JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1992, p. 3258–3262
0095-1137/92/123258-05$02.00/0

Vol. 30, No. 12

An Enzyme-Linked Immunosorbent Assay for Enumeration of *Pneumocystis carinii* In Vitro and In Vivo

MICHELLE M. DURKIN,[1]* MARILYN S. BARTLETT,[1] SHERRY F. QUEENER,[2] MARGARET M. SHAW,[1] CHAO-HUNG LEE,[1] AND JAMES W. SMITH[1]

Departments of Pathology[1] and Pharmacology and Toxicology,[2] Indiana University School of Medicine, Indianapolis, Indiana 46202

Received 8 June 1992/Accepted 21 September 1992

An enzyme-linked immunosorbent assay (ELISA) to quantitate *Pneumocystis carinii* organisms from culture supernatant and rat lung has been developed. A polyclonal antibody specific to *P. carinii* was produced in Sprague-Dawley rats by allowing *P. carinii*-infected animals to recover from infection. This antibody reacted strongly to *P. carinii* proteins of 50 to 55 kDa and weakly to those of 33 and 116 kDa. The ELISA used this convalescent-phase antibody to quantitate the number of *P. carinii* organisms in lung homogenates of infected rats and supernatants from infected tissue cultures which were used to screen drugs for *P. carinii*. The results of the ELISA were compared with those of direct microscopic counting of organisms, and the two methods were highly correlated ($r > 0.9$). Thus, the ELISA can be used as an alternative method for the quantitation of *P. carinii* organisms, and it is superior to the conventional microscopic method because it is easier to perform and less labor-intensive.

Pneumocystis carinii is a significant cause of pneumonia in immunocompromised hosts (6, 8, 11). Both tissue cultures and rat models have been used to determine drug effects on *P. carinii*. Most in vivo *P. carinii* susceptibility testing has used variations of Frenkel's corticosteroid-immunosuppressed rat model (7, 10, 15), which relies upon latent infection becoming manifest. Bartlett et al. (2) developed a transtracheally inoculated corticosteroid-immunosuppressed rat model and adapted it for testing of drugs for treatment and prophylaxis. In assessing reduction of *P. carinii* trophic and cyst forms, it has been necessary to determine numbers of organisms microscopically. Some investigators have assessed infections solely by organism numbers (13), while others have incorporated host response to infection and degree of organ involvement (7). These methods suffer from the variability among samples selected for evaluation, the time-consuming and tedious process of counting organisms, and the experience required for discriminating *P. carinii* from host cell fragments or other organisms, such as yeasts.

In vitro drug testing most often has used feeder cell layers and relied upon demonstrating differences in numbers of organisms in untreated and drug-treated culture wells (1). The method we have developed employs HEL-299 or WI-38 cells grown to confluence in 12- or 24-well plates. Cells are inoculated with *P. carinii* from infected rat lung, and soluble compounds are incorporated into tissue culture media to test effectiveness in inhibiting the growth of *P. carinii*. By sampling and staining a standard volume of culture supernatant, organisms can be counted and averages can be compared with results from positive and negative growth control wells. Microscopic evaluation of these samples is made difficult by the tendency of *P. carinii* trophozoites to clump together as they grow, making it necessary to count many oil immersion fields to establish accurate mean values for growth curves. Our enzyme-linked immunosorbent assay (ELISA) system is a method of quantitation which is less subjective than counting organisms and which accurately and consistently measures *P. carinii* antigen. In order to simplify the quantitation of *P. carinii* organisms, we developed an ELISA system which can accurately and consistently measure *P. carinii* antigen and can be used as an alternative method for evaluating the effect of drugs against *P. carinii* both in vitro and in vivo.

* Corresponding author.

MATERIALS AND METHODS

Inoculation of rats with *P. carinii*. Female Sprague-Dawley rats weighing 125 to 135 g were obtained from barrier 202 of Harlan Sprague-Dawley, Indianapolis, Ind. Animals were immunosuppressed with dexamethasone in drinking water (0.36 mg/kg of body weight per day) for at least 4 days prior to transtracheal inoculation with *P. carinii*-infected rat lung as previously described (3).

Anti-Pneumocystis antibodies produced in rats. Convalescent-phase antibodies were produced in dexamethasone-treated rats that had been infected by transtracheal inoculation with *P. carinii*. Three weeks after inoculation, dexamethasone immunosuppression was stopped and the animals were allowed to recover. To monitor antibody production, the animals were bled every 10 days beginning 3 weeks after immunosuppression was stopped. Approximately 0.2 ml of blood was taken from a lateral tail vein by using a 23-gauge needle fitted onto a 1.0-ml tuberculin syringe. Blood was allowed to clot and then centrifuged in a tabletop microcentrifuge for 5 to 10 s to separate the serum from the clot. Serum from each rat was used as the primary antibody in an ELISA and titrated against *P. carinii* from rat lung to determine the optimum dilution for each serum. Five of the eight rats had titers of 1:400 to 1:3,200. These results were confirmed by an ELISA which used each rat serum at a dilution of 1:400. Again, the same five rats' sera had the highest titers. These five serum specimens were pooled and frozen in the nitrogen freezer in 500-μl aliquots. The total volume of pooled sera was 12 ml.

Inoculation of tissue culture with *P. carinii*. In vitro *P. carinii* cultures for drug studies were prepared with human

embryonic lung fibroblasts (HEL-299 cells [American Type Culture Collection] or WI-38 cells [Whittaker M. A. Bioproducts]). The cells were cultured in 12- or 24-well tissue culture plates with minimum essential medium containing 10% fetal bovine serum, 100 U of penicillin per ml, and 0.1 mg of streptomycin per ml. HEL-299 cells require additional supplements of 1% nonessential amino acids, 0.1% lactalbumin hydrolysate, and 1 mM sodium pyruvate. Drug studies were carried out as previously described (1).

To determine the effectiveness of drugs on *P. carinii*, 10-μl samples of culture supernatant from each of four wells of each test parameter were stained with Giemsa stain and examined as unknowns by two individuals. The means with standard errors were calculated for each set of data for each time point, and the values were used to prepare growth curves.

After the 10-μl samples had been taken from each tissue culture well, 300 μl from each of the four drug dilution wells was placed in a single microcentrifuge tube, making the total sample volume for each drug dilution 1.2 ml. The tubes were centrifuged in a tabletop microcentrifuge at 8,000 × *g* for 2 min to sediment the *P. carinii*. The supernatant was removed, and the pellet was washed with 100 μl of phosphate-buffered saline (PBS) (pH 7.2) containing 0.02% sodium azide. The tubes were vortexed to resuspend the pellets and then centrifuged again at 8,000 × *g* for 2 min. The wash was removed, and 100 μl of PBS was again added to each tube. These samples were stored at 4°C until all samples had been collected before an ELISA was performed on all study samples.

Collection of *P. carinii* and preparation of *P. carinii* antigen from infected rats. Rats for evaluating drugs were used in groups of 10, with an untreated control group and a treated control group receiving trimethoprim-sulfamethoxazole (TMP-SMX) included in each study. Three to 4 weeks after inoculation, treatment was started and continued for 2 to 3 weeks.

To evaluate the extent of infection, rats were anesthetized with 0.2 ml of ketamine cocktail containing 80 mg of ketamine hydrochloride per ml, 0.38 mg of atropine per ml, and 1.76 mg of acepromazine per ml and sacrificed by exsanguination. Lungs were removed and placed in sterile petri dishes, and the weight of each lung was recorded. Numbers of organisms were estimated by ELISA and microscopy.

A portion of each lung was cut, weighed, and ground in PBS to yield a concentration of 10 mg of tissue per ml of solution. One milliliter of homogenized lung from each rat was placed in a sterile microcentrifuge tube and spun at 8,000 × *g* for 3 min. The supernatant was removed, and the pellet was washed with 200 μl of PBS, vortexed, and spun as described above. Two hundred microliters of PBS was added to each pellet, and the antigen was refrigerated until the ELISA was performed.

To evaluate *P. carinii* infection by microscopy, 10 μl of each lung homogenate which was ground for use as the ELISA antigen was placed in a 1-cm^2 area etched onto a microscope slide. The slides were air dried, fixed in methanol, and stained with Giemsa stain. Organisms in 10 ×1,000-magnification oil immersion fields were counted by two individuals, and the counts were averaged.

ELISA procedure for drug study evaluation. An equal volume of a solution containing 1 M urea and 1 mg of dithiothreitol per ml was added to each sample tube. The antigen was vortexed, and then PBS was added to bring the volume to 1.0 ml. One hundred microliters of each antigen solution was placed into each of 3 wells on a 96-well microtiter plate. The plates were incubated for 45 min at 35°C and then washed three times with PBS containing 0.05% Tween 20 and 0.02% sodium azide by using a Corning Immunowash ELISA plate washer.

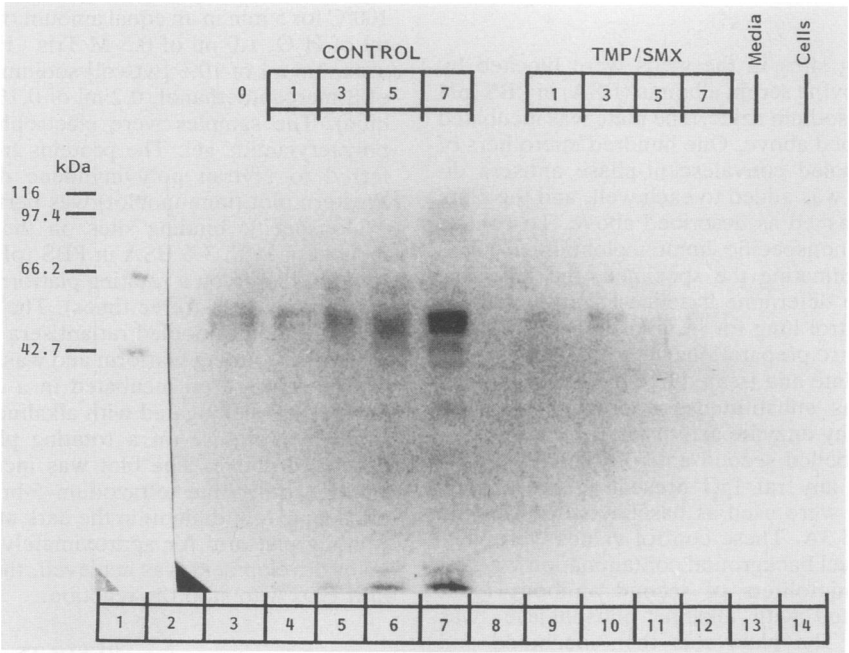
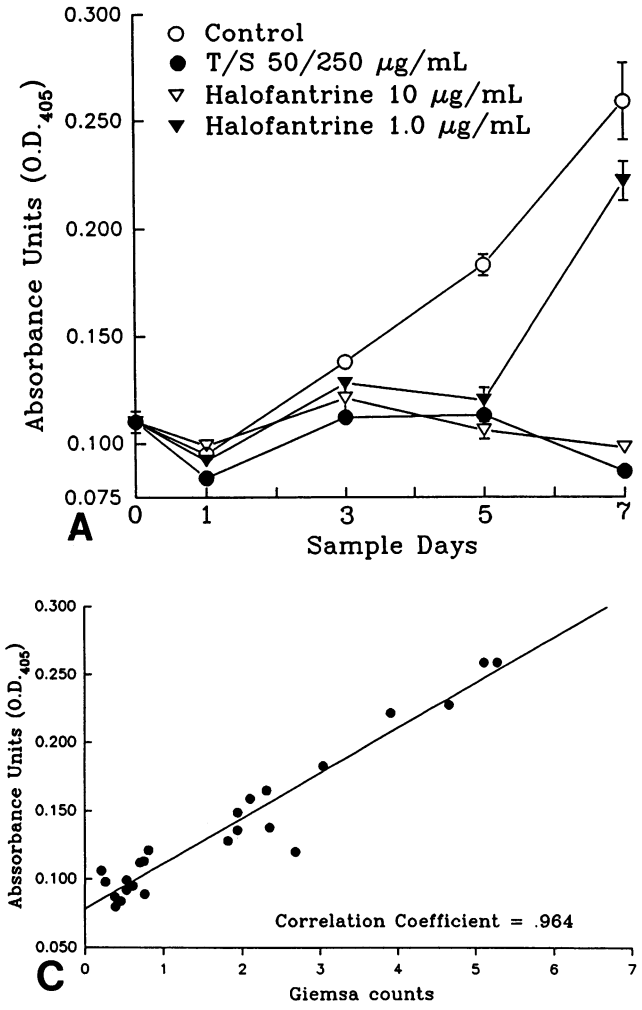


FIG. 1. Western blot of *P. carinii* proteins from an in vitro tissue culture. Lane 1, molecular weight markers; lane 3, culture from day 0 of the drug study; lanes 4 through 7, culture from days 1, 3, 5, and 7, respectively, of untreated control wells; lanes 9 through 12, culture from days 1, 3, 5, and 7, respectively, of TMP-SMX-treated control wells; lane 13, uninoculated tissue culture media; lane 14, uninoculated tissue culture cells.



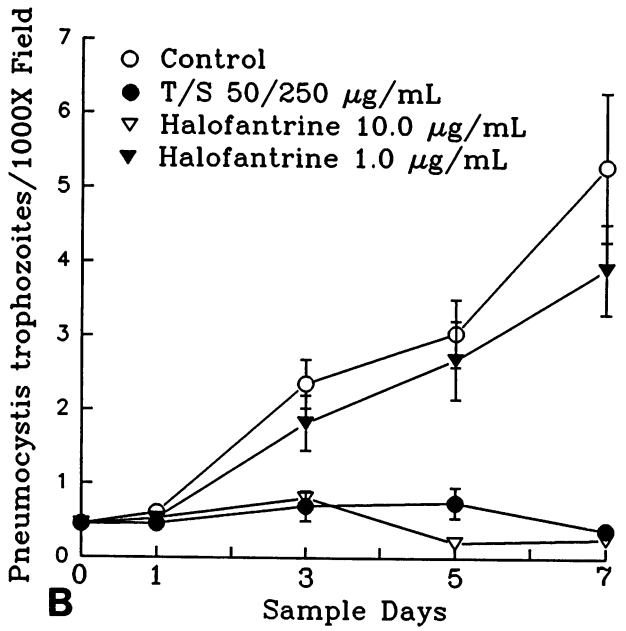


FIG. 2. (A) Growth curves of cultured organisms as measured by absorbance units in an ELISA. The baseline (negative control) value was 0.075. (B) Growth curves of cultured organisms as measured by counting Giemsa-stained culture supernatants. (C) Regression line generated by plotting absorbance units against Giemsa counts of culture supernatants. T/S, TMP-SMX; $O.D._{405}$, optical density at 405 nm.

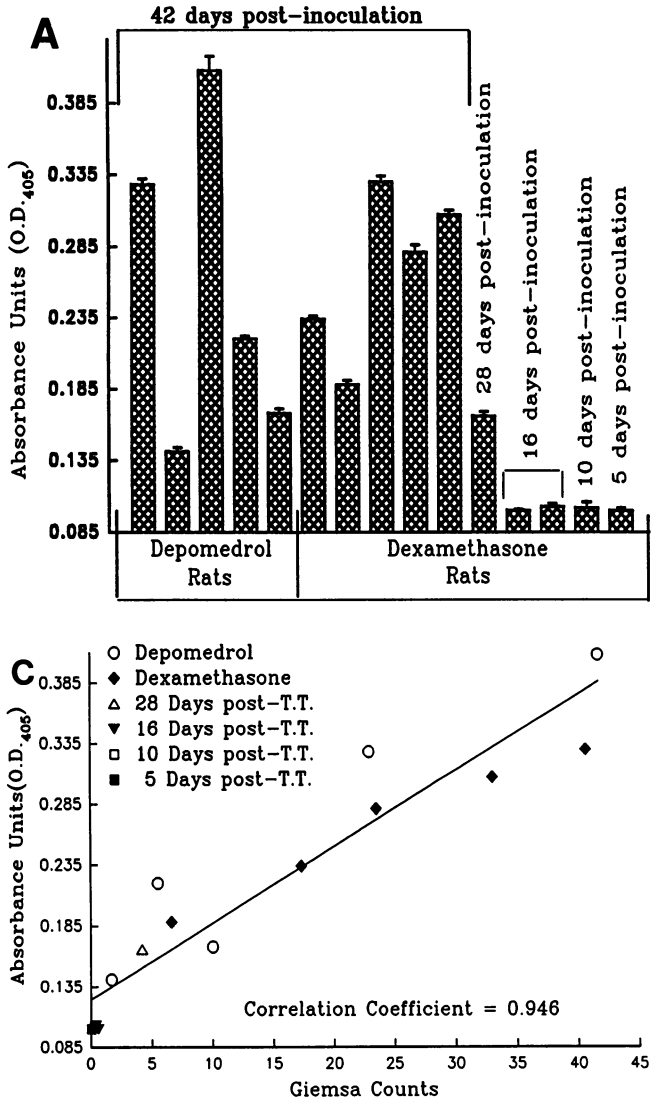
Nonspecific binding sites in the wells were blocked by adding 100 μl of 3% bovine serum albumin (BSA) in PBS (pH 7.2) containing 0.02% sodium azide. The plate was incubated and washed as described above. One hundred microliters of primary antibody (pooled convalescent-phase antisera diluted 1:500 with PBS) was added to each well, and the plate was incubated and washed as described above. To control for the presence of nonspecific immunoglobulin G (IgG) which may be contaminating the specimen, the following controls were used to determine baseline enzyme activity. Antigen (infected control lung for in vivo preparations and day 0 samples for in vitro preparations) was placed into three wells of the ELISA plate and treated like the other antigen, except that PBS was substituted for primary antibody (pooled rat serum). Any enzyme activity in these wells was a result of enzyme-labelled second antibody binding either nonspecifically or to any rat IgG present in the antigen sample. These values were used as baseline values for the graphs in Fig. 2A and 3A. These control values were very low, indicating low-level background contamination with rat IgG. One hundred microliters of second antibody (goat anti-rat IgG conjugated with alkaline phosphatase) was added to each well. The plate was then incubated and washed as above. The wells were washed once with 100 μl of ELISA substrate buffer (100 mg of $MgCl_2$ and 1 ml of diethanolamine per liter of distilled water). One hundred microliters of substrate solution (1 mg of *p*-nitrophenyl phosphate per ml in ELISA substrate buffer) was added to each well. The plate was incubated at 35°C for 20 to 30 min, and the optical density at 405 nm was read on a Molecular Devices ELISA reader.

Western blotting. The antigen was solubilized by heating at 100°C for 5 min in an equal amount of urea sample buffer (4.0 ml of H_2O, 1.0 ml of 0.5 M Tris · HCl [pH 6.8], 800 mg of urea, 1.6 ml of 10% [wt/vol] sodium dodecyl sulfate, 0.4 ml of β-mercaptoethanol, 0.2 ml of 0.1% [wt/vol] bromophenol blue). The samples were electrophoresed through a 10% polyacrylamide gel. The proteins from this gel were transferred to Nytran polyvinylidene difluoride paper, and a Western blot (immunoblot) was performed.

Nonspecific binding sites on the blot were blocked by incubating with 3% BSA in PBS (pH 7.2) at room temperature for 45 min on a rotating platform. The blot was washed for 5 min in PBS (three times). The blot was incubated in a 1:500 dilution of pooled rat antisera for 1 h at room temperature on a rotating platform and washed as described above. The blot was then incubated in a 1:1,000 dilution of goat anti-rat IgG conjugated with alkaline phosphatase for 1 h at room temperature on a rotating platform and washed as described above. The blot was incubated in the substrate solution (nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl-phosphate toluidinium) in the dark at room temperature on a rotating platform for approximately 20 min. When optimal color development was achieved, the blot was washed in 20 mM EDTA to stop the reaction.

RESULTS

To determine whether the convalescent-phase antisera produced were specific for *P. carinii*, Western blot analyses



were performed on antigen from supernatants of untreated and TMP-SMX-treated cultures (Fig. 1). Control antigen included uninfected tissue culture cells and uninfected tissue culture medium. There was a gradual increase of antibody-reactive antigen in the 50- to 55-kDa range over the 7-day period in the untreated control cultures, while there was a diminishing of antibody-reactive antigen at this region in the lanes containing *P. carinii* from TMP-SMX-treated wells (Fig. 1). There was also an increase in bands at 33 and 118 kDa over the 7-day period in the untreated control wells. There was no reaction in lanes containing either tissue culture media or tissue culture cells. This result demonstrates the growth in culture of *P. carinii* organisms and also the specificity of the antibody reaction to the organisms.

Evaluation of in vitro data. The usefulness of the ELISA in quantitating *P. carinii* organisms was evaluated by comparing its results with those of microscopy. HEL-299 cells were infected with *P. carinii* and then treated with the control drugs, TMP (50 μg/ml) plus SMX (250 μg/ml), or with the experimental drug halofantrine at 1.0 and 10 μg/ml. *P. carinii* organisms present in culture supernatants were counted on days 0, 1, 3, 5, and 7 of drug treatment. Culture supernatants

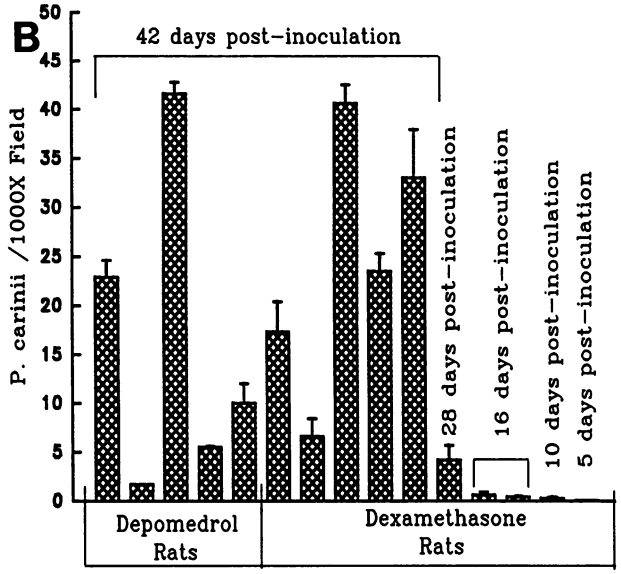


FIG. 3. (A) Organism load of individual rats as measured by absorbance units in an ELISA. The baseline (negative control) value was 0.085. (B) Organism load of individual rats as measured by counting organisms in 10 μl of Giemsa-stained lung homogenate. (C) Regression line generated by plotting absorbance units against Giemsa counts of organisms in homogenized lung. $O.D._{405}$, optical density at 405 nm; T.T., transtracheal inoculation.

were harvested and analyzed by ELISA. The results for quantitation of *P. carinii* obtained from ELISA (Fig. 2A) and from organism counts (Fig. 2B) were plotted. Both methods showed an increase in organisms over time for both the untreated cultures and the cultures treated with halofantrine at a concentration 1.0 μg/ml. Both methods show no increase of organisms for the cultures containing TMP (50 μg/ml) plus SMX (250 μg/ml) and halofantrine at a concentration of 10.0 μg/ml. A linear regression line was plotted for these data, and the *r* value was calculated to be 0.964, indicating that the two sets of data are highly correlated (Fig. 2C).

Evaluation of in vivo data. The ELISA was performed on lung homogenates of *P. carinii*-infected rats to determine its effectiveness in quantitating *P. carinii* organisms in this model. Sprague-Dawley rats were immunosuppressed by dexamethasone or depomedrol and then infected with *P. carinii* by transtracheal inoculation. One each of the rats that were immunosuppressed by dexamethasone and depomedrol were sacrificed to evaluate *P. carinii* infection on days 5, 10, 16, and 28 postinoculation. Five each of depomedrol- and dexamethasone-immunosuppressed rats were sacrificed at 42 days after inoculation. *P. carinii* organisms present in lung homogenates were quantitated by counting and by ELISA. Both methods demonstrated the same low levels of *P. carinii* infection during the first 28 days postinoculation (Fig. 3). At 42 days postinoculation, there were various degrees of infection in different rats. When results from both methods were compared by linear regression analysis, the *r* value was calculated to be 0.946, which again indicates a high degree of correlation.

DISCUSSION

Therapeutic or prophylactic efficacy is most often established by determining numbers of organisms microscopically

in representative portions of lung. Cyst wall stains and organism stains usually are used to evaluate lung impression smears, lung tissue sections, or other preparations from each animal in a study. Gomori methenamine silver nitrate stain (12) or more rapid modifications of this stain (4, 9) and toluidine blue O (5) are the most commonly used cyst wall stains. Organism stains such as Giemsa stain (12) allow detection of trophozoites as well as cyst stages. Hughes et al. (7) used hematoxylin, eosin, and silver-stained lung sections to score infectivity on a stage 0-to-stage III scale. This scoring system was based on numbers of cysts present and the inflammatory response seen in the host tissue. Walzer et al. (14) used silver-stained tissue sections to score infectivity on a scale of 0 to 4 based on the percent involvement of the alveoli with *P. carinii* infection. Bartlett et al. (3) used lung impression smears stained with Giemsa and silver stains to score *P. carinii* infection with a scoring system of 0 to 5. This system is based on numbers of organisms present and is roughly logarithmic.

In this study, we have demonstrated that antibodies specific to *P. carinii* can be produced in rats recovered from *P. carinii* infection. The convalescent-phase antibodies thus produced can be used in ELISAs to quantitate *P. carinii*. We have compared the efficiency of organism quantitation of ELISAs with that of direct counting in 90 drug doses tested in tissue cultures and 39 tested in infected rats. Evaluation of data generated from either method has drawn the same conclusion about the effectiveness of the drugs tested and outcome of the study. The Giemsa stain method has the advantage of allowing direct observation of organisms and surrounding tissue. Cytologic evaluation of the lung specimen and detection of other infectious agents can be accomplished while scoring the smear for *P. carinii* infectivity. Difficulties with microscopic evaluation include the fact that staining and blinding the slides microscopically is very time-consuming. The impression smear on the slide is never homogenous; there are thin and thick areas. Because this can be a subjective way to evaluate lung, three people must read each slide, making the labor cost very high.

With the ELISA, there is less chance that a sampling error may occur, because the results from the ELISA system are based upon an amount of culture supernatant (1.2 ml) or homogenized rat lung (1 ml) that is sizable compared with the small amount placed on a slide for staining (10 μl). Also, there is little manipulation of lung samples compared with that in techniques for fixing, processing, and staining permanent tissue sections, so results can be evaluated more rapidly. We conclude that compared with the microscopic examination of stained samples, the ELISA is a more rapid but equally accurate quantitation method.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants NOI-AI-72647 and UOI-AI-25859-01.

REFERENCES

1. **Bartlett, M. S., R. Eichholtz, and J. W. Smith.** 1985. Antimicrobial susceptibility of *Pneumocystis carinii* in culture. Diagn. Microbiol. Infect. Dis. **3**:381–387.
2. **Bartlett, M. S., J. A. Fishman, M. M. Durkin, S. F. Queener, and J. W. Smith.** 1989. An improved rat model to study efficacy of drugs for treatment of prophylaxis of *Pneumocystis carinii* pneumonia. J. Protozool. **36**:77S–78S.
3. **Bartlett, M. S., J. A. Fishman, S. F. Queener, M. M. Durkin, M. A. Jay, and J. W. Smith.** 1988. New rat model of *Pneumocystis carinii* infection. J. Clin. Microbiol. **26**:1100–1103.
4. **Brinn, N. T.** 1983. Rapid metallic histological staining using the microwave oven. J. Histotechnol. **6**:125–129.
5. **Chalvardjian, A. W., and L. A. Grawe.** 1963. A new procedure for the identification of *Pneumocystis carinii* cysts in tissue sections and smears. J. Clin. Pathol. **16**:383–384.
6. **Fossieck, B. E., Jr., and S. V. Spagnolo.** 1980. *Pneumocystis carinii* pneumonitis in patients with lung cancer. Chest **78**:721–722.
7. **Hughes, W. T., P. C. McNabb, T. D. Makres, and S. Feldman.** 1974. Efficacy of trimethoprim and sulfamethoxazole in the prevention and treatment of *Pneumocystis carinii* pneumonitis. Antimicrob. Agents Chemother. **5**:289–293.
8. **Jarowenko, M., L. Pifer, R. Kerman, and B. D. Kahan.** 1986. Serologic methods for the early diagnosis of *Pneumocystis carinii* infection in renal allograft recipients. Transplantation **41**:436–442.
9. **Musto, L., M. Flanigan, and A. Elbadawi.** 1982. Ten-minute silver stain for *Pneumocystis carinii* and fungi in tissue sections. Arch. Pathol. Lab. Med. **106**:292–294.
10. **Queener, S. F., M. S. Bartlett, M. A. Jay, M. M. Durkin, and J. W. Smith.** 1987. Activity of lipid-soluble inhibitors of dihydrofolate reductase against *Pneumocystis carinii* in culture and in a rat model of infection. Antimicrob. Agents Chemother. **31**:1323–1327.
11. **Rothenberg, R., M. Woelfel, R. Stoneburner, J. Milberg, R. Parker, and B. Truman.** 1987. Survival with the acquired immunodeficiency syndrome. Experience with 5833 cases in New York City. N. Engl. J. Med. **317**:1297–1302.
12. **Smith, J. W., and M. S. Bartlett.** 1982. Laboratory diagnosis of *Pneumocystis carinii* infection. Clin. Lab. Med. **2**:393–406.
13. **Smith, J. W., M. S. Bartlett, S. F. Queener, M. M. Durkin, M. A. Jay, M. T. Hull, R. S. Klein, and J. J. Marr.** 1987. *Pneumocystis carinii* pneumonia therapy with 9-deazainosine in rats. Diagn. Microbiol. Infect. Dis. **7**:113–118.
14. **Walzer, P. D., R. D. Powell, Jr., and K. Yoneda.** 1979. Experimental *Pneumocystis carinii* pneumonia in different strains of cortisonized mice. Infect. Immun. **24**:939–947.
15. **Walzer, P. D., and M. E. Rutledge.** 1980. Comparison of rat, mouse, and human *Pneumocystis carinii* by immunofluorescence. J. Infect. Dis. **142**:449.