Passive intranasal monoclonal antibody prophylaxis against murine Pneumocystis carinii pneumonia

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Passive antibody immunoprophylaxis is one method used to protect patients against infection if they are unable to mount an adequate active immune response. Topical application of antibody may be effective against infections at mucosal sites. Using a SCID mouse model of Pneumocystis carinii pneumonia, we were able to demonstrate protection against an airborne challenge with P. carinii by intranasal administration of antibody. Immunoglobulin M (IgM) monoclonal antibodies to an epitope shared by mouse and human P. carinii organisms reduced organism numbers by more than 99% under the conditions described. An IgG1 switch variant of one of the IgM monoclonal antibodies was also protective. These experiments provide a model for exploring the utility of this approach in protecting at-risk patients from infection with P. carinii.

Because Pneumocystis carinii pneumonia (PCP) is an opportunistic infection affecting individuals with significantly compromised immune systems, passive immunoprophylaxis is a preventive strategy that would be well suited for at-risk patients. Passive immunization is most easily and effectively accomplished with specific polyclonal antibody or monoclonal antibody (MAb) preparations.

CD4 T-cell-dependent immunity to P. carinii is a critical factor in the host’s normal resistance to overwhelming infection with this organism (8, 15), and this immunity can be expressed through antibody-mediated protection (6, 14). The use of passive antibody immunoprophylaxis in humans with compromised immunity is best exemplified by the success of the use of varicella-zoster virus immune globulin (2) and respiratory syncytial virus immune globulin (13) or specific MAbs prepared for staining by cytospinning 0.1 ml of a 1:10-diluted aliquot onto a slide. The homogenate was balanced salt solution (GIBCO, Grand Island, N.Y.). The homogenate was prepared for staining by cytopinning 0.1 ml of a 1:10-diluted aliquot onto a slide. Organisms were stained with Diff Quik (Baxter, Miami, Fla.), and the numbers of P. carinii nuclei in 50 to 100 fields were counted. The lower limits of detection by this method were approximately 3.76 log10 units when only 100 fields were counted and 4.3 log10 units if only 50 fields were counted.

MAbs. A variety of MAb preparations were used for these studies. For experiment 1, we utilized a gpA-specific MAb pool consisting of immunoglobulin G (IgG) MAbs 90-3-2BS and 94-1-3S6 and IgM MAbs 85-1-5E12 (4, 6) and a pool of MAbs specific to P. carinii antigens other than gpA, referred to in this study as anti-P. carinii, consisting of IgG MAbs 92-3-1F5, 95-1-1G12, and 94-2-2C10 and IgM MAbs 90-3-4F11 and 90-3-1G4. In experiments 2 and 3, mice received MAbs that included 92-3-1F5, 95-1-1G12, and 94-2-2C10 used in experiment 1 plus MAbs 96-1-1F1 and 96-1-1F2 to a 30- to 70-kDa group of antigens that have been characterized. This pool was referred to as the anti-30-70-kDa pool. To examine the importance of antibody isotype, some mice were given an IgG1 and IgM isotype switch variant of MAb 4F11 referred to as 4F11(G1), which was derived from the 4F11 cell line by acridine orange mutagenesis (17). Isotype-matched control-irrelevant MAbs were used in experiments 2 and 3. Western blotting was done to compare the binding patterns of the two IgM MAbs directed against KEX1 and to verify that the IgG1 switch variant retained the binding pattern of

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the parent MAb. For experiments 1 through 3, antibodies were prepared by saturated ammonium sulfate precipitation of ascites fluid, which was then resuspended in water to a volume equivalent to the starting volume of ascites fluid and dialyzed against phosphate-buffered saline (PBS). Antibody in these preparations was not quantitated. For experiment 4, all IgM MAb preparations were quantitated by enzyme-linked immunosorbent assay and adjusted to a concentration of approximately 1.0 mg/ml. IgG preparations were isolated by protein A affinity chromatography (Sigma, St. Louis, Mo.) and adjusted to a protein concentration (determined by absorbance at an optical density of 280 nm) of approximately 1.0 mg/ml.

Immunofluorescence. The ability of MAbs 90-3-4F11, IG4, and 4F11(G1) to bind to murine and human *P. carinii* was demonstrated by immunofluorescence. Infected lung homogenates were diluted in PBS dropped onto a microscope slide, air dried overnight, and then lightly heat fixed prior to use. Slides were incubated with test MAbs diluted in PBS containing 0.05% Tween 20 (Sigma) for 60 to 90 min at 37°C in a moist chamber. After being washed in PBS, the slides were then incubated with goat anti-mouse IgM or IgG conjugated to fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories, West Grove, Pa.) for 30 min at 37°C in a moist, dark chamber to detect bound primary antibody. MAbs 2B5 (IgG), which is specific for murine and human *P. carinii* surface antigens, were used as control antibodies for these assays.

Statistical analysis. Data are expressed as mean numbers of *P. carinii* nuclei for each experimental group of mice ± standard deviations. The statistical significance of the differences in *P. carinii* counts between various groups was determined by the Mann-Whitney U test. Chi-square analysis was used to determine MAb isotype effect, if any, a third group of SCID mice received MAb 1G4 or 4F11 rather than both. In addition, to draw rm conclusions about the relative protective capacities of the two IgM MAbs, directed against KEX1, and the anti-gpA pool, and thus, we chose to focus the remainder of our experiments on antibodies other than those specific for gpA.

The experiment was repeated (Table 1, experiment 2), except that the anti-*P. carinii* pool of MAbs was divided into a pool of two IgM MAbs (4F11 and IG4) that bind to *P. carinii* KEX1 and into a pool of the three MAbs from experiment 1 (1F5, 1G12, and 2C10) plus an additional two MAbs (1F1 and 1F2). All five of the IgG MAbs in the pool were directed to the anti-*P. carinii* pool appeared to provide somewhat greater protection than the anti-gpA pool, and thus, we chose to focus the remainder of our experiments on antibodies other than those specific for gpA.

For our initial experiment, we chose to compare the effects of intranasally administered MAbs specific for gpA with the effects of MAbs specific for other antigens of *P. carinii*, since MAbs specific for gpA have been shown to decrease the severity of infection when administered systematically (4, 6). A second group of mice was given a pool of five MAbs specific for surface antigens of *P. carinii* other than gpA. Both pools of MAbs resulted in reduced organism burdens and fewer mice with microscopically detectable organisms per group (Table 1). While only five mice per group were used, the anti-*P. carinii* pool appeared to provide somewhat greater protection than the anti-gpA pool, and thus, we chose to focus the remainder of our experiments on antibodies other than those specific for gpA.

<table>
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<th>Expt</th>
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* Number of organisms detectable by microscopy.
* *P* ≤ 0.03 compared to value for IgG control.
* *P* = 0.008.
* *P* < 0.001 compared to value for IgM control.
* *P* = 0.01 compared to values obtained with control MAbs.

irrelevant IgG and IgM MAbs. These five groups of mice (Table 1, experiment 3) were inadvertently allowed to go 11 weeks after the commencement of cohousing with the *P. carinii*-infected mice and almost 9 weeks after the last dose of antibody before sacrifice and analysis. Despite this prolonged period of time, from the last dose of MAbs to sacrifice, SCID mice receiving MAb 1G4 still had a 90% reduction in the number of *P. carinii* organisms in their lungs (*P* = 0.01). The mice receiving MAb 4F11 had a more than 50% reduction in organism burden, but the large standardized deviation resulted in this reduction being not statistically significant compared to values for the controls (*P* = 0.13).

Since no attempt was made to standardize the various MAb preparations in terms of antibody content, it was not possible to draw firm conclusions about the relative protective capacities of MAbs 4F11, IG4, and 4F11(G1). Experiment 4 (Table 1) was designed to allow us to more accurately compare the capacities of the two IgM MAbs, directed against KEX1, and to also more carefully examine the potential of the IgG1 switch variant to provide protection after topical application. For this experiment, animals were given approximately 50 μg of each antibody/dose (50 μl at a concentration of approximately 1 μg/μl). In addition, because control animals in experiments 1 and 2 were only lightly infected, experiment 4 was planned for 7 weeks’ duration to ensure a higher organism burden in control mice.

By using a standardized dose of 50 μg of MAb/dose, it was found that all three MAbs to *P. carinii* KEX1 were highly protective. The two IgM MAbs resulted in a 99% (2-log-unit) reduction in numbers of *P. carinii* nuclei compared to those of animals receiving an irrelevant IgM. While the mice receiving
the IgG1 switch variant of MAb 4F11 had about the same number of organisms as those receiving the IgM MAb, they had about a 90% reduction in the number of organisms compared to that of mice receiving an irrelevant IgG MAb (P < 0.03) due to the lower organism burden in this control group. This lower number of *P. carinii* organisms in the IgG-treated mice than in IgM-treated negative control animals was unexpected and unexplained. A possible reason for the difference in the observed efficacies of 4F11(G1) between experiments 3 and 4 is the fact that the cell line making antibody 4F11(G1) is a low producer, making it very likely that mice in experiments 3 and 4 received significantly different amounts of IgG because of the protein A purification step used to prepare antibody for experiment 4.

Figure 1 is a scatter plot showing the *P. carinii* nucleus count for each mouse in experiment 4. This plot shows that only three mice (one per each MAb group) failed prophylactic treatment and that no organisms were detectable in the remainder of the mice in the three experimental groups. Because the sequences of the immunoglobulin heavy chain from the hybridoma cell lines producing MAbs 1G4 and 4F11 are the same (light-chain genes have not yet been sequenced), these two MAbs and the IgG1 derivative are either identical or very closely related antibodies (M. Sullivan and F. Gigliotti, unpublished observations). Thus, mice administered any of these three MAbs likely received the same prophylactic treatment. When the results from the three experimental treatment groups were combined, only 3 of 22 mice receiving MAb 1G4, 4F11, or 4F11(G1) had microscopically detectable numbers of organisms compared to 13 of 13 IgG- or IgM-irrelevant control mice (P < 0.0001).

Immunofluorescence was used to demonstrate that the epitope recognized by MAbs 1G4, 4F11, and 4F11(G1) is shared by mouse and human *P. carinii* organisms (Fig. 2 and 3). In addition, by using isotype-specific secondary antibodies, we were able to verify that 4F11(G1) was an IgG switch variant derived from the cell line secreting MAb 4F11. A Western blot of mouse *P. carinii* organisms was used to demonstrate that all three MAbs have similar patterns of antigen recognition (Fig. 4).

**DISCUSSION**

To determine whether topically administered MAb could protect against a natural challenge with *P. carinii*, we admin-

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**Primary monoclonal antibody**

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<th>4F11(G1)</th>
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**FIG. 2.** Immunofluorescence analysis of the binding of the indicated MAb to mouse *P. carinii*. The top-row samples were developed with an anti-mouse IgG secondary antibody (2° Ab), while those in the bottom row were developed with an anti-mouse IgM secondary antibody. MAb 2B5 is an IgG MAb specific for mouse *P. carinii*, and 5E12 is an IgM MAb which binds to both mouse and human *P. carinii*. The analysis shows that MAbs 4F11 and 1G4 are of the IgM isotype and that 4F11(G1) is of the IgG isotype.
istered MAb intranasally to SCID mice which were cohoused with seed mice with active PCP. As has been well demonstrated in the case of respiratory viral pathogens (18, 19), P. carinii-specific MAb delivered to the respiratory tract offered significant protection against the development of PCP in susceptible mice. MAbs to P. carinii gpA (6) and KEX1 (11) were both protective. Since the comparative efficacies of MAbs to these two molecules was not evaluated but rather based on the results of our pilot study (Table 1, experiment 1), we chose to evaluate antibodies to these other molecules of P. carinii.

The IgM MAbs 4F11 and 1G4 given during the 14-day period of exposure to P. carinii and for 3 subsequent days resulted in a 99% reduction in the number of P. carinii nuclei counted in the lungs of the SCID mice when sacrificed 7 weeks after the commencement of exposure (Table 1, experiment 4). Even when the experiment was allowed to run to 11 weeks postexposure (Table 1, experiment 3), MAb 1G4 prophylaxis still resulted in a 90% reduction in the number of organisms. Prophylaxis with an IgG switch variant of MAb 4F11 resulted in an organism burden similar to that seen with the IgM isotype, but because of the unexplained lower organism count in the irrelevant IgG control group, the percent reduction was approximately 90% rather than 99%. Thus, immunoglobulin isotype did not appear to have a major impact on disease outcome.

Several aspects of our experimental design merit comment. First, the experiments included a rigorous challenge with P. carinii. SCID mice were challenged by continuous cohousing for 14 days when as little as 1 day has been shown to be sufficient to transmit P. carinii to SCID mice (16). Furthermore, there was a prolonged period of time from cessation of antibody prophylaxis to time of sacrifice and analysis; thus, any organisms which escaped initial antibody treatment had several weeks to multiply. This very likely explains the lower degree of protection observed in experiment 3, where the animals were allowed to go 9 weeks after the end of cohousing and MAb treatment before being sacrificed. Second, one of our criteria for selecting the MAbs used was that they bound to the surfaces of P. carinii cells. MAbs 4F11 and 1G4 have been shown to bind to the recombinant P. carinii protein KEX1 (11). Through Western blot analysis (Fig. 4), these MAbs were shown to react with several bands, as is common for most anti-P. carinii MAbs, so it is possible that the surface antigen is something other than KEX1 but which shares epitopes with this molecule. Finally, the results reported were based on microscopic analysis, which is the usual method of analysis in such studies of P. carinii. However, we also performed PCR analysis on our samples with primers specific for P. carinii gpA (data...
not shown), which demonstrated that organisms, or at least their DNAs, were usually present when none were visualized. For example, in experiment 4, using 35 cycles of amplification, DNA was detected in 21 of the 22 MAb-treated mice and in all of the control mice. Reducing the number of cycles to 25 resulted in about half of the MAb-treated animals having no visible ampiclon band, while the ampiclon remained visible in all of the control mice. Thus, PCR analysis corroborated our microscopic analysis but also demonstrated that under the conditions of these experiments, intranasal prophylaxis with \(P. carinii\)-specific MAb did not result in sterilizing immunity.

Intranasally administered MAb offered significant protection to SCID mice exposed to \(P. carinii\); however, the mechanism of protection was not addressed in this study. While we delivered antibody to the nasal mucosa, it is likely that by giving a 50-μL drop, a significant portion of the antibody reached the lower respiratory tract. Interaction between antibody and \(P. carinii\) may provide protection in many ways. Antibody may simply interfere with the attachment of \(P. carinii\) to alveolar epithelial cells or prevent \(P. carinii\) from reaching the alveolus by trapping antibody-coated organisms in the mucus, with subsequent clearance by mucociliary clearance mechanisms. Protection may have also involved activation of complement or opsonization of \(P. carinii\), although IgM is less likely than IgG to interact with phagocyte Fc receptors. Since the nature of the transmissible form of \(P. carinii\) is unknown, it is difficult to define the mode of protection more precisely.

As noted above, the MAbs IG4, 4F11, and 4F11(G1) were directed to \(P. carinii\) KEX1 or antigenically related molecules. While protection may have been mediated through a nonimmunologic mechanism such as steric interference with binding, it is important to note that there did appear to be some specificity in the outcome of these experiments in that the anti-30-70-kDa antigen pool of MAbs did not provide significant protection. Whether this relates to MAb affinity, avidity, or concentration or to the distribution of antigens on the surfaces of \(P. carinii\) cells is an area for further investigation.

Previous studies have demonstrated that systemically administered antibody confers protection against the development of PCP (1, 4, 6, 14). Because of variability in experimental design and counting methods, it is difficult to compare our results using intranasal antibody with those achieved with systemic antibody. Our own results with systemically administered MAb (4, 6) generally resulted in an approximately 50 to 90% reduction in organism burden. Our results with intranasally administered MAbs met, and even exceeded, those we achieved with MAbs delivered systemically, even though we used far less antibody and antibody was administered for a shorter period of time.

The use of intranasal antibody for passive immunoprophylaxis has been most extensively studied for the prevention of viral infections (18, 19). Examples of protection against eukaryotic pathogens include the use of orally delivered antibody to protect against \(C. parvum\) and the ability of topically applied antibody to protect against vaginal \(Candida albicans\) infection in mouse models of these diseases (3, 12). Our findings suggest that, in addition to what occurs with \(P. carinii\), mucosal delivery of antibody may be protective for other fungi which enter the respiratory tract via inhalation, such as \(Cryptococcus\) spp., \(Histoplasma\) spp., \(Blastomyces\) spp., and \(Coccidioides\) spp. The potential value of considering such an approach for humans may be dependent on the ability to identify high-risk groups who will benefit from such therapy and on comparison with other available means of protection against any of these infections.

One of the impediments in extrapolating data from animal models of immunoprophylaxis for PCP to the prevention of disease in humans is the strong host species-specific antigenic variation of \(P. carinii\) (5). By focusing our experiments on MAbs specific for epitopes shared by animal- and human-derived \(P. carinii\) organisms, we have the potential to study targets for immunoprophylaxis that could be directly relevant to similar evaluations of humans. Furthermore, molecular technology allows for the “humanization” of murine MAb for use in humans, as has been done for MAb against respiratory syncytial virus, which is currently approved for use in infants (10). To build upon our initial observations, we are in the process of humanizing MAb Ig4 for study in our mouse models of PCP. We plan to evaluate further the humanization of murine MAB, as well as study the effects of varying times of exposure and intranasal prophylaxis and try to localize the site of protection to the upper or lower airway.

In summary, we demonstrated that topical application of \(P. carinii\)-specific MAb to the upper respiratory tract mucosa is quite effective in protecting against infection with \(P. carinii\). This protection was achieved with relatively modest amounts of antibody. If further developed, this approach offers a potential alternative for prophylaxis of patients against PCP by the use of nasal sprays or nebulization devices.

ACKNOWLEDGMENTS

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REFERENCES


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