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Antibody-Mediated Shift in the Profile of Glycoprotein A Phenotypes Observed in a Mouse Model of *Pneumocystis carinii* Pneumonia

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It is well established that *Pneumocystis carinii* has the molecular capability for variation of a major surface antigen, glycoprotein A (gpA). However, the extent of expression of gpA variation among *P. carinii* organisms infecting a single host and whether this variation has any impact on host-parasite immunological interactions is unknown. Using a mouse model of *P. carinii* pneumonia, we were able to demonstrate the expression of more than one gpA phenotype in a closed population of infected mice. Administration of monoclonal antibody (MAb) 2B5, which is specific for one of the gpA phenotypes, resulted in a marked diminution in the frequency of this particular gpA phenotype in the population of organisms. This effect was due to a loss of trophozoites bearing the specific epitope recognized by MAb 2B5; cysts bearing the same epitope appeared unaffected. Interestingly, *P. carinii* was unable to introduce a new phenotype into the population to compensate for the loss of trophozoites bearing the epitope recognized by MAb 2B5. Discontinuing administration of MAb 2B5 allowed the MAb 2B5-binding phenotype to reemerge. This finding suggests that the phenotype recognized by MAb 2B5 was continually produced even when MAb 2B5 was present. Thus, although *P. carinii* exhibited a form of antigenic variation, it did not appear able to rapidly introduce new phenotypes into the population in response to destruction by antibodies.

Glycoprotein A (gpA) is an abundant, immunodominant gp on the surface of the opportunistic pathogen *Pneumocystis carinii* (4, 6, 9). This molecule has been shown to be highly variable in both phenotype (4, 5) and genotype (13, 19, 21, 23, 27). In addition, that genes encoding gpA are highly polymorphic, repeated, and dispersed throughout the genome suggests that antigenic variation could occur within individual organisms of a *P. carinii* population. In this regard, Wada et al. (25) have recently shown that an element of *P. carinii* DNA termed the upstream conserved sequence can have attached to it different gpA genes. This finding suggests that heterogeneity of gpA phenotypes could be generated by recombination between gpA genes at the upstream conserved sequence locus and one or more of the gpA genes located elsewhere in the genome. The potential ability of *P. carinii* to produce variation in gpA raises the obvious speculation that this property could help *P. carinii* evade the host immune system in a fashion similar to that found among other microorganisms which have the capacity to alter surface molecules (1, 2). However, a major difference between *P. carinii* and other pathogenic microorganisms capable of altering their surface molecules is the inability of *P. carinii* to produce disease in immunocompetent hosts. Thus, a better understanding of antigenic polymorphism in *P. carinii* would be helpful in further defining the immunopathogenesis of infection by this and possibly other microorganisms.

Lack of a continuous in vitro culture system and limitations in manipulating *P. carinii* in vivo have made it difficult to define whether there is significant variation in gpA within a population of *P. carinii* and, if there is, how it affects the pathogenesis of disease. For example, although variability in mRNAs encoding gpA has been demonstrated, it is not clear how the expression of their corresponding genes is regulated within either an individual organism or a given population of *P. carinii*. During pilot studies of passive immunoprophylaxis against *P. carinii*, administration of a monoclonal antibody (MAb) specific for rat *P. carinii* gpA resulted in the loss of the epitope recognized by the MAb (8). It was unclear, however, whether the loss of the epitope was a direct result of antibody administration or whether it reflected naturally occurring polymorphism in *P. carinii* gpA. This initial observation, when considered in the context of the highly complex and polymorphic nature of the gene(s) encoding *P. carinii* gpA, suggests that a population of *P. carinii* within a single host may be able to express simultaneously multiple phenotypes of gpA molecules.

To examine more carefully the effect of antibody on expression of *P. carinii* gpA in a closed population of *P. carinii*, we used a SCID mouse model in which animals could be treated with a gpA-specific antibody after *P. carinii* pneumonia (PCP) was induced either by airborne transmission or by direct inoculation of organisms. This model allowed us to maintain a long-term *P. carinii* infection by serial passage of *P. carinii* from animal to animal under conditions which precluded introduction of organisms from the environment. These studies demonstrated that polymorphism of gpA does exist within a given population of *P. carinii*, that antibody can alter the expression of a specific gpA phenotype in the population, and that this effect occurs primarily within the trophozoite life cycle stage of *P. carinii.*

MATERIALS AND METHODS

Mice and experimental design. Male C.B-17 *scid/scid* (SCID) mice were bred and maintained at the Trudeau Institute in micro-isolator cages and given sterilized food and water ad libitum. To induce PCP, weanling SCID mice were cohoused for 6 weeks with *P. carinii*-infected SCID mice (26). After the mice developed PCP, they were given twice-weekly intraperitoneal injections of 0.5 mg of MAb 2B5 (see below), which is specific for mouse gpA (5). Control *P. carinii*-infected SCID mice were either untreated or given twice-weekly intraper-

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itoneal injections of 0.5 mg of an isotype-matched irrelevant MAb (TIB191; American Type Culture Collection, Rockville, Md.). Once the initial infection was established by cohousing, long-term infections were maintained by intratracheal inoculation of *P. carinii* isolated from MAb-treated mice or untreated control mice into *P. carinii*-free SCID mice (14). For these long-term experiments, *P. carinii* was isolated from individual donor mice and inoculated into individual recipient *P. carinii*-free SCID mice. *P. carinii* was prepared for inoculation by pushing infected lungs through a stainless steel screen into 5 ml of Hanks balanced salt solution containing 0.5% glutathione and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 7.2) (18). One milliliter of this homogenate was concentrated by centrifugation, and the pellet was resuspended in 100 μl of phosphate-buffered saline and then inoculated intratracheally. The remaining 4 ml was prepared for analysis as described below. In this manner, we were able to keep a closed *P. carinii* population continually exposed to MAb 2B5 for 6 months. Furthermore, this design ensured that there was active growth of *P. carinii* during exposure to the MAbs, since there is a vast increase in the number of organisms present at the time of sacrifice compared with the number present a few days after inoculation (15). SCID mice were sacrificed at various times after the initiation of MAb treatment, and antigens of *P. carinii* isolated from their lungs were examined by Western blotting (immunoblotting) and flow cytometry.

***P. carinii*-specific MAbs.** Three gpA-specific MAbs were used for various aspects of these experiments (5, 10). MAbs 2B5 (90-3-2B5) and 3D6 (94-1-3D6) are immunoglobulin G1 (IgG1) antibodies specific for mouse *P. carinii* gpA. These two MAbs were shown to recognize different epitopes on gpA by their ability to bind to distinct fragments of gpA when analyzed by Western blotting and by the failure of one of these antibodies to inhibit the binding of the other by flow cytometry analysis. MAb 5E12 (85-1-5E12) is an IgM antibody which recognizes a mannose-containing epitope on gpA of all *P. carinii* isolates examined to date, regardless of the mammalian host from which the organisms are derived. Ascites fluid was prepared in BALB/c mice from these MAbs as well as from the control MAb, TIB 191. The IgG1 MAbs were isolated by saturated ammonium sulfate precipitation and purification by protein G affinity chromatography. MAb 5E12 was isolated by saturated ammonium sulfate precipitation.

Preparation of *P. carinii* for analysis. The remainder of the homogenate prepared for inoculation was further processed prior to analysis. An aliquot of the lung homogenate was removed for quantitation of *P. carinii*. Lung homogenates were pushed through a 22-gauge needle and then through a 26-gauge needle to disperse clumps. Cellular debris was removed from the lung homogenates by a low-speed ($50 \times g$) spin, and the *P. carinii* in the supernatant was then pelleted by centrifugation at $1{,}300 \times g$. Erythrocytes were lysed by hypotonic shock, and *P. carinii* was treated with 0.05 mg of DNase per ml for 30 min at 37°C. Cell debris was removed with a low-speed spin, and *P. carinii* in the supernatant was again pelleted by centrifugation. *P. carinii* was resuspended, filtered through 5-μm-pore-size nylon monofilament screening fabric (Tetko, Inc., Briarcliff Manor, N.Y.), and pushed through a 26-gauge needle several times before phenotypic analysis by flow cytometry. Both cysts and trophozoites were obtained by using this isolation procedure. In some experiments, the isolated *P. carinii* was additionally filtered through 20-μm-pore-size nylon filter membranes (Micron Separations, Inc., Westboro, Mass.) to obtain trophozoites, since cysts adhered to the filter membrane material and were lost from the preparation. Aliquots of purified *P. carinii* were frozen at −70°C until used in Western blot analysis.

Enumeration of *P. carinii* in the lungs of SCID mice. The number of *P. carinii* nuclei in the lungs of SCID mice was determined as previously described (3, 16). Aliquots of lung homogenates or purified *P. carinii* were spun onto glass slides by using a cytocentrifuge (Shandon, Sewickly, Pa.) and stained in Diff-Quik (Baxter Scientific Products, Miami, Fla.). The number of *P. carinii* nuclei and the ratio of trophozoites to cysts were determined by microscopic examination of the slides.

Western blot analysis of *P. carinii* phenotype. Western blotting was performed as previously described after separation of *P. carinii* antigens by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel (5). For these analyses, *P. carinii* was not digested with collagenase; thus, gpA did not appear as a distinctive doublet between 100 and 120 kDa but rather appeared as a slightly larger (≈130-kDa) broad band. Three MAbs, 5E12, 2B5, and 3D6, were used to analyze the expression of *P. carinii* gpA in the various groups. To determine whether administration of MAb 2B5 had any effect on the expression of this epitope in the population of *P. carinii*, organisms were isolated as described above, adjusted to a concentration of 5×10^6 to 10×10^6 nuclei per ml, mixed with sample buffer (2:1), boiled for 5 min, and centrifuged at $14{,}000 \times g$, and 100 μl of the clarified supernatant was loaded in each lane of the gel. As discussed below, because of the effects of MAb treatment, the proportion of cysts and trophozoites varied between the experimental and control groups.

Because analysis of the Western blots depended on the intensity of the band representing gpA, several precautions were taken to avoid artifactual changes in band intensity. First, as noted above, *P. carinii* organisms were carefully enumerated prior to loading onto the gel. Second, preliminary blotting was done to make sure that the reagent concentrations used and the number of organisms analyzed were appropriate to allow detection of gpA without the sample becoming saturated; i.e., if more of the epitope was present in the sample, the band could get darker. Third, all samples from each time point were run on a single gel for blotting with each of the MAbs used. Finally, the blots were processed concurrently to make sure the times for each step in the procedure were precisely the same.

Image analysis of the blots was done to compare the gpA band intensity (darkness) produced after immunoblotting a sample of *P. carinii* isolated from each animal with various MAbs. This analysis was performed with the Metamorph software program (Universal Imaging Inc., West Chester, Pa.) and an image scanner. The integral gray level intensity was calculated after subtraction of local background. These data were then used to calculate the ratio of binding of one MAb to binding of another. For example, the intensity of the gpA band resulting from MAb 2B5 was compared with that resulting from MAb 5E12 and referred to as the 2B5/5E12 ratio.

Flow cytometric determination of *P. carinii* phenotype. Phenotypic analysis of *P. carinii* was performed with MAb 5E12, 2B5, or 3D6. The MAbs were purified from ascites fluid and incubated with *P. carinii* for 30 min at 4°C. *P. carinii* was washed and incubated with isotype-specific secondary antibodies, which included anti-mouse IgM-fluorescein isothiocyanate (PharMingen, San Diego, Calif.), anti-mouse IgG1-biotin (PharMingen), or anti-mouse IgM-phycoerythrin (Cappel, Durham, N.C.). Streptavidin-phycoerythrin was used to detect biotinylated anti-mouse IgG1. To ensure that positive flow cytometry events were due to specific binding by the MAbs, each mouse *P. carinii* sample was also analyzed after incubation with the secondary antibody only. After staining, *P. carinii* was washed and suspended in 200 μl of Hanks balanced salt solution with 0.5% glutathione and HEPES buffer for analysis. Approximately 2×10^5 fluorescein isothiocyanate-labeled 2-μm-diameter beads were then added to each tube prior to analysis. Analysis of *P. carinii* fluorescence was performed with a FACScan cytofluorometer (Becton Dickinson, Mountain View, Calif.). Forward and side scatter were set to a logarithmic scale, and the 2-μm-diameter beads were used as a size marker for locating *P. carinii*. Gates were set to include events falling within forward and side scatter gates, which included particles approximately 2 to 5 μm in size that were positive for fluorescence associated with MAb 5E12, 2B5, or 3D6. The forward scatter threshold was set to exclude any particles less than 2 μm, and 5,000 events were routinely collected. The percentages of *P. carinii* organisms which were both positive for the 5E12 epitope ($5E12^+$) and either $2B5^+$ or $3D6^+$ were reported. *P. carinii* organisms labeled with MAb 5E12 were sorted in a FACStar Plus (Becton Dickinson), spun onto slides, and stained with Diff-Quik. This procedure confirmed that events which fell into the above-noted forward and side scatter genes and which were $5E12^+$ were morphologically identifiable as *P. carinii* (data not shown) and also confirmed that few of the events included clumps of *P. carinii.*

Phenotypic analysis of *P. carinii* cysts by immunocytochemistry. To determine the percentage of cysts expressing gpA epitopes specific for MAb 5E12 or 2B5, purified *P. carinii* was spun onto glass slides and fixed in ice-cold acetone. Two-color immunocytochemistry was performed with MAbs 5E12 and 2B5, using a Vectastain ABC-AP kit (Vector Laboratories, Burlingame, Calif.) as instructed by the manufacturer. As negative controls, samples from each experimental group were also stained with the labeled secondary antibodies only. The percentage of cysts stained by MAb 5E12, MAb 2B5, or both was determined microscopically. At least 100 cysts were counted for each sample by a single investigator. Percentages were confirmed by examination by a second investigator.

Statistics. Data are presented as the mean ± standard deviation of the various groups. Differences between means were determined by using the Mann-Whitney *U* test or Kruskal-Wallis one-way analysis of variance on ranks test followed by Dunn's post hoc test.

RESULTS

The purpose of these experiments was to determine the effect of long-term administration of gpA-specific MAb 2B5 on the phenotype of *P. carinii* present during PCP in SCID mice. To accomplish this, a group of *P. carinii*-free SCID mice were cohoused with *P. carinii*-infected SCID mice. Once PCP developed, the animals were divided into two groups; one group received biweekly injections of 0.5 mg of MAb 2B5; the other remained untreated or in some experiments (Table 3) received an isotype-matched control antibody. At approximately 6- to 8-week intervals, the animals were sacrificed and *P. carinii* was isolated for analysis and further in vivo passage by inoculation into *P. carinii*-free SCID mice. Antibody administration was begun immediately with each passage of *P. carinii* into new SCID mice. Organisms were passaged three times after the initial infection was established so that beginning with the initial infection, *P. carinii* was exposed to MAb 2B5 for 42, 91, 147, or 194 days. Four or five animals per experimental group were evaluated at each time point.

Western blot analysis of epitope expression on *P. carinii* recovered from MAb 2B5-treated or control SCID mice. *P.*

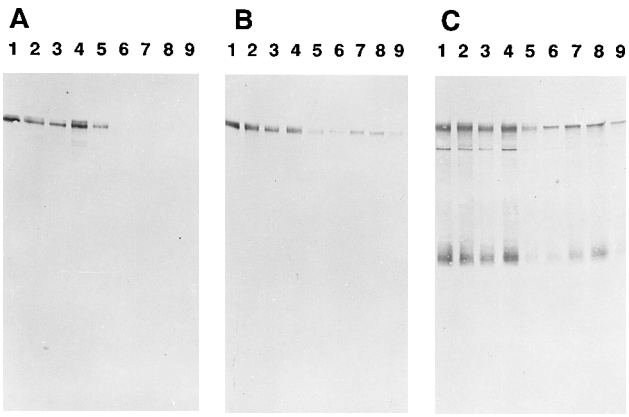

FIG. 1. Western blot analysis of gpA from *P. carinii* obtained after in vivo exposure to MAb 2B5 for a total of 194 days and three passages through MAb 2B5-treated mice (lanes 6 to 9) or obtained after passage three times through untreated control SCID mice for the same length of time (lanes 1 to 5). Panel A was developed after incubation with MAb 2B5, panel B was developed after incubation with MAb 3D6, and panel C was developed after incubation with MAb 5E12.

carinii isolated after three forward transfers and a total of 194 days of in vivo exposure to MAb 2B5 were analyzed by Western blotting for the ability of MAb 2B5 to bind to its target antigen (Fig. 1). MAb 2B5 readily bound to gpA of *P. carinii* isolated from untreated control mice (Fig. 1A, lanes 1 to 5), but binding was below the threshold of detection in the samples isolated from the four MAb 2B5-treated animals (lanes 6 to 9). In contrast, when MAb 5E12, which binds to a carbohydrate epitope on gpAs of *P. carinii* from all hosts analyzed to date, was used to probe a duplicate blot (Fig. 1C), gpA was present in detectable amounts in all samples. To further confirm this apparent selective loss of the 2B5 epitope from the population of organisms analyzed, a third duplicate blot analysis was performed with MAb 3D6, which is specific for mouse *P. carinii* gpA but recognizes an epitope distinct from that recognized by MAb 2B5 or 5E12. This blot (Fig. 1B) demonstrated that MAb 3D6 bound to gpA of *P. carinii* from both the MAb 2B5-treated and untreated control mice. Importantly, image analysis of the band darkness in these blots demonstrated that the levels of binding of MAbs 2B5 and 3D6 to control *P. carinii* gpA were equivalent (Table 1) in that the 2B5/3D6 ratios were 0.9, 0.7, 0.8, 1.3, and 1.4 in the five mice from the control group. However, the 2B5/3D6 and 2B5/5E12 ratios were zero in the samples from the four MAb-treated mice.

Western blotting was also performed with *P. carinii* isolated after 42, 91, and 147 days of exposure to MAb 2B5. At each time point, there was an obvious decrease in the presence of the epitope recognized by MAb 2B5 in *P. carinii* isolated from the MAb 2B5-treated animals relative to the epitopes recognized by MAb 3D6 or 5E12 (data not shown).

Table 1 summarizes the results of Western blot analysis using *P. carinii* isolated after prolonged exposure to MAb 2B5 (194 days and three forward passages). The intensity of binding of MAb 2B5 to gpA decreased from about half of that observed with MAb 5E12 (2B5/5E12 ratio of 0.5) when control *P. carinii* was analyzed to undetectable levels when the *P. carinii* exposed to MAb 2B5 in vivo for 194 days was analyzed. Similar results were noted after the first (91 days of exposure to MAb 2B5) and second (147 days of exposure to MAb 2B5) passages, with an approximately 10-fold decrease in the 2B5/5E12 binding ratio at each time point (data not shown).

Table 1 demonstrates that there was also a decrease, although less pronounced, in the 3D6/5E12 ratio of binding to *P. carinii* from MAb 2B5-treated mice compared with *P. carinii* from control mice, even though MAbs 2B5 and 3D6 bind to distinct epitopes on *P. carinii* gpA. The drop in the 3D6/5E12 ratio noted in the day 194 samples was variable at the other time points and when present was never statistically significant (Tables 2 and 3). Two-color fluorescence-activated cell sorting (FACS) analysis of nonmanipulated *P. carinii* showed that approximately two-thirds of organisms staining with MAb 3D6 also stained with MAb 2B5, which may explain this observation. The marked drop in the 2B5/3D6 binding ratio determined from control and MAb 2B5-exposed *P. carinii* provides strong evidence that the 2B5 epitope is lost preferentially to the 3D6 epitope.

Two additional control Western blot analyses were performed (not shown) to compare the binding characteristics of MAbs 2B5 and 3D6, using an unselected preparation of mouse *P. carinii*. First, the working dilutions of MAbs used in the blots were further serially diluted and then used in the Western blot

TABLE 1. Image analysis of Western blot results comparing the ratios of the intensity of binding of gpA-specific MAbs 2B5, 3D6, and 5E12 to MAb 2B5-exposed (194 days) or control *P. carinii*

Group	Mean binding ratio ± SD		
	2B5/5E12	3D6/5E12	2B5/3D6
Control	0.52 ± 0.15	0.54 ± 0.17	1.01 ± 0.33
MAb 2B5 exposed	0 ± 0[a]	0.25 ± 0.05[a]	0 ± 0[a]

[a] $P < 0.05$ by Mann-Whitney *U* test.

TABLE 2. Image analysis of Western blot results comparing the ratios of the intensity of binding of MAbs 2B5, 3D6, and 5E12 to control *P. carinii*, *P. carinii* exposed to MAb 2B5 for 147 days, or *P. carinii* exposed to MAb 2B5 for 147 days and then released from antibody pressure by passage to new SCID mice for 56 days

Group	Mean binding ratio ± SD		
	2B5/5E12	3D6/5E12	2B5/3D6
Control	4.2 ± 2.4	3.0 ± 2.2	1.5 ± 0.3
MAb 2B5 exposed	0.5 ± 0.3[a]	2.8 ± 0.8	0.2 ± 0.1[a]
Released	1.4 ± 0.5	1.8 ± 0.5	0.7 ± 0.1

[a] $P < 0.05$ by Kruskal-Wallis analysis of variance and Dunn's post hoc test.

TABLE 3. Image analysis of Western blots comparing the ratios of the intensity of binding of gpA-specific MAbs 2B5, 3D6, and 5E12 to *P. carinii* exposed to MAb 2B5 or isotype-matched irrelevant control MAb in vivo for 21 or 42 days

Group	Mean binding ratio ± SD		
	2B5/5E12	3D6/5E12	2B5/3D6
21 days			
Control MAb	5.8 ± 1.9	4.1 ± 1.4	1.5 ± 0.1
MAb 2B5 exposed	2.3 ± 0.6[a]	4.6 ± 1.1	0.5 ± 0.0[b]
42 days			
Control MAb	2.2 ± 0.9	4.0 ± 1.3	0.6 ± 0.4
MAb 2B5 exposed	0.5 ± 0.2[a]	2.8 ± 0.2[c]	0.2 ± 0.1[b]

[a] $P < 0.05$ by Mann-Whitney *U* test.
[b] $P = 0.06$ by Mann-Whitney *U* test.
[c] $P = 0.19$ by Mann-Whitney *U* test.

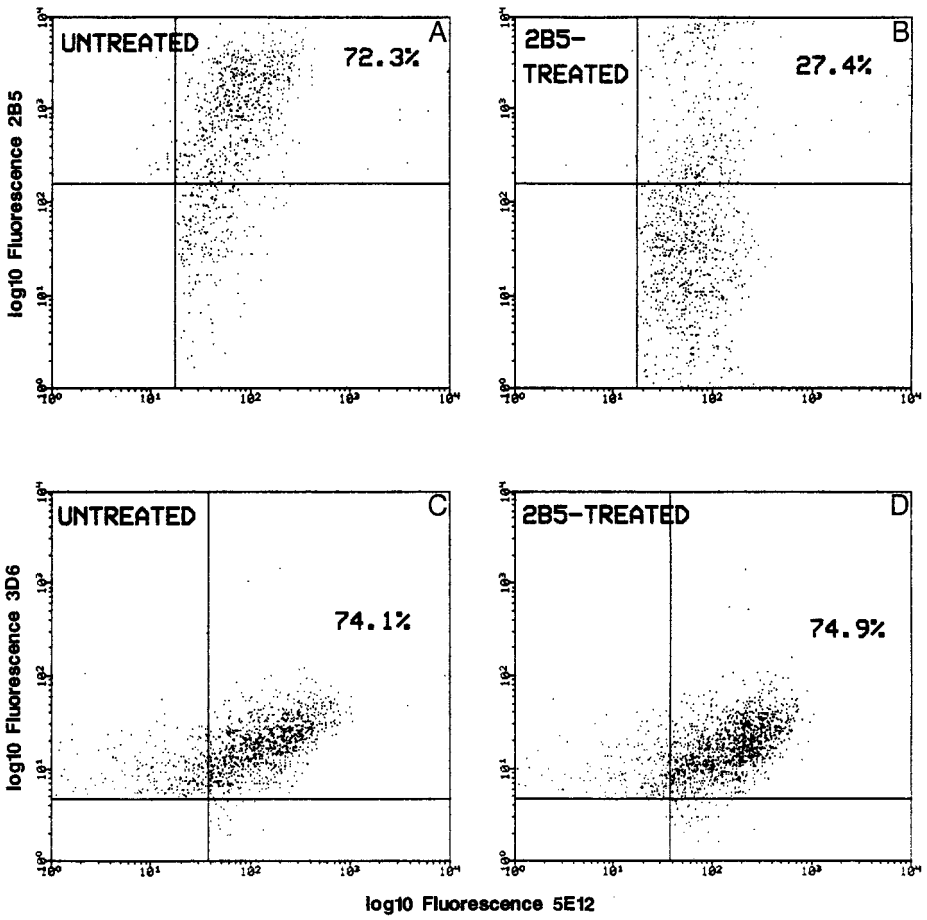

FIG. 2. Flow cytometric analysis of *P. carinii* after in vivo exposure to MAb 2B5. *P. carinii* was isolated from the lungs of SCID mice that had been either untreated or treated with MAb 2B5 biweekly and stained with fluorescence-labeled antibodies 5E12 and 2B5 or 3D6. *P. carinii* had been forward transferred three times and had been under MAb pressure for a total of 194 days. Gates were set to include particles 2 to 5 μm in diameter (compared with those obtained with 2-μm-diameter beads for forward and side scatter) and fluorescence positive for 5E12 (*x* axis), 2B5 (A and B), or 3D6 (C and D) (*y* axis). Data shown are from one mouse in each group but are representative of *P. carinii* from each of four mice in the MAb 2B5 group and five mice in the control group.

format described above. Second, Western blotting was performed on serially diluted samples of *P. carinii*-infected mouse lung homogenates with the same dilutions of MAbs 2B5 and 3D6 as used for Fig. 1 and 4. In both instances, the sensitivity of MAb 2B5 for detecting *P. carinii* gpA was greater than that of MAb 3D6. Therefore, the decreased binding of MAb 2B5 to *P. carinii* gpA in the MAb 2B5-treated mice appears to have been due to the loss of the epitope recognized by MAb 2B5 rather than due to differences in the affinity or avidity of binding between MAbs 2B5 and 3D6. Finally, because the boiling in the presence of detergent used to prepare samples for SDS-PAGE would be expected to dissociate antigen-antibody complexes, masking of the epitope by the in vivo-administered MAb 2B5 would also be highly unlikely. Failure to dissociate antigen-antibody complexes would result in altered mobility of gpA; this was not observed.

Flow cytometric determination of 2B5, 5E12, and 3D6 epitope expression on *P. carinii*. Western blot results reflect the predominant antigenic profile of the entire population of organisms analyzed. Therefore, to confirm the Western blot results and to obtain more precise quantitative data, fluorescence-activated flow cytometry was used to examine the effect of administration of MAb 2B5 on the expression of its epitope on individual *P. carinii* organisms. Figure 2 shows the result of flow cytometric analysis of *P. carinii* isolated after three passages and 194 days of in vivo exposure to MAb 2B5 or isolated from untreated controls. Of the *P. carinii* organisms from control mice, approximately three-fourths bearing the 5E12 epitope ($5E12^+$) were also bound by MAb 2B5 ($2B5^+$) (Fig. 2A). In contrast, after in vivo treatment with MAb 2B5, only one-fourth of *P. carinii* organisms were $5E12^+$ $2B5^+$ (Fig. 2B). To further investigate the specificity of the loss of the 2B5 epitope from *P. carinii* after in vivo exposure to MAb 2B5, flow cytometric analysis was performed with the third gpA-specific MAb, 3D6. As shown in Fig. 2C, approximately three-fourths of $5E12^+$ *P. carinii* from untreated control mice were also $3D6^+$. However, unlike expression of the 2B5 epitope, 3D6 epitope expression did not change on *P. carinii* exposed in vivo to MAb 2B5 (Fig. 2D). Incubation of *P. carinii* from the MAb 2B5-treated mice with secondary antibody only resulted in a number of 2B5 epitope-positive organisms similar to that seen after incubation of these organisms with MAb 2B5 before addition of the secondary antibody. This result indicated that the $2B5^+$ organisms remaining after in vivo antibody treatment were bound by MAb 2B5, from either in vivo or in vitro exposure, and that the decrease in $5E12^+$ $2B5^+$ *P. carinii* was not due to interference by the in vivo-administered MAb 2B5 with subsequent flow cytometry analysis.

The results of flow cytometric analysis of binding of MAb 2B5 after the initial infection and each of the three forward

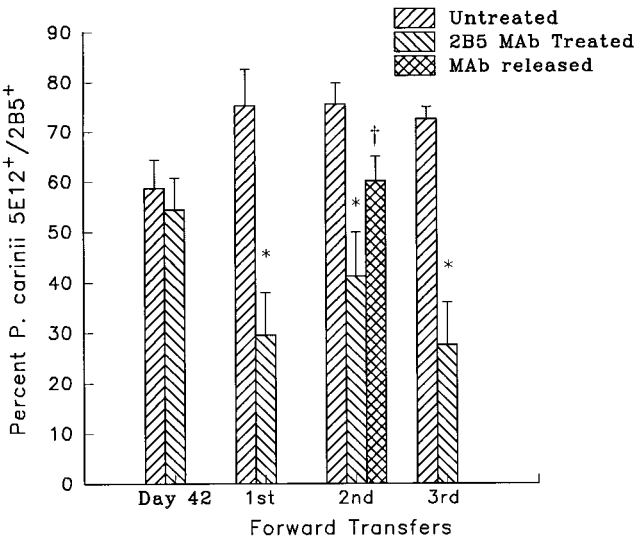

FIG. 3. Percentage of epitope 5E12-positive *P. carinii* that were also epitope 2B5 fluorescence positive by flow cytometry after three transfers into SCID mice. *P. carinii* organisms isolated from the lungs of SCID mice which were either untreated or injected with 2B5 biweekly were transferred into uninfected SCID mice three separate times, and the MAb pressure was either resumed or released (second transfer). Initial data were collected 42 days after initiation of the MAb pressure. Data from the first, second, and third forward transfers were collected at days 91, 147, and 194, respectively, after initiation of administration of MAb 2B5. Data represent the mean ± standard deviation ($n = 4$ to 5). *, $P < 0.05$ compared with untreated mice; †, $P < 0.05$ compared with 2B5-treated mice.

transfers are presented in Fig. 3. As was seen after the third passage, in vivo administration of MAb 2B5 resulted in similar, significant reductions in the number of $5E12^+$ $2B5^+$ *P. carinii* isolated after the first and second forward transfers. Flow cytometry did not demonstrate a significant difference in the percentages of $5E12^+$ $2B5^+$ *P. carinii* after the initial 42 days of in vivo exposure to MAb 2B5 in the series of experiments shown. However, in three other separate experiments, 3 to 6 weeks of in vivo exposure to MAb 2B5 resulted in a 30 to 40% reduction in $5E12^+$ $2B5^+$ *P. carinii* isolated from the MAb 2B5-exposed *P. carinii* compared with unexposed *P. carinii*. This reduction was statistically significant.

Analysis of 2B5 epitope expression after cessation of 2B5 MAb treatment. To determine whether the observed loss of epitope 2B5 from the population of *P. carinii* after administration of MAb 2B5 was reversible, some of the organisms isolated after the first forward transfer from MAb 2B5-treated animals were passed into SCID mice which were not administered MAb 2B5. After 8 weeks, *P. carinii* organisms isolated from these animals, untreated control animals, and a similar number of animals which continued to receive MAb 2B5 were analyzed by flow cytometry and Western blotting. By flow cytometry, the percentage of *P. carinii* organisms which were $5E12^+$ $2B5^+$ was 41.3 ± 8.6 in the mice which continued to receive MAb 2B5, compared with 60.2 ± 4.8 in mice no longer being treated with MAb 2B5 ($P \leq 0.05$) (Fig. 3). The Western blot results from this experiment are shown in Fig. 4. Binding of MAb 2B5 to gpA is much stronger in *P. carinii* isolated from control animals (Fig. 4A, lanes 1 to 5) than in organisms isolated from the MAb 2B5-treated mice (lanes 6 to 9). Cessation of MAb 2B5 treatment resulted in increased binding of MAb 2B5 to *P. carinii* gpA (lanes 10 to 13) compared with the binding observed in *P. carinii* which had been continuously exposed to MAb 2B5. Duplicate blots were incubated with MAb 3D6 (Fig. 4B) and with MAb 5E12 (Fig. 3C). These two MAbs appeared to bind equally well to *P. carinii* gpA in all of the samples, indicating that approximately equal numbers of organisms were present in all samples and confirming that the results observed with MAb 2B5 were due to specific loss of its epitope from the population rather than to differential loading of the gel samples.

These blots were subjected to image analysis to measure objectively the variation in intensity of the binding of MAb 2B5 to gpA and to compare the binding of MAb 2B5 with that of MAb 3D6. Table 2 shows that immunoblot analysis of *P. carinii* from control mice with MAb 2B5 produced a band more than four times the intensity of that observed with MAb 5E12 (2B5/5E12 binding ratio of 4.2 ± 2.4). However, after exposure in vivo to MAb 2B5, the MAb 2B5/5E12 binding ratio was reduced to 0.5. In the *P. carinii* initially exposed to MAb 2B5, the 2B5/5E12 binding ratio increased to 1.4 after passage into SCID mice not treated with MAb 2B5. Image analysis comparing the intensity of binding of MAb 3D6 with that of MAb 5E12 did not show a similar relative decrease of MAb 3D6 binding after in vivo exposure to MAb 2B5, nor was there a

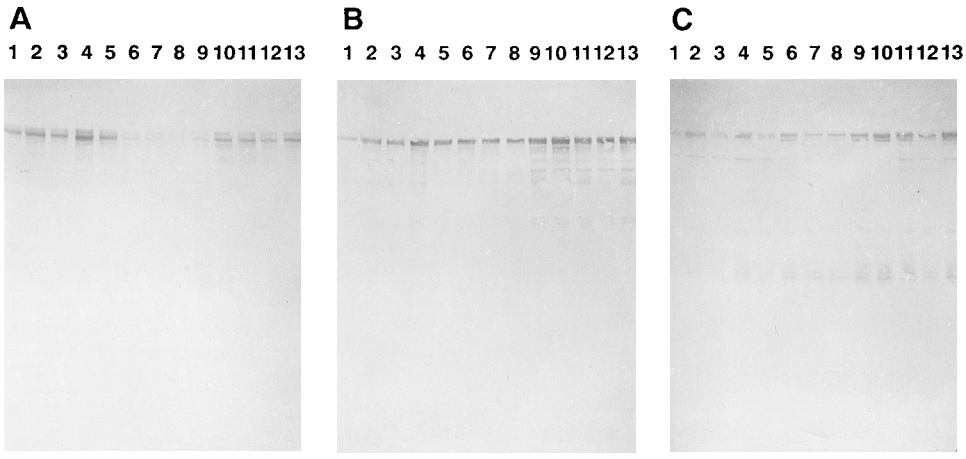

FIG. 4. Western blot analysis of gpA from *P. carinii* obtained from control SCID mice (lanes 1 to 5), from SCID mice treated with MAb 2B5 for a total of 147 days (lanes 6 to 9), or from *P. carinii* exposed in vivo to MAb 2B5 for 91 days and then passaged into SCID mice not receiving any antibody treatment and isolated 56 days later (lanes 10 to 13). Panel A was developed after incubation with MAb 2B5, panel B was developed after incubation with MAb 3D6, and panel C was developed after incubation with MAb 5E12.

TABLE 4. Effect of administration of MAb on the number and life cycle stage of *P. carinii* isolated from MAb 2B5-treated or control SCID mice

Group	Mean no. ($\log_{10}$) ± SD at indicated day after initiation of MAb infusion (forward transfers)			
	42	91 (1st passage)	147 (2nd passage)	194 (3rd passage)
Trophozoites				
Control	7.27 ± 0.50	7.27 ± 0.20	7.22 ± 0.46	7.61 ± 0.31
Treated	6.35 ± 0.22[a]	5.91 ± 0.14	6.29 ± 0.34	6.54 ± 0.33
Released[b]			6.75 ± 0.35	
Cysts				
Control	6.11 ± 0.67	5.92 ± 0.22	5.66 ± 0.64	6.31 ± 0.27
Treated	5.73 ± 0.18	5.90 ± 0.36	5.91 ± 0.36	5.90 ± 0.24
Released			5.87 ± 0.37	

[a] $P < 0.05$ compared with the control.
[b] Isolated from mice treated for 91 days with MAb 2B5 and then passaged into new SCID mice which did not receive any MAb treatments. These mice were sacrificed with the control and treated mice at 147 days.

significant change in this ratio when the organisms which had been released from MAb 2B5 pressure were analyzed. The 3D6/2B5 ratio calculated after image analysis of the blots further confirmed that 2B5 epitope expression was affected to a greater extent than 3D6 epitope expression in these experiments.

Effect of MAb 2B5 treatment on life cycle stages of *P. carinii*. To determine the effect of administration of MAb 2B5 on the number and distribution of life cycle stages of *P. carinii*, cytospin preparations of lung homogenates from the various time points were stained with Diff-Quik, and the trophozoites and cysts were enumerated. Administration of MAb 2B5 to SCID mice with active PCP for 42 days resulted in an approximately 90% reduction (7.27 ± 0.50 [$\log_{10}$] in the untreated mice to 6.35 ± 0.22 [$\log_{10}$] in the MAb 2B5-treated mice; $P < 0.05$) in the number of trophozoites present in the lung tissue (Table 4). Cyst numbers were not reduced by administration of MAb 2B5. This reduction in trophozoite numbers was maintained through the three forward passages, totaling 194 days of exposure to MAb 2B5. When MAb 2B5 administration was stopped in a subgroup of animals after the second forward transfer, the trophozoite numbers increased toward that seen in control mice (Table 4).

Immunohistochemical determination of 2B5 and 5E12 epitope expression on the surface of *P. carinii* cysts. Immunocytochemical analysis of the binding of MAbs 2B5 and 5E12 to the surface of *P. carinii* cysts indicated that 78.0% ± 9.6% of cysts from untreated SCID mice were $5E12^+$ $2B5^+$. *P. carinii* cysts obtained from MAb 2B5-treated mice (194 days) showed similar levels of expression of both epitopes, with 73.9% ± 7.4% of the cysts staining with both MAbs. These data indicate that the loss of 2B5 epitope expression did not occur on the cysts. Direct immunohistochemical analysis of trophozoites was unsuccessful because it was impossible for us to identify trophozoites precisely by this technique. However, FACS analysis of a preparation containing trophozoites only (cysts removed by nylon membrane filtration) from the day 42 time point showed a reduction in $5E12^+$ $2B5^+$ trophozoites from 56.9% ± 26.4% in the untreated mice to 19.1% ± 4.7% in the MAb 2B5-treated mice ($P < 0.05$). This result indicated that the change in 2B5 epitope expression occurred within the trophozoite population.

DISCUSSION

Variation or polymorphism in *P. carinii* gpA, especially in *P. carinii* from different host species, is well documented (5, 13, 19–21, 23, 25, 27). However, whether the organism utilizes this variation in gpA to affect host-parasite interactions is unknown. It is tempting to speculate that *P. carinii* uses antigenic polymorphism to evade destruction by the host; however, *P. carinii* must be limited in its ability to circumvent host defense mechanisms since, unlike other pathogens which employ antigenic variation, *P. carinii* is not known to produce disease in immunocompetent individuals. Once host immunity is impaired, *P. carinii* becomes an effective pathogen capable of killing the host. The experiments described in this report demonstrate that gpA-specific antibodies affect the range of gpA phenotypes present in a SCID mouse model of PCP.

As previously described (7), the administration of a MAb specific for *P. carinii* gpA (MAb 2B5) resulted in partial protection against PCP. In contrast to our previous report (7), MAb treatment in the present study was not begun until after PCP was established and we were able to keep *P. carinii* continually exposed to antibody for a prolonged period of time, approximately 6 months, by repeated passage of *P. carinii* from one MAb-treated infected SCID mouse into another. Passive immunoprophylaxis of *P. carinii*-infected SCID mice with MAb 2B5 resulted in a 90% decrease in the number of organisms relative to that in untreated control mice. More interesting, however, was the fact that the effect of MAb administration appeared to be limited to the trophozoite stage of *P. carinii*. A statistically significant drop in the number of trophozoites was noted in the initial group of treated animals, and this decrease in trophozoite numbers was maintained through three subsequent passages into new MAb 2B5-treated mice. In contrast, cyst counts were relatively unaffected from the beginning to the end of the experimental treatment. It must be noted that the design of these experiments, passaging organisms from one animal directly into another similarly grouped animal, resulted in the forward transfer of *P. carinii* into additional control or MAb 2B5-treated mice with inocula containing equal amounts of lung homogenate but with different numbers and proportions of cysts and trophozoites. However, when antibody pressure was removed from a subset of animals after the second forward transfer (Table 4, day 147), trophozoite numbers increased above those in the animals which continued to receive MAb 2B5 treatments even though all animals in these two particular groups received similar numbers and life cycle stages of organisms. Thus, antibody pressure was necessary to keep trophozoite numbers depressed. This finding along with the observed drop in trophozoite numbers noted after 42 days of antibody treatment of a preexisting naturally acquired PCP (Table 4) strongly supports the conclusion that trophozoites were preferentially removed by MAb treatment.

In addition to determining the effect of in vivo administration of a MAb to gpA on *P. carinii* numbers, the availability of MAbs to different epitopes of mouse *P. carinii* gpA allowed us to examine effects of treatment with these MAbs on the phenotype of gpA. Western blot and FACS analyses of *P. carinii* gpA isolated from mice treated with MAb 2B5 showed a marked decrease in the expression of 2B5 epitope by the *P. carinii* population. In contrast, when the same *P. carinii* preparations were analyzed with two MAbs which recognize epitopes of gpA distinct from that recognized by MAb 2B5, there was no consistent decrease in the expression of these epitopes. When the binding of the two mouse *P. carinii* gpA-specific MAbs, 2B5 and 3D6, was individually compared with that of the cross-reactive MAb 5E12, the ratios of band intensities were very similar in every preparation of control *P. carinii* analyzed (Tables 1 to 3), supporting the consistency and validity of the Western blot analyses.

These data provide direct evidence for antigenic variation or polymorphism in *P. carinii* gpA in vivo. If the changes in antigen expression were merely reflective of decreases in *P. carinii* numbers, either cysts or trophozoites, one would expect that the binding of other MAbs specific for gpA would show a similar decrease in binding. This was not the case in our experiments. Furthermore, direct FACS analysis of randomly obtained *P. carinii* showed that there were both $2B5^+$ $3D6^+$ and $2B5^-$ $3D6^+$ organisms in the lungs of infected animals. This result is consistent with molecular analysis of the genes encoding rat and ferret *P. carinii* gpA showing a 10 to 15% difference in the deduced amino acid sequence of gpA cDNA clones isolated from the same host species (19, 21, 25, 27). Mouse *P. carinii* gpA cDNA has not yet been cloned or analyzed, but analysis of partial genomic clones obtained by PCR shows a similar variation in deduced amino acid sequence (28). An alternative explanation for our results is that the epitopes analyzed are uniformly expressed by all mouse *P. carinii* organisms but are differentially expressed during various life cycle stages. Administration of MAb 2B5 did result in a marked drop in trophozoite numbers while having little effect on cyst counts. Therefore, if more trophozoites expressed the 2B5 epitope than the 3D6 epitope, loss of trophozoites would affect the 2B5 epitope more than the 3D6 epitope. However, if our results were due merely to the fact that trophozoites preferentially express the 2B5 epitope, one would expect that with continued MAb treatment, there would be a continued drop in trophozoite numbers. This was not the case. We noted an initial major drop in trophozoite numbers by 3 to 6 weeks after starting injections of MAb 2B5 and then no further drop over a total of 6 months of antibody treatment. Thus, our interpretation of these results is that administration of MAb 2B5 resulted in immune system-mediated destruction of only a subpopulation of the *P. carinii*, those that were $2B5^+$.

The mechanism by which MAb 2B5 reduced trophozoite numbers and produced a shift in the gpA phenotype of the remaining organisms is unknown. Nucleic acid probes for the genes encoding the specific epitopes recognized by the MAbs used in this study are not yet available, and the inherent variability in the gpA gene would make a molecular analysis of the loss of the 2B5 epitope difficult. Cell-mediated immunity is considered crucial for complete protection against *P. carinii* (16, 22), yet our results demonstrated that antibody can play a role in protection, at least to some extent, in the absence of functional T cells. These results are consistent with our previous observations on aspects of humoral immunity to *P. carinii* (7, 15). A similar phenomenon has been observed with *Giardia lamblia*, in which case cell-mediated immunity is important for control of infection but humoral (antibody) immunity seems to result in a phenotypic change in the variant-specific surface protein of *G. lamblia* trophozoites (11, 12, 17).

It is also of interest that we did not observe repopulation of the SCID mice with a new *P. carinii* phenotype lacking the 2B5 epitope. Since we were unable to start the infection with a carefully defined, i.e., single carefully characterized, clone of *P. carinii*, documenting the emergence of a new phenotype would be difficult. However, if a new phenotype, one that was $2B5^-$, had replaced the $2B5^+$ trophozoites, one would have predicted an increase in trophozoite numbers despite continued exposure to MAb 2B5, since the SCID mice have no other effective defense mechanism against *P. carinii*. A regrowth of trophozoites after the initial drop in $2B5^+$ trophozoites was not observed, suggesting that *P. carinii* is limited in the repertoire of gpA molecules it can express and/or that switching between various gpA molecules is not very efficient in this organism. If this were the case, it could explain, at least in part, why, despite its capacity to undergo phenotypic variation, *P. carinii* is not an effective pathogen for the immunocompetent host (1, 2). Thus, the antigenic variation noted in *P. carinii* is not analogous to that seen in trypanosomes, in which case parasitemia ebbs and flows with the host response to one set of antigens and the emergence of new antigens on the parasite (24). We were also unable to completely extinguish the $2B5^+$ phenotype, and $2B5^+$ cysts appeared to be completely unaffected. When MAb 2B5 administration was stopped, there was an increase in both $2B5^+$ organisms and trophozoite numbers. One explanation for this finding is that the cysts served as a reservoir for $2B5^+$ organisms. Alternatively, $2B5^+$ trophozoites could have been produced continuously but reduced through an antibody-mediated mechanism, and once MAb 2B5 administration ceased, $2B5^+$ trophozoites reemerged. It cannot be ruled out that trophozoites and cysts can replicate independently of each other. Also, if both forms represent mandatory steps in the replication cycle, the phenotype of one stage does not necessarily predict the phenotype of the next; e.g., $2B5^-$ trophozoites could develop into $2B5^+$ cysts. It will be necessary to develop more refined methods of manipulating *P. carinii* to better study these various possibilities.

In summary, we have found that within a population of *P. carinii* from a single host, there are subpopulations of organisms that express phenotypically distinct gpA molecules. Treatment of *P. carinii*-infected mice with a gpA-specific MAb significantly reduced the level of a subpopulation of organisms bearing the epitope recognized by the MAb. The biologic effect of the antibody was directed primarily against the wall-less trophozoite form of *P. carinii*, whereas the level of the thick-walled cysts form bearing the 2B5 epitope was not significantly reduced. *P. carinii* was unable to introduce a new phenotype into the population, over the time course of this experiment, to compensate for the loss of trophozoites bearing the 2B5 epitope. However, release of antibody pressure did result in the rapid reemergence of organisms bearing the epitope. Thus, although *P. carinii* did exhibit a form of antigenic variation, it was not the type described for overtly pathogenic parasites. However, it is possible that such antigenic variation could play a role in the persistence of a subclinical infection.

Although the polymorphism inherent in the gpA molecule may be a major obstacle to the development of immunotherapy targeted at gpA, immunologic and molecular genetic analysis has shown that there are regions of the gpA molecule that are conserved in *P. carinii* derived from a single host or from different host species (5, 20, 28). Hence, directing the immune response against widely distributed or conserved epitopes could effectively reduce organism numbers, providing a useful

adjunct to chemotherapy for PCP. These studies provide the model for such an approach.

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