Br. J. exp. Path. (1987) **68**, 267–276

Experimental *Pneumocystis carinii* pneumonia in the ferret

Dennis C. Stokes, Francis Gigliotti, Jerold E. Rehg, Richard L. Snellgrove and Walter T. Hughes

From the Cardiopulmonary, Infectious Disease, and Comparative Pathology Divisions, St Jude Children's Research Hospital, Memphis, TN, USA

Received for publication 28 April 1986
Accepted for publication 14 October 1986

Summary. *Pneumocystis carinii* pneumonia (PCP) was provoked in the ferret, *Mustela pulorius furo*, by immunosuppression with daily long-term administration of cortisone acetate, 10–20 mg/kg subcutaneously for 9 to 10 weeks, Microscopically *P. carinii* was observed in the lungs of all 11 treated animals: mild to moderate in five and extensive disease in six. The histopathological features of PCP in the ferret included interstitial pneumonitis, scant mononuclear cell alveolitis, with abundant cysts and trophozoites visible in a focal distribution. There were few neutrophils present. Electron microscopy showed large numbers of both cysts and trophozoites in close association with type I cells. No bacterial pathogens were isolated from the lungs of immunosuppressed animals but an unexplained eosinophilic enteritis was present in treated animals. *P carinii* pneumonia developed without significant body weight loss during corticosteroid administration, unlike previously described studies using corticosteroid-treated rodents. Ferrets thus appear to be a 'steroid resistant' animal, like man, and therefore a more suitable model for immunological studies of host response to PCP than rodents. This new model also has practical advantages over previously described animal models of PCP, including larger lung and airway size.

Keywords: *Pneumocystis carinii*, ferret

Pneumocystis carinii resides as a saphrophyte in the lungs of a variety of animal species, including rodents, pigeons, goats, cattle, pigs, primates and man (Walzer 1984; Shimizu *et al.* 1985). In addition, it is a major cause of morbidity and mortality in immunosuppressed hosts, particularly those with the acquired immunodeficiency syndrome (AIDS) (Gottlieb *et al.* 1981; CDC Update 1986). The corticosteroid-treated rodent with *P. carinii* pneumonia developed by Weller (1955) and Frenkel *et al.* (1966) has been used in most experimental studies of this organism. It has proven extremely valuable in elucidating the microbiology and chemotherapy of *P. carinii* pneumonitis (Hughes *et al.* 1974) but better animal models of this important infection are needed.

The ferret, *Mustela pulorius furo*, is a small

Supported in part by American-Lebanese-Syrian Associated Charities and by NIH grant AI-23302.
Presented in part at the 1986 Annual Meeting of the American Thoracic Society, Kansas City, MO, USA.

Correspondence: Dr Dennis C. Stokes, St Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38101, USA.

mammalian carnivore used as an animal model for other respiratory infections, including influenza and respiratory syncytial virus (RSV) (Sweet *et al.* 1985; Porter *et al.* 1980). More recently it has been used in studies of lung growth and physiology (Vinegar *et al.* 1982; McBride 1985). We successfully provoked experimental *P. carinii* pneumonia in the ferret and describe here the major histopathological features of the infection in this animal, including light and electron microscopy. Use of the ferret in studies of *P. carinii* has several major advantages over previously described animal models.

Materials and methods

Animals. The ferrets in the study were young adult males of two different cohort groups, obtained from a commercial breeder (Marshall Farms, North Rose, NY). The initial group was composed of five ferrets, 12-weeks old, weighing 886 ± 44 g and the second group consisted of six ferrets, approximately 8-weeks old, weighing 503 ± 58 g. Each ferret group was housed in a stainless steel rabbit cage placed in a 4ft × 6ft cubicle with dual air flow within a Class P2 infectious containment suite. The cubicle was maintained at 23 ± 1°C, a minimum relative humidity of 50%, 20 air changes per hour and a 12 h light–dark cycle. The animals were provided cat chow (Purina, St Louis, MO) and water *ad libitum*.

Medications. The initial ferret group was administered cortisone acetate (Merck, Sharpe & Dohme, West Point, PA), 10 mg/kg subcutaneously, once daily for 5 days followed by 2 days without drug. Since the animals appeared healthy and had gained weight normally after 4 weeks on the 10 mg/kg dose, the daily dose of cortisone acetate was increased to 20 mg/kg at the same schedule, continued for a total of 5–6 weeks. The second group received the 20 mg/kg daily dose of cortisone acetate for 10 weeks. Tetracycline hydrochloride (E.R. Squibb & Sons, Princeton, NJ), 1 g/l, was also added to both group's drinking water as prophylaxis for bacterial superinfection.

Lung histopathology. Two animals from the first group were killed by pentobarbital overdose after 9 weeks of total corticosteroid therapy and sections of the left lower and right upper lobe were placed in 10% buffered formalin. The remaining three animals in this group were killed by pentobarbital overdose and aortic transection after 10 weeks of steroid therapy. After careful removal of the trachea and lungs of these three animals, the left diaphragmatic lobe bronchus was cannulated and the lobe fixed via the airway with 10% buffered formalin at 25 cm H_2O transpulmonary pressure. Sections of the uninflated right apical and lower lobes were also placed in formalin.

Formalin-fixed, paraffin-embedded sections were stained with haematoxylin and eosin, Gomori's methenanine silver nitrate (GMS), and Giemsa stains. Formalin-fixed lung sections were also embedded in plastic (glycol methacrylate; Fischer, Springfield, NJ) and thin sections (2 μm) were stained with haematoxylin and eosin and Giemsa (Bianco *et al.* 1984). The latter stains were incubated for 2 h to assure adequate staining of trophozoites (Yoshida *et al.* 1981).

Slices of fresh ferret lung were also fixed overnight in 2% glutaraldehyde in 0·1 M cacodylate buffer. Tissue was cut into small blocks and post-fixed in 1% osmium tetroxide in the same buffer for 1 h. The blocks were stained en bloc with 2% uranyl acetate, dehydrated in gradients of ethanol and embedded in Spurr resin (Ladd Research Industries, Inc., Burlington, VT). Ultrathin sections were stained with lead citrate and examined with a Phillips 301 electron microscope.

All six animals in the second group were treated for 10 weeks. Sections were then obtained from the right lower lobe at necropsy and fixed in 10% formalin and lung sections embedded in paraffin and stained

with GMS, haematoxylin–eosin, and Giemsa as noted.

Postmortem examination. Four of six animals from the second group also had postmortem examinations performed by a veterinary pathologist. After gross examination of the organs, sections were removed from the lung, liver, spleen, kidney and intestine and fixed in formalin and embedded in paraffin for haemotoxylin–eosin stains. Cultures of the lung and cecum were inoculated on blood agar and selective media for detection of aerobic and anaerobic pathogens. The aerobic cultures were incubated in atmospheric air at 37°C. The microaerophilic cultures were incubated in 10% CO_2 at 42°C and the anaerobic cultures were processed at 25°C and incubated at 37°C in an anaerobic chamber containing 85% N, 10% H and 5% CO_2.

Results

Prior to necropsy all animals appeared healthy except for a mild coat roughing. Animals in the second group treated at 20 mg/kg appeared to have an increase in coat oil production toward the end of the study, resulting in a 'greasy' character to their coat. Mean body weights for the corticosteroid-treated animals paralleled the expected curve for normal male ferrets, although the second group appeared to show some growth retardation (Fig. 1).

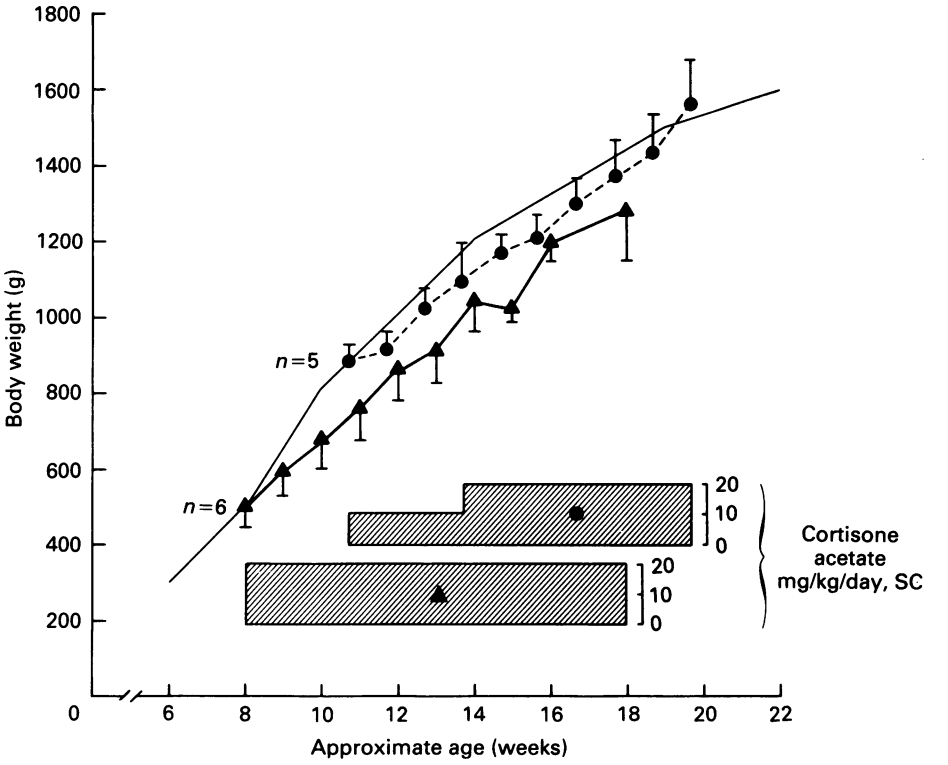


Fig. 1. Mean body weights (± 1 s.d.) for the ferrets during corticosteroid immunosuppression (●, 10 mg/kg/day × 4 weeks, then 20 mg/kg/day; ▲, 20 mg/kg/day). Thin solid line represents growth curve for normal male ferrets (courtesy of Marshall Farms, North Rose, NY; no standard deviations available).

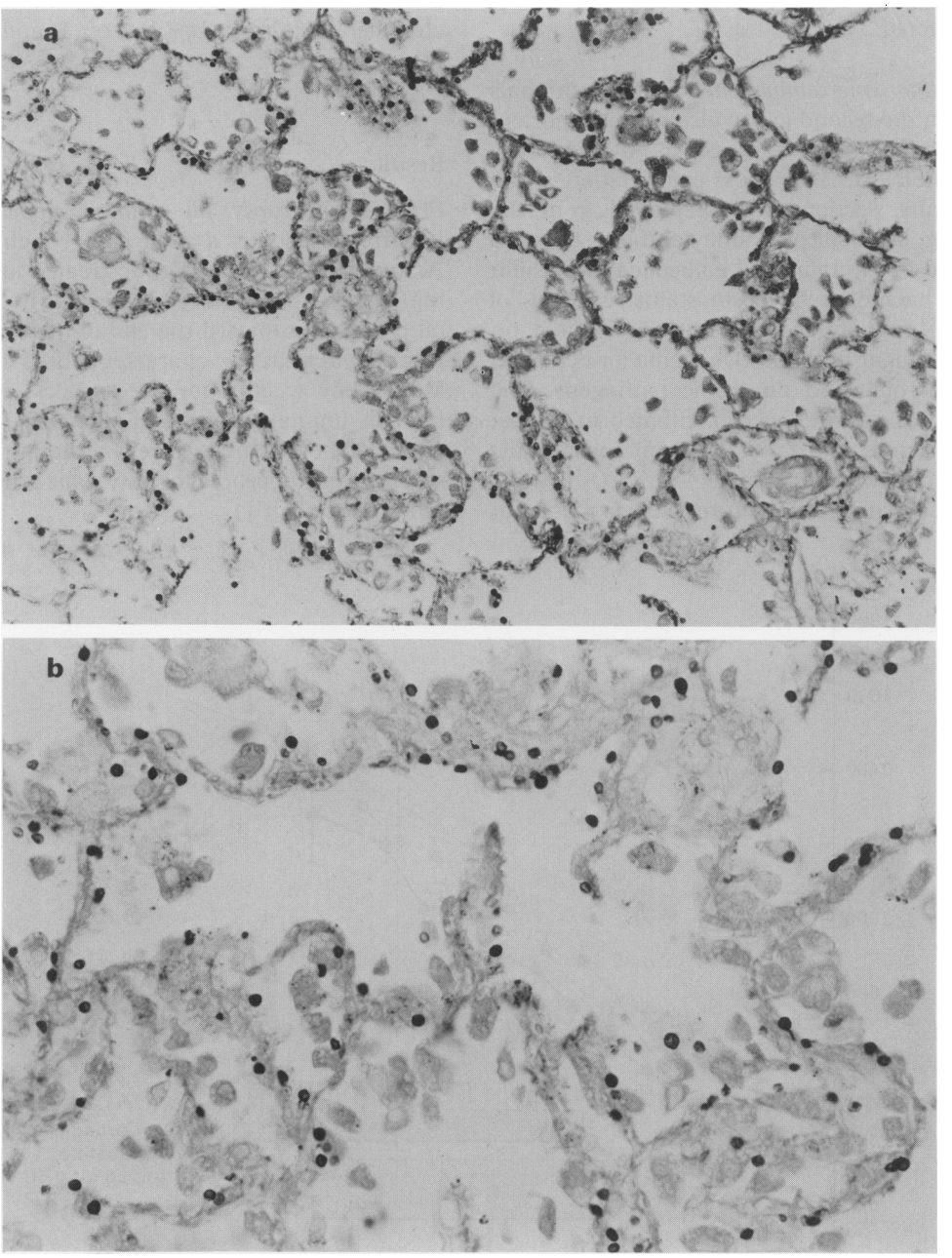


Fig. 2. *a*, Gomori's methenamine silver nitrate-stained section of inflated lung (original magnification, × 200). Numerous *P. carinii* cysts are seen at the alveolar septa with a mild alveolar reaction. *b*, At higher magnification, the cysts are rounded, oval, or cup-shaped structures that stain brownish-black with this stain (× 380).

Postmortem examination

The lungs were pink and normal on gross examination even in the heavily infected animals. Other organs also appeared normal except for the liver which had a yellow-tan appearance. A large amount of fat was present in the abdomen and chest.

Lung: Gomori's methenamine silver nitrate stain (GMS)

All 11 of the experimental animals had evidence of infection with *P. carinii* as demonstrated by GMS stains. Three animals in the first group (two at 9 weeks, one at 10 weeks) showed abundant *P. carinii* cysts with a widespread but patchy distribution (Fig. 2). Two animals at 10 weeks had only scattered focal accumulations of cysts. There was no clear difference between upper and lower lobes in severity of infection. In the second group of animals, *P. carinii* cysts were focally distributed in the lungs of all six, with three having moderate-extensive cysts and three having moderate infection. No pneumocysts were seen in sections from several control, non-immunosuppressed ferrets.

Haematoxylin and eosin (H & E)

H & E stained sections showed focal areas of interstitial pneumonitis and alveolitis, with an intra-septal and intra-alveolar infiltrate of mononuclear cells. Generally, there was little cellular reaction considering the numbers of organisms present in GMS stained sections. Polymorphonuclear leucocytes were rarely seen even in areas of extensive infection. Although thick paraffin-imbedded sections do not generally show *P. carinii*,

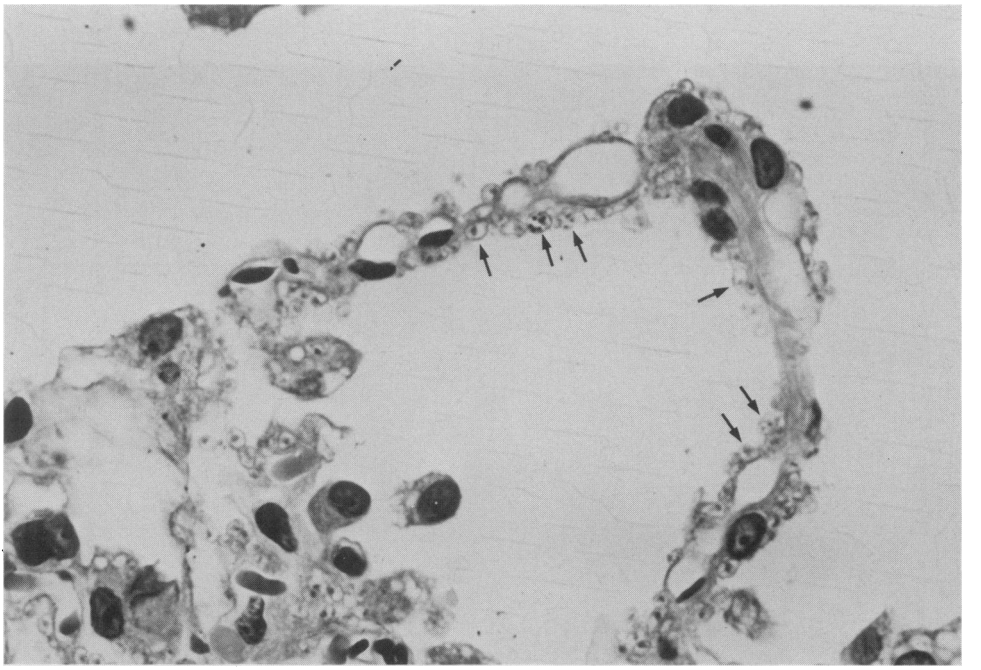


Fig. 3. Detail from haematoxylin and eosin-stained plastic section of inflated ferret lung. Arrows indicate some of the many organisms lining alveolar septum, including intracystic detail. The cyst wall is lightly outlined and intracystic sporozoites are visible within some cysts. Smaller single cells, trophozoites, are seen in large numbers. No organisms are seen within the alveolar space in this section and a few macrophages are evident (original magnification, ×960).

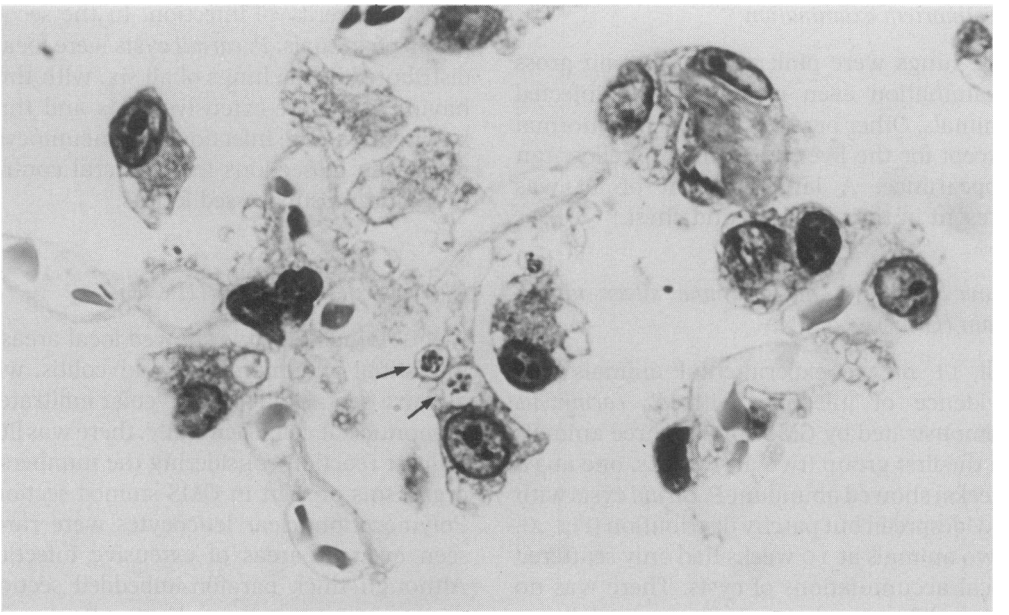


Fig. 4. Detail from Giemsa-stained plastic section, showing two organisms inside a macrophage. The cysts are intact and pleomorphic and crescent-shaped sporozoites are clearly discernible within the cyst. The cyst wall does not stain (original magnification, × 960).

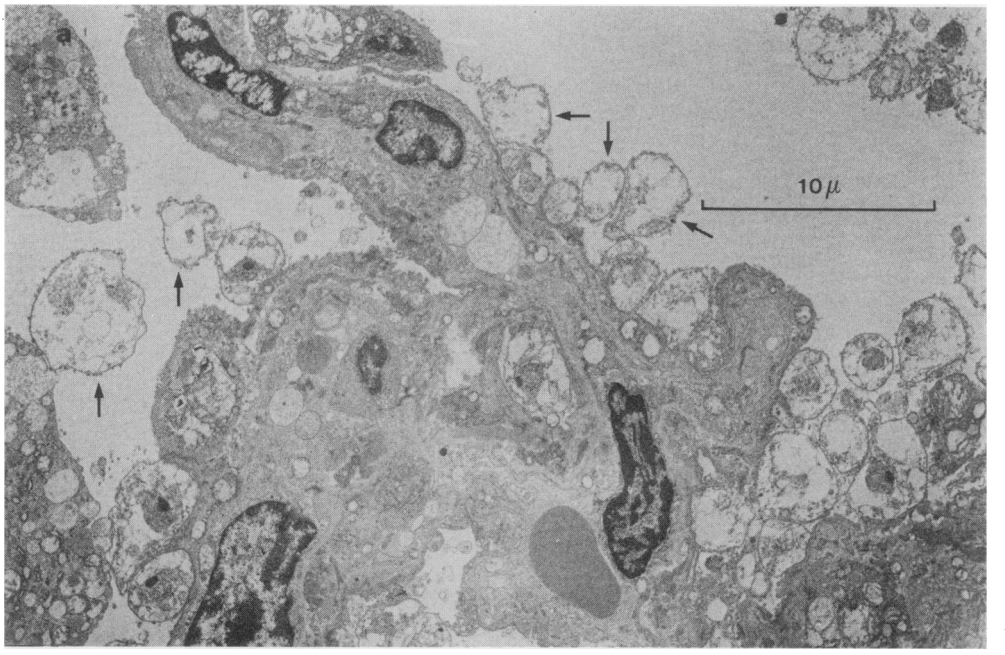


Fig. 5. Ultrastructure of uninflated ferret lung showing abundant forms of different stages in the *P. carinii* life cycle. **Fig. 5.** *a*, Numerous organisms are clearly visible at lower magnification. Essentially all *P. carinii* organisms are in direct proximity to host type I cells. The delicate thin-walled trophozoite is most numerous (arrows) but thickwalled cysts are also visible (en bloc stained only, × 3040).

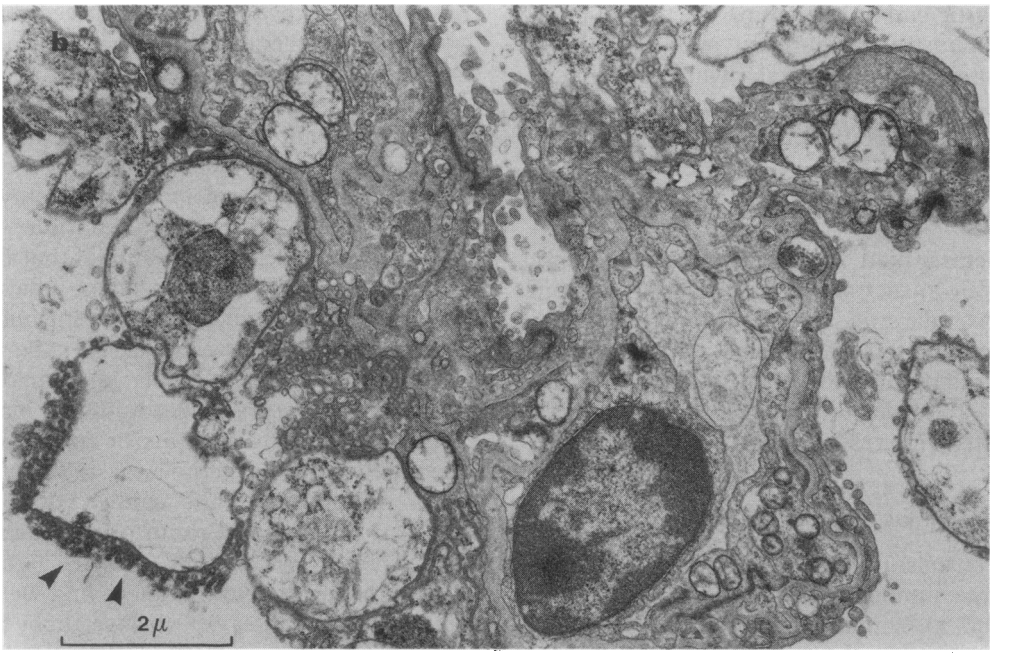
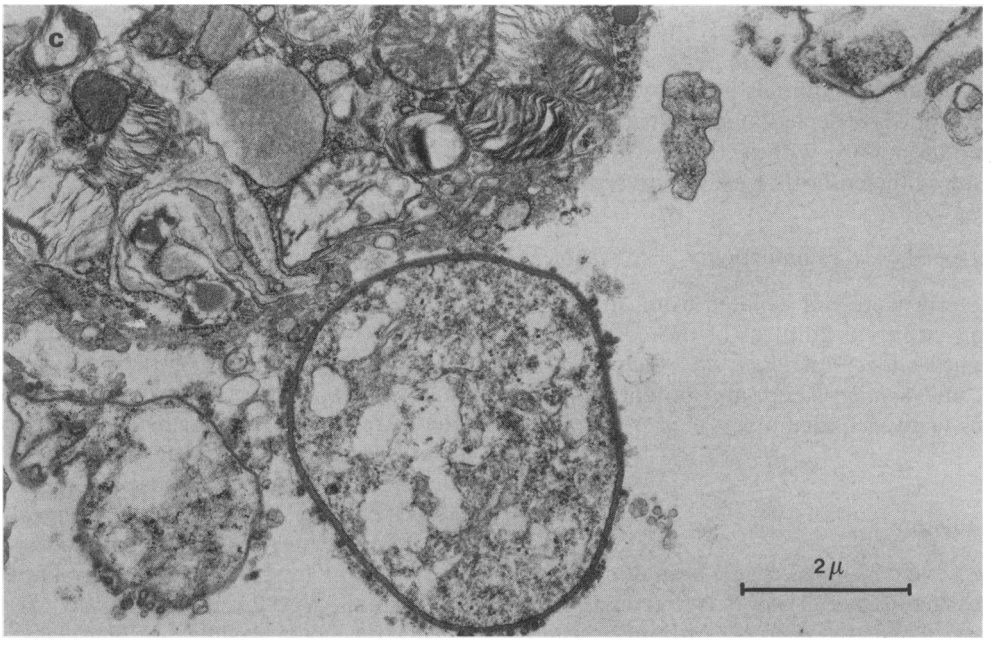


Fig. 5. *b*, Larger magnification of *P. carinii* cysts. One cyst is empty with remnant microtubular structures prominent in its wall (arrows). (× 11360).



c, Thick-walled cyst adjacent to a type II cell without apparent attachment (× 11360).

plastic-embedded thin sections clearly demonstrated abundant cysts and trophozoites lining the alveolar wall, including fine detail usually visible only with Giemsa staining or electron microscopy (Fig. 3).

Giemsa.

Giemsa stain is generally used to demonstrate intracystic structures and trophozoites (Yoshida *et al.* 1981). Although abundant trophozoites were visible in conventional paraffin-imbedded thick sections, fine detail was best demonstrated in thin plastic sections (Fig. 4).

Electron microscopy

Transmission electron microscopy revealed large numbers of both cysts and trophozoites (Fig. 5), similar in appearance and ultrastructure to those previously described in other animals, including man.

Other tissue histopathology

The cytoplasm of the hepatocytes in the four animals examined had multiple fatty vacuoles of variable size. The spleen white pulp in all four animals had varying degrees of lymphocyte depletion and extramedullary haematopoiesis within the red pulp. An eosinophilic enteritis was also present.

Microbiological examination

Bacteria were not isolated from any of the lung cultures. Cultures of the cecum were negative for *Clostridium* sp., *Campylobacter* sp. and *Salmonella* sp. and no enteric parasites were identified.

Discussion

We have developed a new animal model for experimental studies of *P. carinii* using corticosteroid-treated ferrets. Although several rodent species develop *P. carinii* after immunosuppression, better models are needed for pulmonary studies of this infection, the most common life-threatening opportunistic infection in patients with acquired immunodeficiency syndrome (AIDS) (CDC Update 1986). Studies in new species are also useful in probing the immunology and taxonomy of this ubiquitous organism (Gigliotti *et al.* 1986). This model uses an animal that has already proven extremely useful in studies of several respiratory tract infections, including influenza and RSV (Sweet *et al.* 1985; Porter *et al.* 1980), where the ferrets' immunological response appears similar to that of man.

Corticosteroid-treated rats or mice have been used in most experimental studies of *P. carinii* pneumonia (Walzer 1984), but there are several disadvantages in using rodents. Rats typically lose 20–30% of their baseline weight during steroid treatment and weigh only 40–50% of their expected weight by the time *P. carinii* pneumonia develops (Walzer *et al.* 1980; Stokes 1986). Most of this weight loss is due to steroid retardation of body growth in rodents (Claman 1972), but it may also reflect malnutrition due to reduced food intake and poor general condition or superinfection with other organisms, such as *Flavobacterium meningosepticum, Corynebacterium kutscheri* and *Aspergillus* sp., that are common in the rat (Milder *et al.* 1980). Their poor general condition and size precludes serial lung studies and both malnutrition and superinfection complicate interpretation of any studies of *P. carinii* pneumonia in the rat.

The body weight curves shown in Fig. 1 indicate that the ferret, like man, is 'resistant' to body weight loss by corticosteroids (Claman 1972). There is some confusion in the literature regarding classificiation of ferrets as a 'steroid resistant' species. Claman listed the ferret as a 'resistant' species but earlier studies classified ferrets as 'steroid sensitive' like the rat (Shewell & Long 1956) although body weight losses on corticosteroids were very small. Species differences in steroid effects on body weight are paralleled by differences in lymphoid regression with corti-

costeroids and for that reason the ferret's immunological response to steroids may more closely resemble that of man than the rat (Walzer *et al.* 1984). Corticosteroids also retard lung growth in the rat and this is a major factor in interpretation of physiological changes due to *P. carinii* in this animal (Stokes *et al.* 1986).

Despite the ferret's resistance to steroid-induced growth effects, *P. carinii* pneumonia appears to be relatively easy to provoke in this animal. Longer-acting glucorticoid preparations or the addition of other immunosuppressive agents such as cyclosporin A (Hughes & Smith 1982) may prove useful in eliminating the daily injections used in the present study, reducing the time until development of *P. carinii* pneumonia, or increasing the intensity of the infection. The higher initial cost of the ferret is offset by the larger amount of lung tissue available from a single animal for histological or immunological studies compared to the rat (8–12 g *vs.* 1–2 g). Their larger lung and airway size also facilitates use of clinically applicable techniques, such as serial pulmonary function measurements and bronchoalveolar lavage, in an experimental model of PCP. In preliminary studies we have also found that oral prophylaxis against PCP with trimethoprim-sulfamethoxazole appears to be effective in the ferret as it is in man and the rat and additional drug trials using the ferret will be facilitated by the ability to confirm PCP by bronchoalveolar lavage before instituting treatment with experimental therapies.

Ferrets have recently become popular in the United States as exotic household pets. Although animal to man transmission of *P. carinii* has never been demonstrated, normal rats can transmit their latent *P. carinii* infection to immuno-suppressed axenic animals which then develop pneumonia (Hughes 1982). Individuals with immunodeficiency disorders, including AIDS, should probably be discouraged from owning pet ferrets (and rodents) because of this potential risk of transmission of the ferret's latent lung infection.

Acknowledgements

The authors thank Donna Davis and Bonnie Greer for their preparation of the electron microscopy and light microscopy sections. The authors also thank Mike Straign and Marsha Kuhns for invaluable technical assistance.

References

BIANCO P., PONZI A. & BONUCCI, E. (1984) Basic and 'special' stains for plastic sections in bone marrow histopathology, with special reference to May-Grunwald Giemsa and enzyme histochemistry. *Bas. Appl. Histochem.* **28**, 265–279.

CDC UPDATE (1986) Acquired immunodeficiency syndrome (AIDS)—United States. *MMWR* **35**, 17–21.

CLAMAN H.N. (1972) Corticosteroids and lymphoid cells. *N. Engl. J. Med.* **287**, 388–397.

FRENKEL J.K., GOOD J.T. & SCHULTZ J.A. (1966) Latent pneumocystis infection in rats, relapse, and chemotherapy. *Lab. Invest.* **15**, 1559–1577.

GIGLIOTTI F., STOKES D.C., CHEATHAM A.B., DAVIS D.S. & HUGHES W.T. (1986) Development of monoclonal antibodies for the study of *Pneumocystis carinii*. *J. Infect. Dis.* **154**, 315–322.

GOTTLIEB M.S., SCHROFF R., SCHANKER H.M., WEISMAN J.D., FAN P.T., WOLF R.A. & SAXON A. (1981) *Pneumocystis carinii* pneumonia and mucosal candidiasis in previously healthy homosexual men. *N. Engl. J. Med.* **305**, 1425–1431.

HUGHES W.T. & SMITH B. (1982) Provocation of infection due to *Pneumocystis carinii* by cyclosporin A. *J. Infect. Dis.* **145**, 767.

HUGHES W.T. (1982) Natural mode of acquisition for de novo infection with *Pneumocystis carinii*. *J. Infect. Dis.* **145**, 842–848.

HUGHES W.T., MCNABB P.C., MAKRES T.D. & FELDMAN S. (1974) Efficacy of trimethoprim and sulfamethoxazole in the prevention and treatment of *Pneumocystis carinii* pneumonitis. *Antimicrob. Agents Chemother.* **5**, 289–293.

HUGHES W.T., PRICE R.A., SISKO F., HAVROM W.S., KAFATOS A.G., SCHONLAND M. & SMYTHE P.M. (1974) Protein calorie malnutrition. *Am. J. Dis. Child*, **128**, 44–52.

MCBRIDE J.T. (1985) Postpneumonectomy airway growth in the ferret. *J. Appl. Physiol.* **58**, 1010–1014.

MILDER J.E., WALZER P.D., COONROD J.D. & RUTLEDGE M.E. (1980) Comparison of histological

and immunological techniques for the detection of *Pneumocystis carinii* in rat bronchial lavage fluid. *J. Clin. Microbiol.* **11**, 409–417.

PORTER D.D., MUCH K.B. & PRINCE G.A. (1980) The age dependence of respiratory syncytial virus growth in ferret lung can be shown in organ and monolayer cultures. *Clin. Immunol. Immunopathol.* **15**, 415–423.

SHEWELL J. & LONG D.A. (1956) A species difference wth regard to the effect of cortisone acetate on body weight, gamma-globulin and circulating antitoxin levels. *J. Hyg.* **54**, 452–460.

SHIMIZU A., KIMURA F. & KIMURA S. (1985) Occurrence of *Pneumocystis carinii* in animals in Japan. *Jpn. J. Vet. Sci.* **47**, 309–311.

STOKES D.C., HUGHES W.T., ALDERSON P.O., KING R.E. & GARFINKEL D.J. (1986) Lung mechanics, radiography, and Ga-67 scintigraphy in experimental *Pneumocystis carinii* pneumonia. *Br. J. exp. Pathol.* **67**, 383–393.

SWEET C., BIRD R.A., HOWIE A.J., OVERTON H.A., COATES D.M. & SMITH H. (1985) Further studies of the reasons for the lack of alveolar infection during influenza in ferrets. *Br. J. exp. Path.* **66**, 217–231.

VINEGAR A., SINNETT E.E. & KOSCH P.C. (1982) Respiratory mechanics of a small carnivore: the ferret. *J. Appl. Physiol.* **52**, 832–837.

WALZER P.D. & YOUNG L.S. (1984) Clinical relevance of animal models of *Pneumocystis carinii* pneumonia. *Diagn. Microbiol. Infect. Dis.* **2**, 1–6.

WALZER P.D. (1984) Experimental models of *Pneumocystis carinii* infections. In: *Pneumocystis carinii pneumonia* Ed. L. S. Young. New York: Marcel Dekker. pp. 7–76.

WALZER P.D., LABINE M., REDINGTON T.J. & CUSHION M. (1984) Lymphocyte changes during chronic administration of and withdrawal from corticosteroids: relation to *Pneumocystis carinii* pneumonia. *J. Immunol.* **133**, 2502–2508.

WALZER P.D., POWELL R.D. & YONEDA K. (1980) Growth characteristics and pathogenesis of experimental *Pneumocystis carinii* pneumonia. *Infect. Immunol.* **27**, 928–937.

WELLER R. (1955) Zur Erzeugung von Pneumocystosen in Tierversuch. *Z. Kinderheilk.* **76**, 366–378.

YONEDA K. & WALZER P.D. (1980) Interactions of *Pneumocystis carinii* with host lungs: an ultrastructural study. *Infect. Immun.* **29**, 692–703.

YOSHIDA Y., SHIOTA T., YAMADA M. & MATSUMOTO Y. (1981) Further light microscopic studies on morphology and development of *Pneumocystis carinii*. *Zbl. Bakt. Hyg., I. Abt. Orig.* **A250**, 213–218.