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Active Immunization against *Pneumocystis carinii* with a Recombinant *P. carinii* Antigen

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Mice immunized with recombinant mouse *Pneumocystis carinii* antigen A12-thiredoxin fusion protein developed an antibody response that recognized *P. carinii* antigens, as determined by Western blotting and immunofluorescence analysis. Compared to mice immunized with thioredoxin alone, mice immunized with A12-thioredoxin had significantly reduced lung *P. carinii* burdens after CD4$^+$ T-cell depletion and challenge with *P. carinii*.

An infection caused by *Pneumocystis carinii*, *P. carinii* pneumonia (PCP), remains an important cause of morbidity and mortality in immunocompromised hosts. Although there are drug treatments for *P. carinii* pneumonia, poor compliance, adverse side effects, and recurrent infections remain a problem (12). Therefore, other treatments and strategies to prevent PCP merit further investigation.

Immunization of BALB/c mice with intact *P. carinii* cells provides protection against PCP (5, 9, 14). Since *P. carinii* cannot be satisfactorily cultivated, development of a subunit vaccine is essential. However, there are no convincing data showing that a protective immune response has been elicited after active immunization with an isolated antigen of *P. carinii*.

Here we describe active immunization of mice against *P. carinii* with a recombinant *P. carinii* antigen that we designated A12 (13). A12, which exhibits homology to *P. carinii* Kex1 (7, 13), was selected because it is recognized by a monoclonal antibody that has been shown to provide passive prophylaxis against development of PCP (3).

Immunization with recombinant A12 produces an antibody response to native mouse *P. carinii*. To investigate the immunogenicity of a recombinant fusion protein encoded by a portion of *P. carinii* cDNA clone A12, a PCR amplicon encoding the first 142 amino acids of the A12 polypeptide (GenBank accession no. AY371664) was cloned into pBAD:THIO, a thioredoxin six-His fusion vector (Invitrogen, Carlsbad, CA). $A12_{1\text{-}142}$:THIO and a thioredoxin control were expressed in *Escherichia coli* and purified under denaturing conditions by metal ion affinity chromatography. BALB/c mice were immunized subcutaneously three times at 4-week intervals with 25 μg of either $A12_{1\text{-}142}$:THIO or thioredoxin in TiterMax gold adjuvant (Sigma, St. Louis, MO). Two experiments were performed using the same lots of recombinant A12, control antigen, and adjuvant. *P. carinii*-specific serum antibody responses were determined by immunofluorescence analysis and Western blotting of *P. carinii*-infected and normal mouse lung homogenates (2, 3, 5). Serum antibodies obtained after the third immunization (and prior to infection) from $A12_{1\text{-}142}$:THIO-immunized mice bound to the surface of *P. carinii* cysts in an immunofluorescence analysis up to a 1:400 dilution (Fig. 1A), while sera from control mice did not recognize *P. carinii* cysts at dilutions as low as 1:25 (data not shown). Therefore, the antibody to recombinant A12 was capable of recognizing native *P. carinii* antigens.

In Western blots with *P. carinii*-infected mouse lung homogenates and noninfected mouse lungs, anti-$A12_{1\text{-}142}$:THIO immune serum antibodies detected several bands in the range from 50 to 120 kDa in *P. carinii*-infected lung homogenates (Fig. 1C, lane 1). The major bands detected were at M_rs of 65,000 and 120,000. Anti-A12 sera from a pilot study with six mice and from both of the immunized cohorts described in this report gave similar Western blot banding profiles. The $A12_{1\text{-}142}$:THIO postimmune sera did not show enhanced binding to any antigens in lanes containing an equal amount of uninfected mouse lung homogenate (Fig. 1C, lane 2). Preimmune sera from the same mice did not recognize *P. carinii* antigens (Fig. 1B, lane 1). The thioredoxin fusion partner did not induce antibody reactivity to *P. carinii* (Fig. 1D and E, lane 1).

The recognition of multiple bands by the $A12_{1\text{-}142}$:THIO immune sera may have been due to epitopes shared by A12 and other *P. carinii* antigens, proteolytic processing, or degradation. Cross-reactivity to a number of shared epitopes in different *P. carinii* antigens has been demonstrated with anti-mouse *P. carinii* monoclonal antibody 4F11, which is capable of recognizing at least two *P. carinii* antigens, A12 and Kex1 (13). It may be beneficial that immunization with A12 produces a response to a number of *P. carinii* antigens, as this may enhance the repertoire of T-cell responses and/or the opsonic capacity of the immune sera.

Immunization with recombinant *P. carinii* A12 significantly reduces the organism burden in CD4$^+$ T-cell-depleted mice. To determine whether the immune response to recombinant $A12_{1\text{-}142}$:THIO reduced the organism burden, groups of $A12_{1\text{-}142}$:THIO- and thioredoxin-immunized mice were depleted of CD4$^+$ T cells and challenged by cohousing them with *P. carinii*-infected source mice. Six weeks following the third immunization, mice were depleted of CD4$^+$ T cells by

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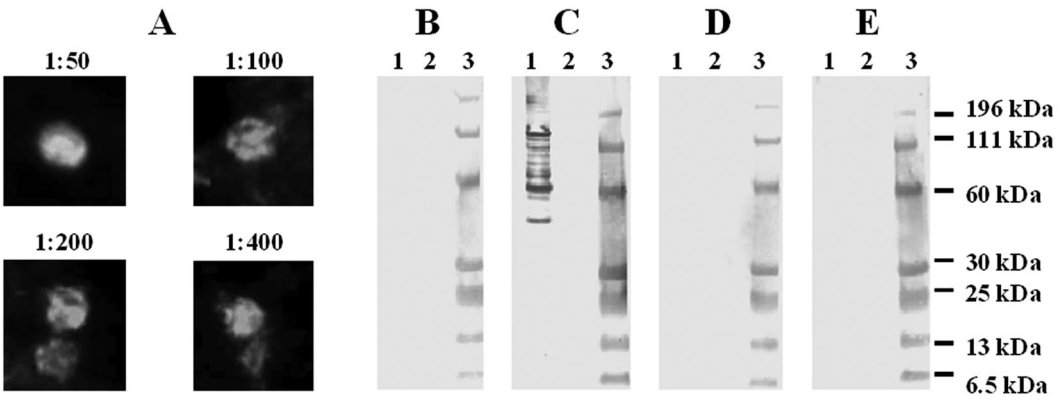

FIG. 1. Immunization with recombinant A12 fusion protein produces a specific antibody response to native *P. carinii* antigens. (A) Immunofluorescence assay showing *P. carinii* cysts in *P. carinii*-infected mouse lung homogenates using different dilutions of pooled anti-*P. carinii* $A12_{1-142}$:THIO antisera and goat anti-mouse immunoglobulin G:BODIPY-Fl secondary antibody. (B to E) Western blots for *P. carinii* isolated from infected mouse lungs and normal lung homogenates probed with $A12_{1-142}$:THIO preimmune sera (B), $A12_{1-142}$:THIO postimmune sera (C), thioredoxin preimmune sera (D), and thioredoxin postimmune sera (E). All sera were diluted 1:250. Lane 1, *P. carinii*-infected mouse lung homogenate; lane 2, normal lung homogenate; lane 3, molecular mass markers.

twice-weekly administration of 250 μg anti-CD4 monoclonal antibody (clone GK 1.5; ATCC, Manassas, VA). To simulate natural exposure to *P. carinii*, the immunized mice were exposed to *P. carinii*-infected SCID mice 1 day following the second administration of monoclonal antibody GK1.5 for 5 days. Two cohorts of seven recombinant *P. carinii* antigen-immunized mice and six control-immunized mice were studied. The mice were sacrificed 6 weeks after termination of cohousing, and the *P. carinii* burden was determined by real-time PCR quantification of the single-copy *kex1* gene as described previously (4). Because the outcomes of the two experiments were identical, the results were combined for statistical analysis. As shown in Table 1, only 4 of the 14 (29%) $A12_{1-142}$:THIO-immunized mice contained detectable *P. carinii*, whereas 8 of the 10 (80%) thioredoxin-immunized mice contained detectable *P. carinii* ($\chi^2 = 6.17$; $P \leq 0.025$). (Two control mice died during the retroorbital bleeding procedure before they were exposed to *P. carinii*.) The organism burden was also analyzed. Using a conservative assumption (9.99×10^3 *P. carinii* copies in the lungs of mice without PCR-detectable *P. carinii* [the limit of detection for this assay was 10^4 *kex1* copies per mouse lung]), there was an approximately 1-log reduction ($P \leq 0.005$) in the average *P. carinii* burden in mice immunized with $A12_{1-142}$:THIO compared to mice immunized with thioredoxin. Since organisms were not detected by PCR in a majority of the $A12_{1-142}$:THIO-immunized mice, we also used a censored regression method of statistical analysis (SAS PROC LIFETEST) with the assumption that the distribution of the undetectable values was log normal between 10^2 and 10^4. This increased the *P* value to 0.0005. A more sensitive qualitative PCR assay targeting the multicopy *P. carinii* gene, *gpA*, was positive for the mice with infections that were not detectable by the quantitative real-time assay. Thus, we did not achieve sterilizing immunity.

TABLE 1. Effect of immunization on *P. carinii* burden

Immunogen	*P. carinii* burden[a]	No. of animals immunized	No. of animals with detectable *P. carinii*
A12:THIO	$4.83 \times 10^4 \pm 9.81 \times 10^4$[b]	14	4[c]
THIO	$3.52 \times 10^5 \pm 3.42 \times 10^5$	10	8

[a] The data are means ± standard deviations and are the *P. carinii kex1* copy numbers obtained in triplicate analyses of boiled lung homogenates by real-time PCR. Mice with no detectable *P. carinii* as determined by real-time PCR were assigned a value of 9.99×10^3 copies, which was just below the limit of detection (1×10^4 copies).

[b] The value is significantly different from the value for THIO-immunized mice as determined by Student's *t* test ($P \leq 0.005$).

[c] The number of mice with detectable *P. carinii* is significantly different from the number of THIO-immunized mice with detectable *P. carinii* ($\chi^2 = 6.17$; $P \leq 0.025$).

In the second experiment, mice were tagged and sacrificed so we could compare the serum antibody response after the final boost, which was determined by an enzyme-linked immunosorbent assay (ELISA) as previously described (5), and the *P. carinii* count for each mouse. Sera were diluted 1:50. The background optical density in the ELISA in which uninfected mouse lungs were used was subtracted from the optical density obtained using *P. carinii*-infected lungs. In the $A12_{1-142}$:THIO-immunized group, the resulting optical density values were 0.02 and 0.03 for the two mice with detectable *P. carinii* and 0.07, 0.14, 0.24, 0.59, and 0.62 for the five mice with undetectable *P. carinii*. Thus, higher ELISA values appeared to be associated with lower organism burdens. There was no detectable optical density above the background level for any of the four control thioredoxin-immunized mice. A limitation of this ELISA format for the purposes of this experiment was that we were unable to determine the amount of "A12" that was bound to the plate.

The degree of protection observed in our study was not as pronounced as the degree of protection achieved by immunization with *P. carinii* cells (5). It may be possible to achieve greater protection by varying the dose and adjuvant, by immunizing with a larger portion of the A12 molecule, or by using a combination of A12 and additional *P. carinii* antigens. Previous attempts to elicit protective immunity against PCP with recombinant or purified native *P. carinii* antigens were unsuccessful unless the immunized mice were also cohoused with *P. carinii*-infected mice, which confounds the analysis of the vaccine effect (2, 6, 10, 11).

Zheng et al. have recently published results similar to those described here, which were obtained by using DNA immunization with the gene for *P. carinii* Kex1 (15). Because kexins are generally intracellular molecules (1), the success of vaccination with Kex1 is somewhat surprising and interesting. Alignment of the primary amino acid sequence of the immunogen $A12_{1\text{-}142}$ with the primary amino acid sequence of mouse *P. carinii* kexin revealed 54% identity and 67% similarity in a region where 137 amino acids overlap. A comparison of the cross-reactive antibody responses to the two antigens using either the A12 polypeptide or kexin protein as the immunogen may help to more precisely define critical protective epitopes that can be exploited for vaccine development. Given our results, further defining the antigenic relationship between A12, Kex1, and possibly *P. carinii* protease 1 (7, 8) discussed above is necessary.

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