INFECTION AND IMMUNITY, Feb. 1982, p. 620–626
0019-9567/82/020620-07$02.00/0

Vol. 35, No. 2

Serum Antibody Responses to *Pneumocystis carinii* Among Different Strains of Normal and Athymic Mice

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Received 14 August 1981/Accepted 20 October 1981

Pneumocystis carinii infection was produced in normal mice by the administration of corticosteroids and in athymic mice by the transmission of exogenous mouse- or rat-derived organisms. Serum antibodies to *P. carinii*, measured by an indirect flourescent-antibody technique, were found in five of six strains of normal mice. Although antibody titers varied widely among the control and steroid-treated mice, they were inversely proportional to the intensity of *P. carinii* infection in the lungs. In sequential studies, antibody titers were low during steroid administration but rose with steroid withdrawal. Antibodies were mainly of the immunglobulin G (IgG) class; IgM antibodies also occurred, but IgA antibodies were rare. nu/nu mice rarely produced serum antibodies to *P. carinii* despite the fact that one strain was sensitive and the other was resistant to exogenous infection. nu/+ mice produced specific antibodies to rat and mouse *P. carinii* which were primarily IgG. Thus, the host produces antibodies to *P. carinii* which are mainly IgG and T-cell dependent; yet, these antibodies do not appear to be important in susceptibility or resistance to *P. carinii* infection.

Pneumocystis carinii is a well-recognized cause of pneumonia in immunocompromised patients. Animal models have provided valuable data on the pathogenesis of the disease. In the basic model, rats that are administered corticosteroids for about 8 weeks spontaneously develop *Pneumocystis* pneumonia, a process which represents reactivation of latent infection (6, 8, 28). Recent studies have shown that mice can also be used in this model: steroid-treated normal mice develop pneumonia in a manner similar to that in rats (27); *P. carinii* infection has been produced in congenitally athymic (nude) mice with exogenously derived organisms (25, 32; P. D. Walzer and R. D. Powell, *in* N. Reed [ed.], *Proceedings of the Third International Workshop on Nude Mice*, in press). The data so far suggest that there may be strain differences among mice in susceptibility to *P. carinii*.

Little is known about the nature of the host immune responses to *P. carinii* or about the roles of these responses in susceptibility or resistance to infection with the organism. We have recently conducted detailed studies of the humoral immune responses to *P. carinii* in the steroid-treated rat model by using an indirect fluorescent-antibody (IFA) technique (30). The results indicated that the host develops local and systemic immunoglobulin G (IgG) antibodies to *P. carinii*, with both active infection and prolonged environmental exposure. However, the lack of readily available reagents limited investigation of the antibody responses of other immunoglobulin classes.

In the present study we examined the serum antibody responses to *P. carinii* in mice, with emphasis on the following: determination of the immunoglobulin class (i.e., IgG, IgM, or IgA) involved in the antibody response, determination of whether there are strain differences among mice in the antibody response, and determination of whether the type or degree of antibody response can be related to susceptibility or resistance to infection with *P. carinii*.

MATERIALS AND METHODS

Normal mice. The following strains of normal mice were used: C3H/HeN, BALB/cAnN, AKR/J, B10.A (2R), C57BL/6N, and DBA/1J. These mice were part of a larger study, described in detail previously (27), which was designed to develop the mouse as an experimental model for *P. carinii* infection. Members of each mouse strain were divided into two basic groups: steroid treated and non-steroid treated (control). The mice were sacrificed at 8 to 12 weeks or sooner if they appeared ill. A variety of protocols were studied to establish the optimal steroid regimen. Since the number of serum specimens in these protocols available for analysis was often small, the effects of individual differences in steroid regimens on antibody responses could not be compared.

The optimal regimen to induce *P. carinii* pneumonia

was cortisone acetate (1 mg) injected subcutaneously twice weekly, a low (8%) protein diet, and tetracycline (1 mg/ml) in the drinking water. In a separate study, BALB/cAnN mice were randomly divided into two groups. Group 1 mice were placed on the optimal steroid regimen for 12 weeks. Group 2 mice received the same regimen for 4 weeks; a regular diet was then instituted, and the steroids were tapered to zero over the next 3 weeks. Mice were sacrificed at weekly intervals. A few non-steroid-treated BALB/cAnN mice that were administered a regular diet and tetracycline in the drinking water served as reference controls.

Nude mice. Initial studies were performed at Memorial Sloan-Kettering Cancer Center (MSKCC) as described elsewhere (32). Outbred Swiss female nude (nu/nu) mice and haired (nu/+) litter mates were used; the mice were of an ICR background and were raised and maintained in an area of MSKCC apart from other animals. Transmission studies included intrapulmonary injection of *P. carinii*-infected rat lung homogenates and exposure to steroid-treated rats in isolators. The mice were sacrificed at 4 to 8 weeks. The results indicated that rat *P. carinii* could be readily transmitted to nu/nu mice.

Later studies were performed at the Lexington Veterans Administration Medical Center and at the University of Kentucky with an outbred Swiss nude mouse strain developed at the National Institutes of Health (Walzer and Powell, in press). Transmission experiments and sacrifice times were similar to those performed at MSKCC. Other transmission studies were carried out by exposing nu/nu and nu/+ mice to steroid-treated normal C3H/HeN mice. The results indicated that neither rat nor mouse *P. carinii* infection could be consistently established in nu/nu mice, suggesting considerable innate resistance to the organism on the part of these animals.

Specimens. The mice were sacrificed by exsanguination under halothane anesthesia. Serum and lung specimens were usually obtained from each mouse; in a few instances, lung specimens were unavailable for examination. Serum specimens were stored at −20°C.

Histopathology. Lung sections were stained with methenamine silver, coded, and read in a blind manner to determine the presence of *P. carinii*. The following semiquantitative scoring system, described previously (27, 28), was used to grade the intensity of *P. carinii* infection in the lungs: 0, no *P. carinii* found; 0.5+, minimal infection, <1% alveoli involved; 1+, light, 1 to 25% alveoli involved; 2+, moderate, 25 to 50% alveoli involved; 3+, heavy, 50 to 75% alveoli involved; 4+, very heavy, >75% alveoli involved. The mean score for each group of mice was calculated for those members with demonstrable *P. carinii* (i.e. with a score ≥0.5+).

Reagents. The following fluorescein-conjugated antisera were used: $F(ab)_2$ fragment goat anti-mouse IgG and goat anti-mouse IgA from Cappel Laboratories (Cochranville, Pa.) and goat anti-mouse IgG and goat anti-mouse IgM from Meloy Laboratories (Springfield, Va.).

Immunological tests. Serum antibodies to *P. carinii* were measured by an IFA technique, as detailed previously (15, 30, 31). Briefly, mouse *P. carinii* served as the principal antigen; rat *P. carinii* was also used in some studies. Organisms obtained from infected lungs by digestion with collagenase and hyaluronidase and by Ficoll-Hypaque density gradient centrifugation were added to the wells of Teflon-coated glass slides and heat fixed. The test mouse serum was added in serial dilutions (beginning at 1:4) to the wells, incubated in a moist chamber at 37°C for 45 min, and washed with phosphate-buffered saline (pH 7.2 to 7.4). Fluorescein-conjugated goat anti-mouse IgG, IgM, or IgA was then added to each well, and the slide was incubated and washed as described above. The slide was then mounted with glycerin-phosphate-buffered saline and read with a Leitz Orthoplan fluorescence microscope. The intensity of fluorescence was graded on a scale from 0 (negative) to 4+ (maximum); the highest dilution with a 1+ intensity of fluorescence was considered to be the peak antibody titer.

Controls. Tests of specificity of the IFA technique have been reported previously (15, 30, 31). No immunofluorescent staining with fluorescein-conjugated goat anti-mouse IgG, IgM, or IgA antiserum occurred when phosphate-buffered saline was substituted for the test mouse serum. The antisera were also reacted against normal mouse serum by Ouchterlony immunodiffusion or immunoelectrophoresis or both. Reproducibility of the IFA system was checked by testing the same mouse serum on different days. The potency of the goat anti-mouse IgM and IgA antisera was investigated further in the rat study; these antisera were used to demonstrate IgM and IgA on the surface of *P. carinii* by direct immunofluorescence in rat bronchial lavage fluids (30).

Statistics. The nonparametric Kendall-Tau test (23) was used to determine the correlation between serum antibody titers in normal mice and histological assessment of the intensity of *P. carinii* infection.

RESULTS

Normal mice. Of the 73 mouse sera tested, 53 (73%) had demonstrable serum antibodies (i.e., IFA titers ≥ 1:4) to *P. carinii* with at least one of the immunoglobulin classes (Table 1). Of these 53 mice, 28 (53%) had serum antibodies of more than one Ig class; these antibodies were primarily IgG and IgM. Sixteen (30%) mice had antibodies only of the IgG class and nine (17%) mice had antibodies only of the IgM class.

Serum antibodies to *P. carinii* were detected in five of the six strains of mice. IgG was the predominant immunoglobulin class of antibody found among C3H/HeN, BALB/cAnN, AKR/J, and B10.A(2R) mice as judged by the frequency and level of titers achieved. Figure 1 shows the distribution of serum antibody titers in BALB/cAnN mice; similar results were found with the other three mouse strains. By contrast, IgM antibodies were more prominent than IgG antibodies in steroid-treated C57BL/6N mice (Fig. 2). No serum antibodies of any immunoglobulin class were found among eight control DBA/1J mice; however, the survival of these mice was shorter than that of other groups of mice.

Histopathologically, *P. carinii* was found in the lungs of most of the steroid-treated mice,

TABLE 1. Serological and histological features of *P. carinii* infection in normal mice

Group[a]	Survival (Days)[b]	Serum antibodies[c]			*P. carinii* infection	
		IgG	IgM	IgA	No.[d]	Mean score
C3H/HeN						
Rx	62 ± 15	5/10	4/10	1/10	10/10	2.2
C	74 ± 2	6/6	3/6	0/6	1/6	0.5
BALB/cAnN						
Rx	48 ± 4	7/11	5/11	0/11	5/9	1.0
C	74 ± 2	6/6	5/6	1/5	0/5	0.0
AKR/J						
Rx	56 ± 0	3/3	3/3	0/3	3/3	2.3
C	80 ± 9	6/7	5/7	1/7	2/7	0.5
B10.A(2R)						
Rx	56 ± 0	1/1	1/1	0/1	1/1	2.0
C	64 ± 1	6/6	1/6	1/6	1/6	0.5
C57B1/6N						
Rx	65 ± 2	4/13	8/13	1/13	12/13	1.0
C	80 ± 17	1/2	1/2	1/2	1/2	0.5
DBA/1J						
Rx						
C	42 ± 3	0/8	0/8	0/8	2/7	0.5

[a] Rx, Steroid treated; C, control.
[b] Mean ± standard error of the mean.
[c] Number positive (titer ≥ 1:4)/number tested.
[d] Number positive for *P. carinii*/number tested. In a few instances, lung specimens were not available for examination.

and the intensity of the infection varied among the different mouse strains (Table 1). *P. carinii* was infrequently found among control mice; when present, the infection was of minimal intensity. Antibody responses varied widely among control or steroid-treated mice at any given level of the intensity of the infection (Fig. 3). When all mice were considered, an inverse relationship was found between serum IgG antibody titer and semiquantitative assessment of the intensity of *P. carinii* infection ($P < 0.03$).

IgG was the principal antibody class found in BALB/cAnN mice sacrificed at weekly intervals (Fig. 4). Group 1 mice on the optimal steroid regimen sacrificed early in the study had no demonstrable serum antibodies to *P. carinii*; serum antibody levels rose slightly over time but only reached a peak titer of 1:16 in one mouse. In contrast, serum antibody titers increased markedly in group 2 mice after steroids had been discontinued. Serum IgM and IgA antibodies were low or absent in both groups of mice.

Nude mice. Serum antibodies to both rat and mouse *P. carinii* were tested (Table 2). The number of specimens examined sometimes varied for different immunoglobulin classes depending on the availability of sera. Among MSKCC nu/nu mice, serum antibodies were rare: IgG antibodies of low titer (1:4) to rat or mouse *P. carinii* were found in only three mice. These antibodies were unrelated to type of exposure to the organism.

Serum antibodies were not found among Kentucky nu/nu mice. By contrast, nu/+ mice exposed to rat or mouse *P. carinii* produced serum antibodies which were mainly IgG (Fig. 5). In both cases the immune response was quite specific for the exposure source (i.e., rat or mouse) of *P. carinii*.

DISCUSSION

Investigation of humoral immune responses to *P. carinii* infection has usually been performed in humans, and has involved either the IFA or complement fixation techniques (26). In earlier studies performed in this country, serum antibodies to the organism were found in 30 to 40% of immunosuppressed patients with *P. carinii* pneumonia and in 5 to 10% of close contacts of these patients, but were rare in the general population (17, 18). Thus, whereas serology was of limited value in clinical diagnosis, it has been used to investigate hospital epidemics of *Pneumocystis* pneumonia (19, 21, 24). On the other hand, more recent IFA studies have found a high prevalence of serum antibodies among the general populations of the United States, Great Britain, and The Netherlands (14, 20, 22), sug-

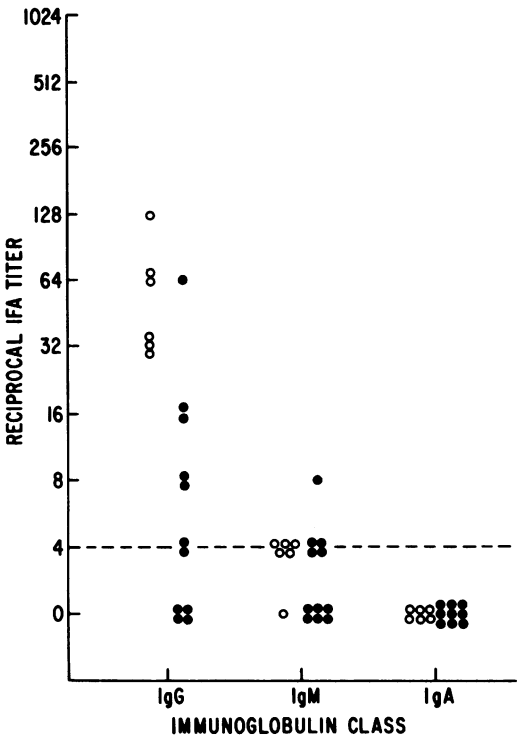

FIG. 1. Immunoglobulin class of serum antibody titers to *P. carinii* in BALB/cAnN mice. O, Control mice; ●, steroid-treated mice.

gesting that the organism is widely encountered in nature. Additional serological techniques are needed to help resolve the conflicting results of these surveys.

Among rats, serum antibodies to *P. carinii* were usually absent in healthy young animals but were present in retired breeders obtained from standard commercial laboratories (30). Healthy rats, bled sequentially, developed serum antibodies to the organism after prolonged (≥10 weeks) residence in a colony room containing *P. carinii*-infected rats. Antibody levels were suppressed in rats that were administered steroids but increased when steroids were tapered and *P. carinii* was cleared from the lungs.

The present study has extended these observations to mice. Serum antibodies to *P. carinii* were detected by the IFA technique in five of six strains of normal mice. Antibody titers varied considerably among the different mouse strains and among individual steroid-treated and control mice; however, when all mice were considered, an inverse correlation was observed between antibody titer and intensity of *P. carinii* infection in the lungs. We have found that the optimal steroid dose (on a milligram-per-kilogram basis) to provoke *P. carinii* pneumonia in mice is only about one-half that used in rats, because at higher doses the mice frequently die from overwhelming bacterial infection (27). Nevertheless, serum antibody titers in mice that were administered this steroid regimen and sacrificed sequentially were low, but titers rose when the steroids were withdrawn.

Serum antibodies in the mice were primarily of the IgG class, as evidenced by both the frequency and level of titers achieved. IgM antibodies were present, but IgA antibodies were uncommon and the titers were low. Similar findings have been reported in humans (2, 13, 14, 20, 22). Although the histopathological features of rat, mouse, and human *P. carinii* pneumonia are similar, the fact that rats and mice are steroid sensitive and humans are steroid resistant (5) suggested that these animals might not be useful in studying host immune responses to *P. carinii*. The data from our studies indicate that the serum antibody responses in rats and mice to *P. carinii* have considerable potential applicability to the human disease, including the possible development of new serological techniques. The prominence of IgG antibodies in this immune response also supports the hypothesis that *P. carinii* pneumonia, which develops in patients receiving immunosuppressive therapy,

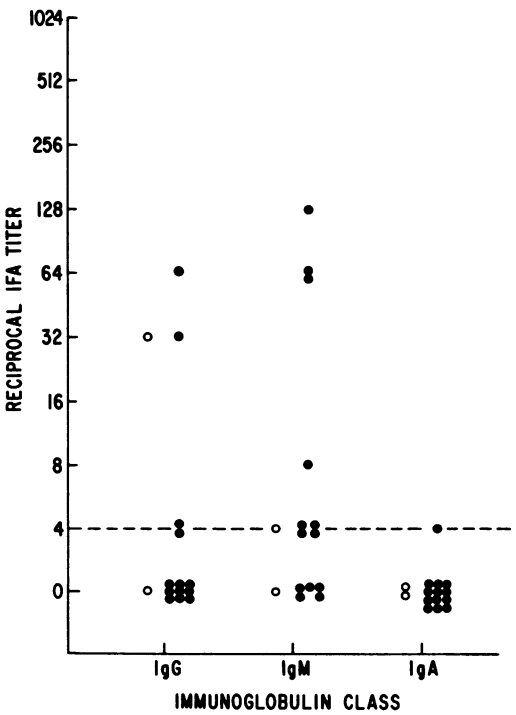

FIG. 2. Immunoglobulin class of serum antibody titers to *P. carinii* in C57BL/6N mice. O, Control mice; ●, steroid-treated mice.

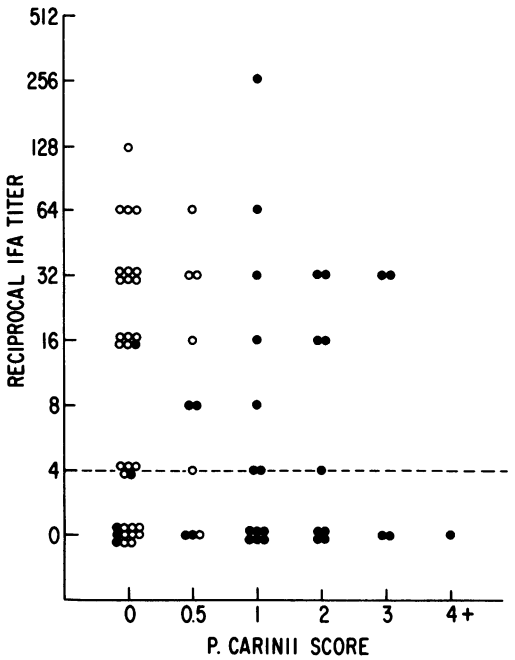

FIG. 3. Correlation between the histopathological assessment of the intensity of *P. carinii* infection and serum IgG antibody titers. ○, Control mice, ● steroid-treated mice.

usually results from reactivation of latent infection rather than from contagion (19, 22).

nu/nu mice were unable to produce serum antibodies to *P. carinii*; however, nu/+ mice mounted a vigorous antibody response, primarily of the IgG class. These data suggest that *P. carinii* antigens as measured by the IFA technique require an intact functioning thymus for antibody production. Similarly, the in vitro proliferative responses of lymphocytes to *P. carinii* appear to mainly involve T lymphocytes (7). nu/nu mice can respond to T-cell-independent antigens and produce IgM antibodies to a few microorganisms (3, 10, 34); yet, in most studies IgG antibody responses are lacking (1).

In contrast to their inability to produce serum antibodies to *P. carinii*, the outbred nude mouse strains differed markedly in their susceptibility to infection with exogenous organisms. Thus, in this animal model, serum antibodies do not have a major role in host susceptibility or resistence to *P. carinii* infection. The resistance of nude mice to some microorganisms is incompletely understood, but may be related to the enhanced activity of macrophages (4, 16). Among patients with primary immunodeficiency disorders, *P. carinii* pneumonia occurs most often in severe, combined immunodeficiency disease, which is characterized by profound defects in B and T lymphocytes (33).

nu/+ mice were able to selectively produce antibodies to rat or mouse *P. carinii* in response to exposure to exogenous sources of organisms. These findings extend our previous studies of the specific nature of host humoral immune responses to the organism in rats and humans (29). They also suggest that there may be species or strain differences in *P. carinii*. Controversy over the antigenic properties of *P. carinii* as determined by immunoflourescence has arisen largely because of differences in methodology and reagents (9, 11–13, 18). We used organisms derived from lungs by digestion with collagenase and hyaluronidase followed by Ficoll-Hypaque density gradient centrifugation. The process does not appear to alter the characteristics of *P. carinii* as judged by IFA staining and electron microscopy (31), but other enzymes (e.g., trypsin, pronase) produce noticeable changes in the organism (24a).

In conclusion, this study and our previous study (30) have provided data on the serum antibody responses in mice and rats to experimental *P. carinii* infection. The choice of the animal model system depends on the needs of the investigator. Rats have been the principal

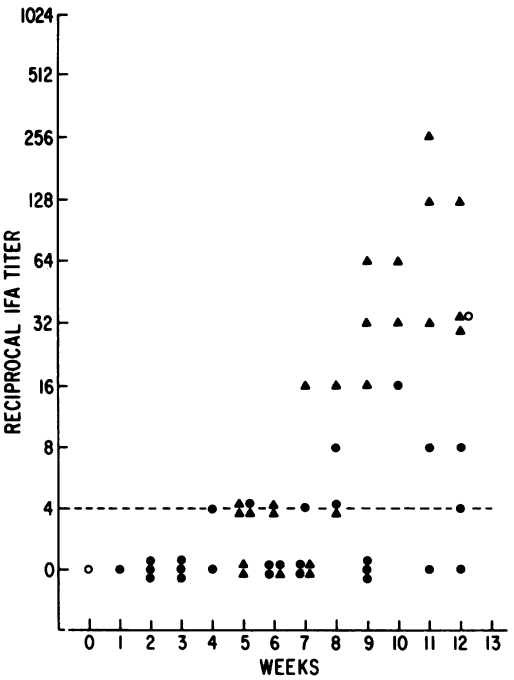

FIG. 4. Serum IgG antibody titers to *P. carinii* in BALB/cAnN mice sacrificed at weekly intervals. ●, Group 1 mice on optimal steroid treatment regimen throughout. ▲, Group 2 mice administered this regimen during weeks 1 to 4; the steroid dose was tapered to zero during weeks 5 to 7. ○, Control mice.

TABLE 2. Serum antibodies to *P. carinii* in nu/nu and nu/+ mice

Group	Exposure	Test source of *P. carinii*					
		Rat IgG	Rat IgM	Rat IgA	Mouse IgG	Mouse IgM	Mouse IgA
MSKCC							
Nu/nu	Variable[a]	3/89[b]	0/89		3/89	0/80	0/37
Kentucky							
Nu/nu	Rat	0/82	0/82	0/77	0/82	0/82	0/63
Nu/+		19/40	5/40	0/32	4/40	0/40	0/23
Nu/nu	Mouse	0/4	0/4	0/4	0/4	0/4	0/4
Nu/+		0/8	0/8	0/8	7/8	3/8	0/8
Nu/nu	Control	0/23	0/23	0/22	0/23	0/23	0/22
Nu/+		0/16	0/16	0/16	0/16	1/16	0/15

[a] Includes animals exposed to *P. carinii* and controls.
[b] Number positive (titer ≥ 1:4)/number tested.

experimental model for *P. carinii* and usually develop heavier degrees of infection, but have been limited by a relatively small supply of commercially available immunological reagents. Mice offer the advantages of a variety of well-characterized inbred strains and more plentiful immunological reagents, but are susceptible to overwhelming bacterial infection with small alterations in the steroid dose.

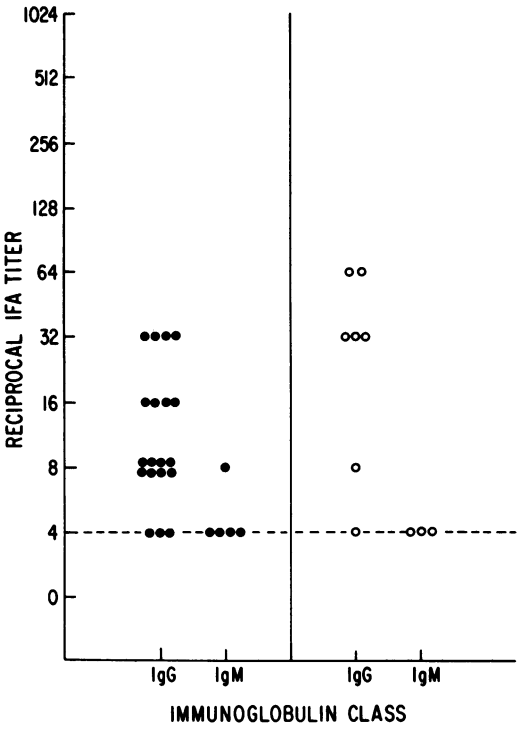

FIG. 5. Immunoglobulin class of serum antibody titers to *P. carinii* in Kentucky outbred Swiss nu/+ mice. ●, Antibody titers to rat *P. carinii* in mice exposed to rat sources of the organism; ○, antibody titers to mouse *P. carinii* in mice exposed to mouse sources of the organism.

ACKNOWLEDGMENTS

This study was supported by the Veterans Administration and by the Ida C. Hagman grant for cancer research from the American Cancer Society.

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