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Immunization with *Pneumocystis carinii* gpA Is Immunogenic but Not Protective in a Mouse Model of *P. carinii* Pneumonia

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Immunization with whole *Pneumocystis carinii* has been shown to protect mice from the development of *P. carinii* pneumonia (PCP) when they are subsequently immunosuppressed and challenged with viable organisms. To determine whether these results could be duplicated by using a subunit vaccine, we examined the immunogenicity and efficacy of an immunization strategy based on *P. carinii* gpA. This antigen was chosen for study because passive immunoprophylaxis, based on gpA, has been shown to be partially protective in various animal models of infection. Immunization with gpA produced an anti-gpA specific antibody response comparable to that resulting from immunization with whole organisms. However, in contrast to immunization with whole *P. carinii*, which was protective, immunization with gpA did not protect T-cell-depleted mice from the development of PCP. These studies suggest that other antigens in addition to gpA need to be evaluated for their role in protective immunity against *P. carinii*.

Recent experiments have demonstrated that it is possible to prevent immunosuppressed mice from developing *Pneumocystis carinii* pneumonia (PCP) by immunizing them with a crude preparation of *P. carinii* prior to immunosuppressing them (5, 14). This ability to confer immunity into the period of immunosuppression raises the interesting possibility of trying to develop passive or active immunization for use in the prevention of human PCP. This could be especially useful in oncology patients, for example, who are at risk of developing PCP only for a limited period of time.

There is an established track record of successful immunotherapy in immunocompromised patients. For example, passive immunoglobulin (Ig) therapy has demonstrated efficacy in the treatment of varicella-zoster virus (20), cytomegalovirus (16, 21), and enterovirus (2) infections in immunocompromised patients. Interestingly, there is evidence that cytomegalovirus hyperimmune globulin has a secondary effect in reducing fungal infections (including PCP) in renal transplant patients (16). There are also human studies providing support for active immunization as a means to prevent infections in the immunocompromised host. Vaccination with a live attenuated varicella vaccine is quite effective in either preventing or significantly reducing the severity of varicella in children with leukemia who are on chemotherapy (15). Vaccination against the bacterial pathogen *Haemophilus influenzae* type b induces protective antibody levels both in human immunodeficiency virus-infected men early in the course of their disease (17) and in children with leukemia while receiving chemotherapy (3).

As mentioned above, the mouse model of active immunization against *P. carinii* is based on immunization with a crude unfractionated preparation of whole viable organisms. Two critical factors make the development of such a whole-cell vaccine for use in humans unlikely at present. First, *P. carinii* cannot be propagated in culture; thus, the only source of organisms is the lung of an infected mammalian host. Second, the host species-specific antigenic variation in *P. carinii* is likely to be an impediment in using organisms (antigens) isolated from one mammalian species as a vaccine in a second mammalian species (9). Therefore, the first step in developing a vaccine for the prevention of PCP will be to identify the antigen(s) responsible for eliciting the protective immune response in the mouse model of PCP. Subsequent studies could then be done to determine whether any such antigen(s) or its homolog from human *P. carinii* could be used in the prevention of PCP in humans.

A candidate protective antigen of *P. carinii* is gpA (also referred to as major surface glycoprotein). Studies in mice have shown gpA to be an immunodominant molecule after immunization with (11–13) or exposure to (19) *P. carinii*. Animal models of passive immunoprophylaxis using monoclonal antibodies (8, 10) or sensitized lymphocytes (18) provide suggestive data that immune recognition of gpA may be important in clearance of *P. carinii*. However, direct proof that an immune response directed specifically against gpA is necessary or sufficient to confer complete protection against the development of PCP is lacking. Therefore, to assess the immunogenicity and efficacy of a vaccine based on *P. carinii* gpA, we undertook a series of experiments using gpA in both soluble and particulate forms in an attempt to duplicate the protection observed after immunization with whole *P. carinii*.

MATERIALS AND METHODS

Preparation of gpA. Two forms of gpA were used as immunogens: lectin affinity-purified (4) and nitrocellulose antigen-bearing particles (1, 6). Both methods result in an enriched, but not homogeneous, preparation of gpA. Affinity chromatography was performed with lentil lectin-Sepharose followed by succinyl-concanavalin A-agarose (Sigma, St. Louis, Mo.) as described previously (4). *P. carinii*-infected CB-17 SCID mice, from the Trudeau Institute colony, were used as a source of organisms for these experiments. *P. carinii* infection has been maintained in this colony since 1990. Thus, the same source of organisms was used to prepare gpA and as the inoculum for the challenge studies. Briefly, *P. carinii*-infected lung homogenates were solubilized with 1% sodium dodecyl sulfate (SDS), clarified by centrifugation and microfiltration (0.45-μm-pore-size filter), and then sequentially passed over the lentil and succinyl-concanavalin A columns. After extensive washing (50 to 100 column volumes), the succinyl-concanavalin A column was eluted with 0.5 M NaCl–0.5 M α-D-methylmannopyranoside (Sigma). Fractions containing gpA (determined by SDS-polyacrylamide gel electrophoresis [PAGE] and Western blotting) were pooled, and the total protein content of this final preparation was estimated by measuring its A_{280} and A_{260}, using the formula $1.55 \times A_{280} - 0.77 \times A_{260}$ = mg of protein/ml.

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A particulate form of gpA was produced by making gpA-bearing nitrocellulose particles (1, 6). This was done by separating antigens in solubilized *P. carinii*-infected mouse lungs by SDS-PAGE (10% gel), transferring the separated components onto nitrocellulose, and then probing a strip cut from the left and right sides of the membrane with monoclonal antibody 90-3-2B5, which is specific for mouse *P. carinii* gpA. Once the position of gpA was identified, that portion of the membrane was cut into fine pieces, dried, solubilized in dimethyl sulfoxide (Sigma), and then caused to reaggregate by slowly (dropwise) adding an equal volume of 0.015 M (pH 9.7) carbonate buffer to the dimethyl sulfoxide while continually vortexing the mixture. This causes the nitrocellulose to re-form into fine particles which bear the antigen of interest on their surface. The particles were then washed with phosphate-buffered saline (PBS) and resuspended (10%, vol/vol) in physiologic saline for injection into the mice. Because of the way these particles are produced, it is impossible to quantitate the amount of gpA present on the particles, but it is possible to verify that gpA is present on the particles by probing them with a monoclonal antibody specific for gpA.

***P. carinii* immunization and challenge.** Three types of immunizations were used for these studies. As previously described, immunization with whole *P. carinii* is protective and was used as a positive control for these experiments (5, 14). CB-17 mice, bred at the Trudeau Institute, were immunized with 10^7 *P. carinii* nuclei by the intratracheal (i.t.) route prior to $CD4^+$ T-cell depletion. Other groups of CB-17 mice were immunized with lectin-isolated gpA in doses of either 10 or 40 μg mixed with 10 μg of the adjuvant Quil A. Control CB-17 mice were given an equal amount of bovine serum albumin (BSA) in Quil A. Immunizations were given by the intraperitoneal (i.p.) route 30 days and again 14 days before commencing T-cell depletion. The final experimental group consisted of CB-17 mice immunized with gpA in the form of gpA-bearing nitrocellulose particles. These animals were given a 100-μl i.p. injection of gpA-bearing nitrocellulose particles 30 days and again 14 days before T-cell depletion. Control mice for this experimental group were immunized with an equal volume of saline or BSA-bearing nitrocellulose particles.

After immunization, the mice were depleted of T cells by a twice-weekly injection of 200 μg each of anti-CD4 monoclonal antibody GK1.5 and anti-Thy 1.2 monoclonal antibody 3OH12, both obtained from American Type Culture Collection, Rockville, Md.

Seven days after commencement of T-cell depletion, mice were challenged with 10^7 freshly obtained *P. carinii* by direct i.t. inoculation. The mice were maintained depleted of T cells until sacrificed. Animals immunized with whole *P. carinii* or gpA-nitrocellulose particles and their corresponding controls were sacrificed 10 days after challenge. Experimental groups of animals receiving gpA Quil A, and their corresponding controls, were sacrificed either 14 (two experiments) or 20 (one experiment) days after challenge. After sacrifice, the number of *P. carinii* in the lungs of the mice was determined by making cytospins of lung homogenates and staining them with Diff-Quik (Baxter Scientific Products, Miami, Fla.). Mice were considered to have developed PCP if they had microscopically detectable organisms. The limit of detection by this method is $\log_{10}$ 4.0 *P. carinii* nuclei per lung (14).

Analysis of *P. carinii* gpA-specific antibody response. Pre- and postimmunization sera were assayed for *P. carinii* gpA-specific antibody responses by using two enzyme-linked immunosorbent assay (ELISA) protocols, one measuring total Ig and the second measuring the Ig subclass response (5, 11). Briefly, flat-bottomed microtiter plates (Flow Laboratories, McLean, Va.) were coated with a 1:10 dilution of gpA isolated by lectin affinity chromatography as described above. This resulted in a gpA concentration of approximately 1 to 10 μg/ml. After blocking of the wells with 5% nonfat powdered milk in PBS, sera diluted 1:100 in PBS–0.05% Tween 20 (Sigma) were added to the wells and incubated overnight at 4°C. Total *P. carinii* gpA-specific Ig was detected by using goat anti-mouse IgG, IgM, and IgA conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories Inc., West Grove, Pa.). Controls for this assay included the preimmune sera as well as gpA-specific monoclonal antibodies of known subclasses. After documentation of a response in the total Ig ELISA, specific subclass responses were measured by using rabbit anti-mouse IgG subclass-specific antisera (Bionetics, Charleston, S.C.) followed by goat anti-rabbit Ig conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories).

RESULTS

Over a 3-year period, six separate immunization experiments were performed to determine the immunogenicity and efficacy of immunization with gpA: three using gpA-nitrocellulose and three using gpA in Quil A. One additional experiment was done with gpA in Freund's adjuvant, but because control mice immunized with BSA in Freund's adjuvant also developed an antibody response to gpA which indicated that the mice had likely been exposed to *P. carinii* in the environment, results of that experiment are not included. Further studies using gpA in Freund's adjuvant were not pursued.

For these experiments, groups of 5 to 10 mice were immunized with gpA, either in adjuvant or in the form of nitrocellulose particles, assayed for an antibody response, then $CD4^+$ cell depleted, and challenged with *P. carinii*. To ensure that we were getting an adequate response to isolated gpA, we designed the immunization regimen to produce an antibody response to gpA equivalent to or greater than that resulting from immunization with whole *P. carinii*, as determined by the ELISA for total IgG. These experiments included animals immunized with whole *P. carinii* as a positive control or with BSA or saline as a negative control. The mean optical densities at 405 nm (OD_{405}) in the total Ig gpA ELISA were 1.33 ± 0.48 for mice immunized with whole *P. carinii*, 1.61 ± 0.62 for mice immunized with gpA-nitrocellulose antigen-bearing particles, and >2.0 for animals immunized with gpA in Quil A. Representative sera from the gpA-immunized mice were also shown to react with whole *P. carinii*, as judged by immunofluorescence, obtained from the general infected SCID mouse colony and from the lungs of the immunized animals which were not protected after challenge (data not shown), indicating that the two immunization regimens resulted in gpA-specific antibody which recognized gpA on the surface of *P. carinii*. Control animals remained seronegative (except for two mice in one of the BSA-nitrocellulose groups) after immunization, as documented by the total-Ig ELISA.

TABLE 1. Characterization of the antibody response to *P. carinii* gpA

Form of gpA	Isotype response[a]				
	IgG1	IgG2a	IgG2b	IgG3	IgM
Nitrocellulose	++/+++	−	−	−	−
With Quil A	+++	−	++/+++	−/+	−
Whole *P. carinii*	++/+++	−	++	−	−

[a] Expressed relative to background OD: −, <3×; +, 3× to 5×; ++, 5× to 10×; +++, >10×.

Because experiments were designed to present gpA in various forms, we also performed an Ig subclass-specific ELISA to determine how the use of different antigen preparations and adjuvants affected the humoral response. Representative subclass-specific results from each of the experimental groups are shown in Table 1. For ease of comparison, the ELISA data are indicated in a semiquantitative manner. This was necessitated by the fact that the assays were performed over a 3-year period, thus making it problematic to directly compare results. Immunization with gpA in the form of nitrocellulose antigen-bearing particles produced an antibody response restricted to the IgG1 subclass. When administered in Quil A, gpA resulted in an antibody response profile similar to that resulting from immunization with whole *P. carinii*, with a prominent IgG1 and IgG2b response. These results showed that while all forms of gpA were immunogenic, the form of presentation did influence the subclass distribution of the response.

Eighty percent of the mice immunized with gpA-nitrocellulose developed PCP when immunosuppressed and challenged with *P. carinii* (Table 2). In addition, the organism burden was similar to that for the control animals that received BSA-nitrocellulose or saline. As expected, the majority of those mice immunized with whole *P. carinii* were protected.

Experiments were also performed with lectin-isolated gpA mixed in the adjuvant Quil A. An initial experiment used an immunizing dose of approximately 10 μg of gpA in Quil A. For subsequent experiments, animals were immunized with 40 μg of gpA in Quil A; controls received an equivalent amount of BSA in Quil A. For these experiments, immunized mice were included for analysis (considered to have responded to immu-

TABLE 2. Effects of various immunizations on the development of PCP in a $CD4^+$ cell-depleted mouse model of PCP

Immunogen	No. of mice	Developed PCP	
		No. (%) that did	No. (%) that did not
Whole *P. carinii*	15	3 (20)	12 (80)
Saline	15	14 (93)	1 (7)
gpA-nitrocellulose	15	12 (80)	3 (20)
BSA-nitrocellulose	11	11 (100)	0
gpA-Quil A	22	16 (73)	6 (27)
BSA-Quil A	18	12 (67)	6 (33)

nization) only if their serum, diluted 1:100, gave an ELISA OD of >1.0 (16 of 20 immunized mice responded to immunization by this criterion, with an average OD of >2.0). This was done to ensure high levels of gpA-specific antibody in the animals at the time of immunosuppression and challenge. Serum from BSA-immunized controls resulted in an OD of 0.1 or less by this ELISA. When immunosuppressed and challenged, both high- and low-dose gpA-Quil A-immunized animals developed PCP at the same rate as the BSA-Quil A group (73% versus 67%) (Table 2). Again, there were no differences in the organism burdens between the two groups. Thus, as summarized in Table 2, we were not able to protect mice against PCP by immunizing with isolated gpA in a model where immunization with whole PCP consistently protects. Overall, only 3 of 15 mice immunized with whole *P. carinii* developed PCP, compared to 28 of 37 mice immunized with gpA ($P < 0.001$).

DISCUSSION

Having established that immunization with whole *P. carinii* is protective in the $CD4^+$ cell-depleted mouse model of PCP (5, 14), we wanted to determine whether we could duplicate our results with isolated antigens of *P. carinii*. These experiments were deemed important because whole-cell vaccine against *P. carinii* is currently not feasible given that this organism cannot be propagated in culture. Studies of host species specificity in the immune response to *P. carinii* further complicates the concept of developing a whole-cell vaccine because animal-derived organisms may not be able to elicit protective immunity against the development of PCP in humans (6, 9). Therefore, given the passive immunoprophylaxis data suggesting that gpA might be a target for the development protective immunity (8, 10, 18), we undertook a series of experiments designed to examine whether gpA could be used as a vaccine in our mouse model of PCP.

As expected, when used as an immunogen, gpA elicited a strong specific antibody response on par with that observed when whole organisms are used to immunize the mice. Not surprisingly, the isotype distribution of the antibody response was influenced by the form of gpA used for immunization. While we did not measure the cytokine profiles after immunization, the finding of only an IgG1 response to gpA-nitrocellulose suggests that this form of gpA induced a Th2-like antibody response. The isotypic antibody response to gpA mixed in Quil A was similar to that produced by immunization with whole organisms and appeared to be consistent with a mixed Th1/Th2-like response.

Despite eliciting an antibody response to gpA after immunization with this antigen that was at least equivalent to that observed after immunization with whole organisms, we were unable to demonstrate protection in our animal model. In contrast, and as previously published (5, 9, 14), immunization with whole *P. carinii* was quite effective in protecting animals against the development of PCP. One obvious difference in the immunization described in this report is that gpA was given i.p., while whole *P. carinii* organisms were given i.t. However, we have previously shown that whole *P. carinii* organisms elicit a protective response even when given i.p. (9). Thus, we are left with an as yet unexplained difference in the protective capacity of passive versus active immunization based on gpA (8, 10, 18).

There are several possibilities which could account for our inability to protect $CD4^+$ cell-depleted mice from a challenge with *P. carinii*. One possibility would be that isolated gpA did not elicit the correct type (Th1 versus Th2) of antibody response. However, both Th1- and Th2-type antibody responses to whole *P. carinii* have been shown to be effective in protecting against PCP in the model used for the present study (5). Thus, the isotype of antibody response elicited is an unlikely explanation for our results. It is also possible that active immunization with gpA failed to induce a protective concentration of specific antibody, which could explain the differences between our active and passive immunization experiments. However, we designed the active gpA immunizations so that the concentration of gpA-specific antibody was at least equal to that resulting after immunization with whole *P. carinii*. Therefore, if gpA was the critical target for protective immunity in this model, the gpA-immunized mice should have been protected since they had a gpA-specific antibody concentration on par with that of the whole-*P. carinii*-immunized mice, which were protected. Another possibility is that critical protective epitopes of gpA were denatured in our processing of the molecule. To address this issue, we chose two different methods of preparing the gpA vaccine, and neither was protective. The only way to definitively address the issue of potential denaturation of gpA, if it were the critical protective antigen, would be to continue to pursue other methods of antigen preparation or to produce a mutant *P. carinii* lacking gpA which could be used to define the importance of a response to gpA when a whole-cell vaccine is used to protect these mice. The former approach would be cumbersome given our present results, and the latter is currently impossible. It is possible that a gpA nucleic acid vaccine, with resultant in vivo production of gpA, could be used to circumvent the problem of antigen denaturation if desired. Finally, even though we cannot rule out the possibility that some form of gpA could be protective in our model, we feel that we should begin to consider it more likely that gpA is not the critical target antigen in protection against PCP. gpA has been shown to be an immunodominant antigen after immunization or exposure to *P. carinii* based on analysis of monoclonal antibodies produced from splenic lymphocytes or serum antibody (11–13, 19). However, we have recently made the observation that during recovery from PCP, B cells isolated from tracheobronchial lymph nodes are much more likely to produce antibodies specific to *P. carinii* antigens other than gpA (7). These antigens, recognized by the local pulmonary immune system, deserve further investigation with regard to their potential role in protection against infection with *P. carinii.*

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