-cells for 60 min caused no changes in the relationship of trophozoites to the L-cells and no morphological changes in the trophozoites or the L-cells.

Discussion

Pneumocystis is an important cause of pneumonia in immunologically altered patients. However, little is known about its transmission, life cycle, biochemistry, or its response to host cellular and humoral immune mechanisms (1-4, 8-12). The organism has been observed in the lungs of many animals, including rodents, goats, horses, monkeys, and man (1-3). Over 20 yr ago it was implicated as a cause of pneumonia in institutionalized infants (10). Recently,
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*Pneumocystis* was recognized as a cause of interstitial pneumonia in patients receiving cancer chemotherapy or corticosteroid therapy, and in patients with hereditary deficiencies of B or T lymphocytes (1-3, 11, 12).

Observations in this report indicate that *Pneumocystis* cysts and trophozoites can be obtained in large numbers by bronchial lavage. Because *Pneumocystis* maintained a typical morphology while cultured in vitro for several days, and 10-20% took up radiolabeled nucleotides, the relationship of these organisms and macrophages or L-cells could be studied. *Pneumocystis* adhered in a similar manner to L-cells, alveolar macrophages from normal or steroid-treated rats, and peritoneal macrophages. After the addition of antipneumocystis serum, rapid interiorization of the surface organisms occurred when they were adherent to macrophages but not to L-cells. The opsonic titer of this antiserum was determined to be 1:256. Organisms adherent to L-cells were not altered morphologically after the addition of antipneumocystis serum. They were promptly destroyed within macrophages after ingestion. Persisting or multiplying intracellular forms were not seen. These studies suggest that humoral and cellular mechanisms may be necessary in combination to deal with *Pneumocystis* infection.

Previous investigators have identified two basic forms of *Pneumocystis* by bright field, phase contrast, and electron microscopy (3, 8, 9). One form has been designated the cyst, and the other the trophozoite. A life cycle has been proposed (8, 9). During a 4- to 6-h period, a trophozoite presumably develops into a thick-walled cyst and six to eight sporozoites then develop within the cyst. Sporozoites are subsequently released into the surrounding medium and develop into trophozoites (3, 8, 9).

The cyst is the form of *Pneumocystis* usually demonstrated in clinical specimens, and the form studied recently in other in vitro studies (3, 4). Investigations reported here show that in rat bronchial lavage fluid, the trophozoite is by far the predominant form (over 99%). Other investigators have obtained large numbers of cysts from minced lung preparations, but the presence of trophozoites was not evaluated (4, 5). The relative abundance of cysts to trophozoites has not been assessed (3-5, 13, 14). Whether preparations of minced lung actually contain a larger yield of cysts than does bronchial lavage fluid is uncertain. It is conceivable that the cysts are more tightly adherent to alveolar tissue, and therefore less abundant in lavage fluid than in minced preparations. The predominance of trophozoites in rat bronchial lavage fluid suggests that this form of the organism is most important in transmission of *Pneumocystis* among susceptible species of mammals.

Phase contrast microscopy is superior to bright field microscopy for determining the relationship of *Pneumocystis* cysts and trophozoites to host cell membranes and vacuoles. Under phase contrast microscopy, the two forms of *Pneumocystis* can be readily recognized, and they are distinct from erythrocytes, platelets, and mononuclear cells.

When examined by transmission electron microscopy, the appearance of cysts and trophozoites in infected monolayers corresponded to morphologic descriptions in tissue (3, 8, 9). A variety of forms were seen which had features of both the cyst and trophozoite. This is consistent with gradual transition of one form into the other.
Close adherence of *Pneumocystis* to phagocytic and nonphagocytic cells was common, not easily reversed, and morphology of the organism or cell was not changed by the attachment. The limiting membrane of the trophozoite was separated from the macrophage surface by about 100 Å, and there was no evidence of fusion or junctional areas. Pseudopodial extensions or filaments between the trophozoite and host macrophage or the L-cell were observed by scanning electron micrographs (15). The origin of these extensions and their function are unknown.

Alveolar macrophages have IgG and complement receptors characteristic of phagocytic cells, and trophozoites from steroid-treated rats may be coated with immunoglobulin or complement in the rat alveoli before lavage (16). Attachment of the trophozoites to the alveolar macrophage by these molecules has been considered (17). In addition, Lobuglio et al. (18) demonstrated binding of IgG-coated red cells to mononuclear cells. Free gamma G inhibited attachment, and papain treatment released the red cell from the macrophage. It is unlikely that *Pneumocystis* trophozoites attach by a similar mechanism because attachment occurs to both phagocytes and nonphagocytes. The nature of host cell receptors for *Pneumocystis* is unknown.

Engulfment of trophozoites does not follow attachment in the absence of antipneumocystis serum. Other systems in which engulfment does not rapidly follow attachment have been described. Engulfment of glutaraldehyde-treated red cells followed attachment only when serum, proper temperature, and divalent cations were provided (19). These manipulations, as well as addition of metabolic inhibitors, can prevent engulfment when specific antibody or serum containing "natural" antibody is used to promote attachment (20–22). *Mycoplasma pulmonis* has been shown to attach to macrophage surfaces, but ingestion does not occur rapidly without the addition of anti-mycoplasma antibody (6). *Pneumocystis* trophozoites appear to interact with macrophages in a similar fashion. Further studies will be needed to elucidate which component of the *Pneumocystis* surface membrane participates in the attachment process.

Observation of living cells in the Sykes-Moore chamber confirmed that *Pneumocystis* trophozoites were extracellular, and there was no evidence for intracellular persistence or replication of any form of *Pneumocystis*. These studies do not support a role for intracellular *Pneumocystis* in the pathogenesis of *Pneumocystis* (3, 23). The *Pneumocystis* used in these studies were viable. Organisms remained adherent to well-spread macrophages for at least 72 h, resisted engulfment, and most showed few morphologic alterations. Because few trophozoites remained free in the medium after the first 12 h in culture, macrophages were not phagocytizing surface trophozoites and accumulating newly adherent organisms from the medium. In addition, phase contrast and electron microscopy did not reveal significant numbers of intracellular organisms. The ability of 10–20% of the trophozoites and cysts to take up radiolabeled uridine and thymidine supports the viability of these organisms in this system. We have not substantiated a recent report that *Pneumocystis* can take up radiolabeled nucleotides and amino acids from a monolayer that had been labeled before the addition of organisms (4).
Phase contrast observations of heavily infected alveolar macrophages allowed evaluation of the effect of antipneumocystis serum. Within 30 min after the addition of antisera, each adherent trophozoite was engulfed by macrophage surface membrane, entered a phase-lucent vacuole, and was rapidly destroyed. The importance of antibody in the immune response to *Pneumocystis* has been suggested by the clinical settings in which the disease occurs, and by a few experimental observations (1-3).

Addition of 10% antipneumocystis serum for 1-8 h to *Pneumocystis*-infected L-cells induced no change in the morphology of the trophozoites or the L-cells, and no ingestion of the organisms. The inability of L-cells to ingest and digest trophozoites emphasizes the difference between "professional" and "nonprofessional" phagocytes, and parallels similar results obtained with *M. pulmonis* and glutaraldehyde-fixed erythrocytes (6, 19). These results also suggest that antipneumocystis serum does not substantially alter the viability of trophozoites directly.

Brzosko (17) has suggested that a deficiency in complement interaction with organisms was responsible for disease because he found large amounts of immunoglobulin in the lungs of children with *Pneumocystis*. The predominant immunoglobulins in the lung exudate were IgG and IgM, with only small amounts of IgA. *Pneumocystis*-immunoglobulin conglomerates had rheumatoid factor activity and B-1-C globulin. Our studies did not confirm a requirement for complement in the antibody-induced phagocytosis of *Pneumocystis*.

To investigate the effect of corticosteroids on alveolar macrophages in *Pneumocystis* pneumonia, studies were performed comparing alveolar macrophages obtained from nonsteroid-treated rats, with those obtained from steroid-treated rats with *Pneumocystis* pneumonia. Heavily infected monolayers of normal rat alveolar macrophages demonstrated no ingestion of trophozoites after 1 h unless specific antiserum was added. There were no qualitative differences in the ability of the two macrophage populations to ingest and digest *Pneumocystis*.

Absence of an effect by corticosteroids on the phagocytic function of macrophages is consistent with previous observations in this laboratory and others (24-25). Infiltration with mononuclear cells is a part of the pathologic appearance of pneumocystosis (26). The well-described effects of corticosteroids on lymphocytes (i.e. release of lymphokines chemotactic for monolayers and cytotoxic substances) and on migration of inflammatory cells from the vascular system appear more significant than a direct effect of steroids on macrophage function (27).

Taken together, these observations support a role for opsonic antibody and macrophages in the control of *Pneumocystis* infection in the lung. They are consistent with the clinical observation of disease in both hypogammaglobulinemic and corticosteroid-treated patients.

**Summary**

A model was developed for studying the interaction between *Pneumocystis*, rat-derived cells, and humoral factors. *Pneumocystis* were obtained in large quantity by bronchial lavage of steroid-treated rats. The trophozoite was the predominant form obtained, and it could readily be recognized by phase
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contrast microscopy. Organisms maintained a typical morphology for at least 3 days in culture, and 10–20% took up radiolabeled nucleotides. *Pneumocystis* readily adhered to cell surfaces in a similar manner in alveolar macrophages from steroid-treated or normal rats, mouse peritoneal macrophages, and L-cells. Adherent organisms were not interiorized to a significant degree in the absence of antipneumocystis serum. After addition of rabbit antipneumocystis serum, rapid interiorization of organisms occurred from the surface of macrophages but not L-cells. Organisms appeared to be promptly destroyed within macrophages after interiorization. Persisting or multiplying intracellular forms were not seen. Antipneumocystis serum did not morphologically alter *Pneumocystis*.

These observations suggest a role for antibody and mononuclear phagocytes during the immune response to *Pneumocystis*.

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