INFECTION AND IMMUNITY, June 1999, p. 2935–2940 Vol. 67, No. 6
0019-9567/99/$04.00+0

Resistance to *Coccidioides immitis* in Mice after Immunization with Recombinant Protein or a DNA Vaccine of a Proline-Rich Antigen

RAED O. ABUODEH,[1] LISA F. SHUBITZ,[1] ERIN SIEGEL,[1] SHANNON SNYDER,[1] TAO PENG,[1] KRIS I. ORSBORN,[1] ELMER BRUMMER,[2] DAVID A. STEVENS,[2] AND JOHN N. GALGIANI[1]*

Valley Fever Center for Excellence, Veterans Affairs Medical Center, and University of Arizona, Tucson, Arizona,[1] *and Santa Clara Valley Medical Center, Stanford University School of Medicine, Stanford, California*[2]

Received 28 January 1999/Returned for modification 20 February 1999/Accepted 15 March 1999

Two inbred strains of mice (BALB/c and C57BL/6) were vaccinated with either recombinant expression protein of a *Coccidioides immitis* spherule-derived proline-rich antigen (rPRA) in monophosphoryl lipid A-oil emulsion adjuvant or a DNA vaccine based on the same antigen. Four weeks after vaccination, mice were infected intraperitoneally with arthroconidia. By 2 weeks, groups of mice receiving saline or plasmids with no PRA insert exhibited significant weight loss, and quantitative CFUs in the lungs ranged from 5.9 to 6.4 $\log_{10}$. In contrast, groups of mice immunized with either rPRA or DNA vaccine had significantly smaller pulmonary fungal burdens, ranging from 3.0 to 4.5 $\log_{10}$ fewer CFUs. In vitro immunologic markers of lymphocyte proliferation and gamma interferon (IFN-γ) release after splenocytes were stimulated with rPRA correlated with protection. Also, plasma concentrations of rPRA-specific total immunoglobulin G (IgG), IgG1, and IgG2a showed increases in vaccinated mice. These studies expand earlier work by demonstrating protection in mice which differ in *H-2* background, by using an adjuvant that is potentially applicable to human use, and by achieving comparable protections with a DNA-based vaccine. Our in vitro results substantiate a Th1 response as evidenced by IFN-γ release and increased IgG2a. However, IgG1 was also stimulated, suggesting some Th2 response as well. PRA is a promising vaccine candidate for prevention of coccidioidomycosis and warrants further investigation.

Coccidioides immitis causes a systemic fungal infection in approximately 100,000 persons each year (14). Whereas a small percentage of infections result in progressive and debilitating illness, most stimulate an immune response in the host which effectively controls the disease process and affords long-lasting protection against future infection (46). The remarkable effectiveness of the immune response has sustained a prolonged search for a means to produce protection equivalent to that which follows naturally acquired infection (40).

Four decades ago, formalin-killed whole-cell vaccines made from spherules of *C. immitis* were demonstrated to protect mice from lethal intranasal infection (31). However, induction of protection required repeated administrations of several hundred milligrams of vaccine per mouse (32). In a field trial using the maximally tolerated dose of the whole-cell vaccine, humans who received vaccine acquired little or no protection against subsequent coccidioidal infection compared to controls (41). Because of these findings, recent efforts to improve vaccine efficacy have focused attention on subcellular fractions and recombinant antigens as vaccine candidates (26, 27, 56).

One vaccine candidate is a proline-rich antigen (PRA) identified in spherules of *C. immitis* (12, 16). Previous studies demonstrated that as a purified deglycosylated protein, it can stimulate peripheral blood lymphocytes from humans with prior coccidioidal infections. We cloned and reported the sequence for the cDNA encoding this protein, which we referred to as the PRA (13). Subsequently, the deduced amino acid sequence of another antigen, antigen 2, was shown to be identical to that of PRA (55). We have also shown that recombinant PRA (rPRA) with Freund's adjuvant afforded BALB/c mice significant protection from intraperitoneal (i.p.) challenge (26). We have now extended the original findings by decreasing the number of immunizations, increasing the interval between immunization and challenge, and substituting a monophosphoryl lipid A (MPL)-oil emulsion adjuvant that is potentially applicable to human use (42, 52). Additionally, we have expanded these observations by studying a second inbred strain of mouse with a different *H-2* background and by testing a DNA vaccine prepared with PRA cDNA. The results from this work and their correlations with several in vitro immunologic measurements are the subject of the current report.

(This work was presented in part at the Annual Meeting of the Infectious Diseases Society of America, San Francisco, Calif., 14 September 1997.)

MATERIALS AND METHODS

Animals. Six-week-old female BALB/c and C57BL/6 mice were obtained from Harlan Sprague-Dawley (Indianapolis, Ind.). All animals were housed in pans with microisolator lids at a density of three to four per pan. Mice were immunized within 1 week of arrival.

Fungal culture. Strain Silveira of *C. immitis* was used to prepare arthroconidial suspensions as previously described (15, 16). The concentration of CFUs was determined by enumerating colonies that grew in culture on agar, and diluted suspensions were transferred to sterile serum vials for subsequent use. All handling of viable arthroconidia and transfer of cell suspensions between institutions followed National Institutes of Health-Centers for Disease Control and Protection guidelines for biosafety level 3 containment (7).

Antigen preparations. The cloning, expression, and purification of rPRA have been previously described (38). Antigen protein content was determined by bicinchoninic acid assay (Pierce, Rockford, Ill.).

DNA vaccines. The full-length transcript encoding PRA was used to construct the DNA vaccine. A mammalian expression vector, VR1020 (Vical, Inc., San Diego, Calif.), was used in these studies (19, 34). This vector has a cytomegalovirus promoter-enhancer, followed by a signal sequence to direct the protein through the secretory pathway. Also, VR1020 contains the poly(A)-terminator

* Corresponding author. Mailing address: Valley Fever Center for Excellence (1-111), 3601 South Sixth Ave., Tucson, AZ 85723. Phone: (520) 792-1450, ext. 6793. Fax: (520) 629-4738. E-mail: spherule@u.arizona.edu.

sequence of bovine growth hormone and the kanamycin gene for selection in bacteria. An amplimer of 597 bp, containing the entire translated sequence for PRA, was created by PCR from pCiAg33.41 (13) and cloned into the *Bam*HI/*Bgl*II site downstream of the signal sequence of VR1020 to create pCVP20.17 (44). Orientation and reading frame were verified by DNA sequencing. Plasmid DNA was prepared for injection by using the Qiagen (Chatsworth, Calif.) Gigaprep kit, adjusted to a concentration of 1 mg/ml with sterile saline, and stored at −20°C.

Immunization procedures. For immunization with rPRA, mice were vaccinated subcutaneously with 5 μg of rPRA emulsified in 100 μg of MPL per ml of 1% oil as adjuvant (Ribi Immunochem Research, Inc., Hamilton, Mont.), 5 μg of rPRA without adjuvant, or sterile saline in a volume of 200 μl by injection into the inguinal area. Four weeks later, vaccination was repeated on the opposite side.

For DNA vaccination, a plasmid encoding PRA (pCVP20.17; 100 μg/mouse), a plasmid with no PRA cDNA insert (VR1020; 100 μg/mouse), or sterile saline was injected into the cranial tibial muscle. Both hind legs were swabbed with a solution of Betadine, and 50 μl was delivered into the muscle of each leg with a 30-gauge needle. A single booster injection was given 4 weeks later.

Animal inoculation and quantitative assessment of fungal burden. Infections were carried out in a class III safety cabinet (The Baker Co., Sanford, Maine). At the time of infection, mice were weighed and inoculated by injecting 0.1 ml of arthroconidia i.p. BALB/c mice (seven per group) received 50 spores, and C57BL/6 (eight per group) received 500 spores. Preliminary studies demonstrated that these numbers of arthroconidia resulted in equivalently large fungal burdens. At 14 days after infection, mice were weighed and sacrificed, after which the spleens and right lungs were removed for quantitative culture. Tissues were homogenized, and saline dilutions were plated on 2× glucose yeast extract medium by the method of Kirkland et al. (27). Colonies were enumerated 3 days after plating.

In vitro immunologic studies. Four weeks after the last vaccination, mice (four per group) were anesthetized with a ketamine-xylazine mixture given i.p. and exsanguinated from the retro-orbital plexus; this was immediately followed by cervical separation. Spleens were harvested aseptically and pooled in pairs. Pairs of spleens were processed by gentle disruption with a sterile syringe plunger and suspension of splenocytes in RPMI base medium (Gibco BRL, Grand Island, N.Y.). Cells were centrifuged for 5 min at 200 × *g* in a Sorvall T-6000 centrifuge. Erythrocytes were lysed with an ammonium chloride-based hypotonic lysis buffer for about 60 s and the cell suspension was washed twice with RPMI base medium at 200 × *g* and then resuspended in 5 ml of RPMI complete medium (CM; including 10% heat-inactivated fetal bovine serum, L-glutamine, 0.01% β-mercaptoethanol, penicillin-streptomycin, and amphotericin B deoxycholate). Cells were counted on a hemocytometer using trypan blue viable stain (viability was usually higher than 90%).

Lymphocyte stimulation was performed by transferring cells to 96-well tissue culture plates (5×10^5 cells/well in CM). Stimulating agents, in CM, were rPRA (10 μg/ml), bovine serum albumin (BSA; 25 μg/ml), concanavalin A (5 μg/ml), and a medium-only control. Concanavalin A and BSA were used as controls (data not shown). For each pair of spleens, six replicates were plated for each antigen. Plates were incubated for 4 days at 37°C and 6% CO_2. Approximately 9 h before completion, 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, Mass.) per well was added. Cells were then harvested by using a 96-well FilterMate harvester (Packard Instruments Co., Meriden, Conn.) and read with 96-well TopCount reader (Packard). Results were expressed as a stimulation index (SI) (mean counts per minute from wells of test antigen/mean counts per minute of medium-only control wells).

For assays of in vitro cytokine stimulation, splenocytes (10^7 cells/well in CM) were plated into 24-well tissue culture plates and test antigens in CM were added to a final volume of 1 ml. Cells were incubated as described above for 3 days, after which the supernatants were harvested into sterile cryotubes and stored at −70°C until analyzed. Quantitation of gamma interferon (IFN-γ) and interleukin-4 (IL-4) release was performed on culture supernatants using capture enzyme-linked immunosorbent assay (ELISA)-based kits (Endogen, Inc., Woburn, Mass.) and following the manufacturer's instructions. The samples were diluted in assay buffer at 1:10 for IFN-γ and 1:5 for IL-4. Ranges for standard curves were 17 to 5,000 pg/ml for IFN-γ and 21 to 900 pg/ml for IL-4. Each sample, in triplicate, was added to capture antibody-coated wells at 100 μl/well, and six blank wells were also included in each assay. Results are reported as averages of replicates.

Immunoglobulin concentrations were determined in plasma collected at the time of sacrifice. Plasma samples were analyzed for anti-PRA total immunoglobulin G (IgG), IgG1, and IgG2a by using an indirect ELISA technique as previously described with minor modifications (38). Linbro EIA II Plus 96-microwell plates (Flow Laboratories, Inc., McLean, Va.) were coated with 100 ng of rPRA per well and incubated at 4°C overnight. Fourfold serial dilutions (1:10 to 1:10,240) of the heat-inactivated plasma samples from the mice were tested in triplicate. Plates were incubated with anti-isotype-specific conjugated antibodies (IgG, IgG2a, and IgG1; Zymed, Inc., South San Francisco, Calif.), and reactions were detected with *o*-phenylenediamine substrate. The reaction was stopped by using 1.0 N H_2SO_4, and the A_{490}s of plates were read within 15 min. Titers were expressed as the lowest dilution which was twice the background.

Statistical analysis. The significance of differences between groups was assessed with the Wilcoxon signed rank test. Differences of $P = 0.05$ or less were taken as significant.

RESULTS

Result of i.p. infection. By 10 days after infection, mice receiving placebo vaccinations exhibited lethargy, anorexia, and emaciation. In the studies reported here, saline- or vector-immunized BALB/c and C57BL/6 mice lost 13 to 26% of their baseline body weight between infection and sacrifice ($P < 0.05$ for each group). Of the 90 mice infected in this study, 2 died between 12 and 14 days after infection. One BALB/c mouse which received pCVP20.17 appeared to have died of coccidioidal infection and was assigned a result of 5×10^6 CFU per organ. The second was a C57BL/6 mouse that had received saline and suffered from malocclusion. Since this death may not have been related to infection, it was excluded from analysis.

Protection of mice. BALB/c and C57BL/6 strains of mice were immunized with rPRA (with or without MPL-oil emulsion adjuvant), with plasmid DNA (with or without a PRA insert), or with saline, and 4 weeks after the booster injection, they were challenged. The fungal burdens of the groups are shown in Fig. 1.

For protein immunizations, the lungs and spleens from both BALB/c and C57BL/6 mice immunized with rPRA in adjuvant had significantly fewer CFU than those of mice who received saline vaccinations. The median ($\log_{10}$) for lungs of BALB/c mice was 2 compared to 5.9 ($P = 0.043$), and for those of C57BL/6 mice it was 1.7 compared to 6.1 ($P = 0.028$). For spleens of BALB/c mice receiving rPRA with adjuvant vaccine, the CFU count was 4 compared to 6.5 for those of saline-vaccinated mice ($P = 0.043$), and for those of C57BL/6 mice it was 2.7 compared to 6.2 ($P = 0.028$). In addition, C57BL/6 mice vaccinated with rPRA with no adjuvant produced colony counts in the lungs and spleens of 4.2 and 4.7, respectively, which were also significantly lower than those of saline-vaccinated controls ($P = 0.046$ and 0.028, respectively).

When the DNA vaccine was used, there emerged a pattern very similar to that obtained with the recombinant protein. The median CFU ($\log_{10}$) count for lungs of BALB/c mice vaccinated with pCVP20.17 was 3.6 compared to 6.4 for animals receiving saline ($P = 0.028$). Compared to animals vaccinated with the vector alone, there was a difference of 2.6 $\log_{10}$, although this did not reach statistical significance ($P = 0.161$). For C57BL/6 mice, the median lung CFU count was 1.7 for pCVP20.17, compared to 6.0 ($P = 0.028$) for mice given the vector alone and 6.2 ($P = 0.017$) for saline-vaccinated mice. Comparisons of spleen CFU counts showed differences that went in the same directions as those obtained with lungs but did not achieve statistical significance.

In no vaccine group where protection was observed was there a significant decrease in mouse weights. In C57BL/6 mice immunized with rPRA and adjuvant, weight actually increased an average of 12.3%.

Lymphocyte stimulation studies. The capacity of splenocytes to mount an immune response after vaccination with rPRA or a PRA-containing plasmid was tested in vitro by stimulating splenocytes 4 weeks after immunization with medium, BSA, or rPRA (Fig. 2). Splenocytes from BALB/c mice receiving rPRA in MPL-oil emulsion adjuvant gave a significantly higher response when stimulated in vitro with rPRA ($P = 0.028$); geometric mean SIs were 0.92, 1.81, and 4.69 in mouse groups receiving saline, rPRA, and rPRA with adjuvant, respectively. Protein vaccines evoked a similar pattern with

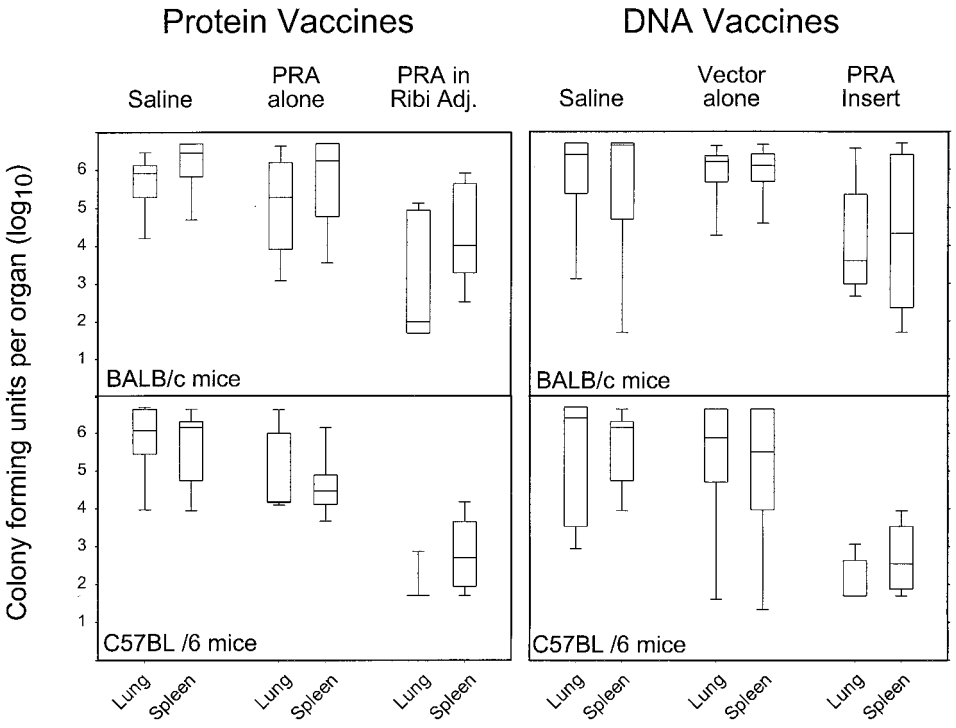

FIG. 1. Protective efficacy of vaccine in mice immunized twice (4 weeks apart) with saline (control) or an rPRA or DNA vaccine and challenged 4 weeks after a boost with viable arthroconidia. Two weeks later, mice were sacrificed and tissues (lung and spleen) were removed and processed as described in Materials and Methods. The box plot data represent CFU (log_{10}) counts per organ. Each box represents percentiles 25 to 75, and the line inside indicates the 50th percentile. Caps represent the 10th and 90th percentiles. Ribi Adj. denotes the MPL-oil emulsion.

splenocytes from C57BL/6 mice. With splenocytes from DNA-vaccinated animals, SIs of pCVP20.17-vaccinated mice were higher than those of mice immunized with the vector alone. With C57BL/6 mice, the difference achieved statistical significance ($P = 0.04$).

In vitro cytokine stimulation studies. Splenocytes were also tested 4 weeks after vaccination with respect to their capacity to produce IFN-γ. Similar findings were obtained with both mouse strains, and those obtained with C57BL/6 mice are shown in Fig. 3. Medium or BSA produced little or no IFN-γ

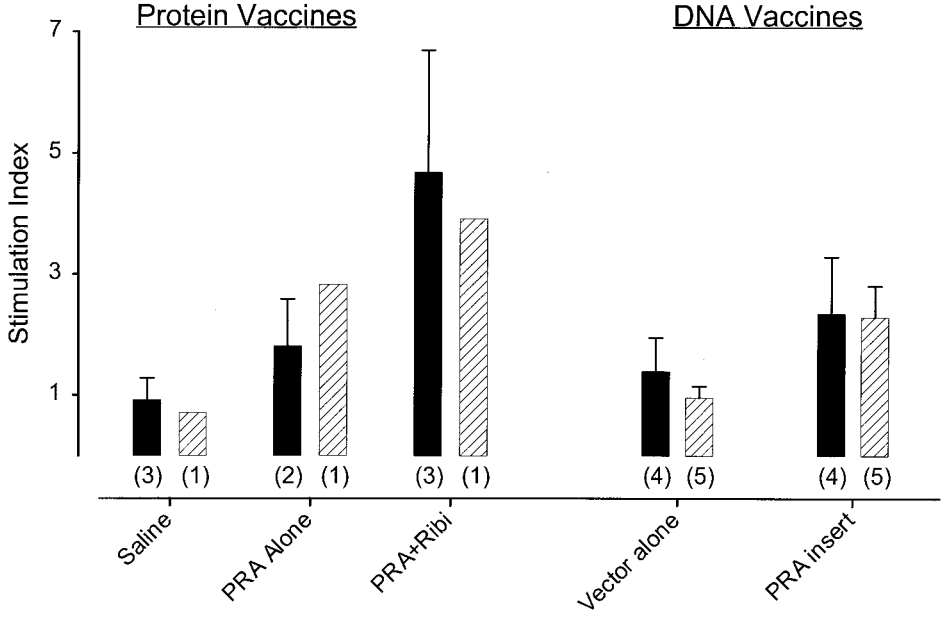

FIG. 2. Proliferative responses of splenocytes from immunized mice to antigens. Splenocytes from BALB/c (filled bars) or C57BL/6 (hatched bars) mice immunized with saline, rPRA (with or without adjuvant), or DNA were tested in vitro by using rPRA (10 μg/ml in CM). Each bar represents the mean (± the standard error of the mean) SI. The numbers in parentheses are the numbers of experiments done.

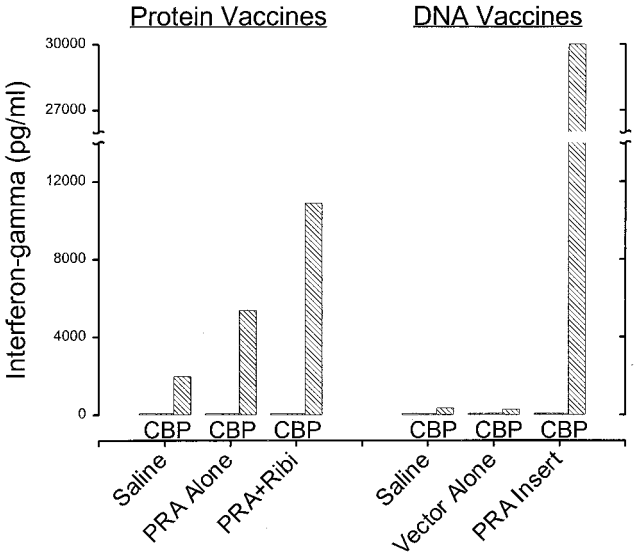

FIG. 3. Production of IFN-γ in stimulated splenocytes from immune mice. Spleen cells from C57BL/6 mice immunized twice (4 weeks apart) with saline, rPRA (with or without adjuvant), or DNA were tested in vitro 4 weeks after boosting. Below the bars are the stimuli used (medium control [C], BSA [B], and rPRA [P]). Cells were incubated for 3 days before they were harvested, and IFN-γ was quantitated by using an ELISA kit.

response from splenocytes of any animals (less than 128 pg/ml). In studies of the protein vaccine, larger amounts of IFN-γ were produced by splenocytes from mice immunized with rPRA in adjuvant than by those from mice that received either saline or rPRA with no adjuvant. Similar differences were obtained from splenocytes of mice immunized with pCVP20.17 compared to the vector alone.

Measurements were made on IL-4 released into culture supernatant by splenocytes after stimulation in a fashion identical to that used for IFN-γ. However, in all cases, stimulation was below the threshold of detection of our assay (21 pg/ml).

Circulating-immunoglobulin measurements. Plasma from immunized mice was tested for rPRA-specific total IgG, IgG1, and IgG2a. The results of these experiments with BALB/c mice are shown in Fig. 4. Immunization either with rPRA in adjuvant or with pCVP20.17 produced measurable immunoglobulin levels, but immunization with either saline or the vector alone did not. Comparable results were obtained with C57BL/6 mice.

DISCUSSION

The studies reported here extend the work of Kirkland et al., which first demonstrated that rPRA could protect mice from infection with *C. immitis* (26). In that study, BALB/c mice were immunized with three injections of rPRA over 4 weeks by using complete and incomplete Freund's adjuvant and infected i.p. 2 weeks later. Fourteen days after infection, the median lung CFU count of rPRA-vaccinated mice was 2.7 $\log_{10}$ lower than that of unvaccinated mice. The current protection study using BALB/c mice differs from the original in that two rather than three vaccinations were employed, challenge was 4 rather than 2 weeks after the last immunization, the infecting inoculum was prepared from the Silveira strain rather than the RS strain of *C. immitis*, and MPL-oil emulsion adjuvant was substituted for Freund's adjuvant. Although Freund's adjuvants have been very useful experimentally, they are not approved for human use, whereas the MPL adjuvant formulation used in our study has been reported as safe in clinical trials of vaccines under consideration for licensing (30, 47, 48). With these differences, the lung CFU count of rPRA-immunized BALB/c mice in the current work was 3.9 $\log_{10}$ lower than that of mice receiving saline injections, which agrees well with the first study. It is also better than the protection reported by another group, who carried out immunization by a very different protocol and used the identical recombinant antigen and its fusion partner (referred to as antigen 2-GST) (23). In addition, measurements of animals demonstrated significant weight loss only in groups of mice in whom protection was not observed. Prevention of weight loss has been noted previously to be associated with vaccine protection against experimental murine influenza virus infection (9, 50, 51).

Having corroborated the initial evidence for protection in the inbred BALB/c mouse strain, the current report expands this observation to a second inbred mouse strain, C57BL/6. Since C57BL/6 mice have a different *H-2* background, protection of this strain suggests that immunity produced by PRA is not restricted to a unique repertoire of antigen recognition. C57BL/6 mice have previously been shown to be more resistant to i.p. infection with arthrospores of *C. immitis* (25). That 10-fold more arthrospores produced an equivalent fungal burden in our results is consistent with the previously published experience. This difference in intrinsic susceptibility to infection between the two inbred mouse strains may also be responsible for the significant protection afforded by rPRA with no adjuvant in C57BL/6 mice which was not observed with BALB/c mice. This is concordant with the impression that C57BL/6 mice mount a stronger Th1 response than does the BALB/c strain (20). Adjuvants may engender nonspecific resistance to infectious agents unrelated to specific antigen sensitization. When such effects occur, they usually are minimal by 4 weeks after vaccination (11, 29, 33). For this reason, we delayed infection in the current studies for a longer interval than was used previously (26). That significant protection could be demonstrated with no adjuvant in C57BL/6 mice is further evidence that rPRA confers antigen-specific immunity.

We have also demonstrated in this report that using the

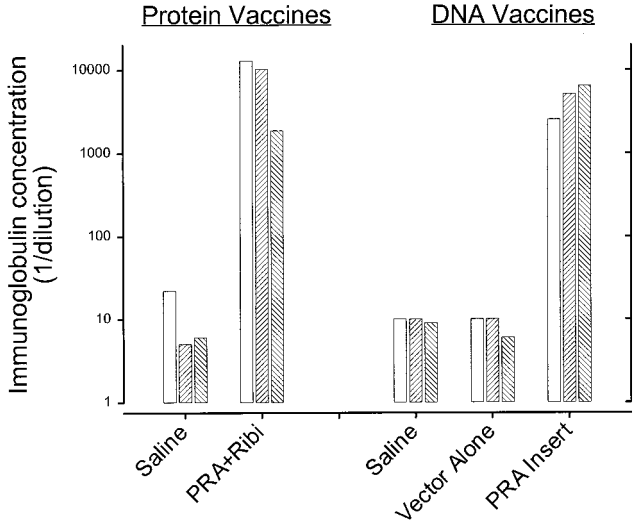

FIG. 4. Total-IgG and IgG subclass responses of immunized BALB/c mice. Mice were immunized as described in Materials and Methods and terminally bled, and plasma samples were tested for PRA-specific total IgG (open bars), IgG1 (rising diagonal bars), and IgG2a (falling diagonal bars) by ELISA. Results represent reciprocal titers (1/dilution).

PRA cDNA as a DNA vaccine can also produce protection comparable to that achieved by using the recombinant protein. Naked-DNA immunization against influenza virus was first reported in 1993 (50) and has since been reported for other viruses (8, 28), bacteria (21, 39), mycoplasmas (3), protozoa (53), and trematodes (54). Compared to protein vaccines, DNA vaccines are easier to prepare and purify, potentially reducing the costs of production. Another potential advantage of DNA immunization is major histocompatibility complex presentation similar to the way antigens are presented during infection without the risk of infection (49). It has been suggested that this difference in presentation might circumvent haplotype restriction (45). Our experience serves to encourage further investigations of coccidioidal DNA vaccines.

In relation to the results of the protection study, we examined several in vitro markers of an immune response. Although our current study was not intended to determine which surrogate immunologic markers would predict protection, future development of predictive markers would be valuable for any vaccine candidate which approaches consideration for human clinical trials. Toward that ultimate goal, identifying markers that correlate with protection is a necessary first step. In our studies, a correlation was found between lymphocyte stimulation and in vitro-stimulated IFN-γ and total IgG, IgG1, and IgG2a.

SIs of PRA-immunized mice ranged from two- to fivefold greater than those of mice receiving saline. These results need to be cautiously examined because splenocytes represent a mixed population of cells. Greater differences were observed with in vitro release of IFN-γ; levels in supernatant collected from rPRA-stimulated splenocytes showed increases of 3 to 4 $\log_{10}$. For example, stimulated cells from unvaccinated control BALB/c mice were near the background for the assay, while the levels averaged over 10,000 pg/ml for mice vaccinated with rPRA in MPL-oil emulsion adjuvant and with pCVP20.17 plasmid DNA. These findings are consistent with a correlation of IFN-γ production and protection in several other experimental infections, including tuberculosis (37), listeriosis (22), leishmaniasis (18, 35), toxoplasmosis (17), blastomycosis (5), paracoccidioidomycosis (6), candidiasis (24), histoplasmosis (1), cryptococcosis (43), and coccidioidomycosis (4). Our findings encourage us to continue to include assays of IFN-γ release in relation to protection in future studies of coccidioidal vaccines.

Specific anti-rPRA total IgG, IgG1, and IgG2a were also stimulated by immunization with PRA. These findings are consistent with stimulation of both Th1 and Th2 immune responses (21). Whether both Th1 and Th2 are stimulated by the same or different regions of PRA remains to be elucidated. As shown in Fig. 4, with DNA vaccination, the concentration of IgG2a was relatively greater than that resulting from rPRA immunization, whereas IgG1 concentrations were nearly equal. It is possible that this pattern reflects an increased Th1 response with the DNA vaccine, as has been found with other antigens (10). IL-4 levels were also tested in these mice, and responses were below the detection limit of our assay. This is also consistent with the paradigm that both of the vaccines tested here push the immune response toward Th1 (36). Alternatively, it is possible that our assay for IL-4 was simply insensitive to small but significant levels of response in splenocytes from vaccinated animals. In support of this possibility, substantial differences in cytokine levels were reported recently when ELISA kits from different vendors were used (2). Further work is needed to clarify this question.

Developing a vaccine candidate to prevent human or veterinary disease resulting from coccidioidal infection has been the long-term goal of several currently active research groups. The studies reported here contribute additional support to the potential application of PRA as a component of an eventual vaccine candidate.

ACKNOWLEDGMENTS

This work was supported in part by the U.S. Department of Veterans Affairs and the California Health Care Foundation.

We thank J. T. Ulrich of Ribi Immunochem Research, Inc., for making PML-oil emulsion adjuvant available for these studies and R. H. Zaugg of Vical, Inc., for supplying the VR1020 plasmid used to prepare the DNA vaccine. We also express our appreciation to Jeffrey Ulmer for his many helpful suggestions during the course of our studies.

REFERENCES

1. **Allendoerfer, R., and G. S. Deepe, Jr.** 1997. Intrapulmonary response to *Histoplasma capsulatum* in gamma interferon knockout mice. Infect. Immun. **65:**2564–2569.
2. **Aziz, A., P. Nishanian, R. Mitsuyasu, R. Detels, and J. L. Fahey.** 1999. Variables that affect assays for plasma cytokines and soluble activation markers. Clin. Diagn. Lab. Immunol. **6:**89–95.
3. **Barry, M. A., W. C. Lai, and S. A. Johnston.** 1995. Protection against mycoplasma infection using expression-library immunization. Nature **377:** 632–635.
4. **Beaman, L.** 1991. Effects of recombinant gamma interferon and tumor necrosis factor on in vitro interactions of human mononuclear phagocytes with *Coccidioides immitis*. Infect. Immun. **59:**4227–4229.
5. **Brummer, E., L. H. Hanson, and D. A. Stevens.** 1993. IL-4, IgE, and interferon-gamma production in pulmonary blastomycosis: comparison in mice untreated, immunized, or treated with an antifungal (SCH 39304). Cell. Immunol. **149:**258–267.
6. **Cano, L. E., S. S. Kashino, C. Arruda, D. André, C. F. Xidieh, L. M. Singer-Vermes, C. A. C. Vaz, E. Burger, and V. L. G. Calich.** 1998. Protective role of gamma interferon in experimental pulmonary paracoccidioidomycosis. Infect. Immun. **66:**800–806.
7. **Centers for Disease Control-National Institutes of Health.** 1993. Biosafety in microbiological and biomedical laboratories, p. 1–177. U.S. Government Printing Office, Washington, D.C.
8. **Cox, G. J., T. J. Zamb, and L. A. Babuik.** 1993. Bovine herpesvirus 1: immune response in mice and cattle injected with plasmid DNA. J. Virol. **67:**5664–5667.
9. **Deck, R. R., C. M. DeWitt, J. J. Donnelly, M. A. Liu, and J. B. Ulmer.** 1997. Characterization of humoral immune responses induced by an influenza hemagglutinin DNA vaccine. Vaccine **15:**71–78.
10. **Donnelly, J. J., J. B. Ulmer, J. W. Shiver, and M. A. Liu.** 1997. DNA vaccines. Annu. Rev. Immunol. **15:**617–648.
11. **Dubos, R. J., and R. W. Schaedler.** 1956. Reversible changes in the susceptibility of mice to bacterial infections. I. Changes brought about by injection of pertussis vaccine or of bacterial endotoxins. J. Exp. Med. **104:**53–72.
12. **Dugger, K. O., J. N. Galgiani, N. M. Ampel, S. H. Sun, D. M. Magee, J. Harrison, and J. H. Law.** 1991. An immunoreactive apoglycoprotein purified from *Coccidioides immitis*. Infect. Immun. **59:**2245–2251.
13. **Dugger, K. O., K. M. Villareal, A. Ngyuen, C. R. Zimmerman, J. H. Law, and J. N. Galgiani.** 1996. Cloning and sequence analysis of the cDNA for a protein from *Coccidioides immitis* with immunogenic potential. Biochem. Biophys. Res. Commun. **218:**485–489.
14. **Galgiani, J. N.** 1999. Coccidioidomycosis: a regional disease of national importance. Rethinking approaches to its control. Ann. Intern. Med. **130:** 293–300.
15. **Galgiani, J. N., K. O. Dugger, N. M. Ampel, S. H. Sun, and J. H. Law.** 1988. Extraction of serologic and delayed hypersensitivity antigens from spherules of *Coccidioides immitis*. Diagn. Microbiol. Infect. Dis. **11:**65–80.
16. **Galgiani, J. N., S. H. Sun, K. O. Dugger, N. M. Ampel, G. G. Grace, J. Harrison, and M. A. Wieden.** 1992. An arthroconidial-spherule antigen of *Coccidioides immitis*: differential expression during in vitro fungal development and evidence for humoral response in humans after infection or vaccination. Infect. Immun. **60:**2627–2635.
17. **Gazzinelli, R. T., M. Wysocka, S. Hayashi, E. Y. Denkers, S. Hieny, P. Caspar, G. Trinchieri, and A. Sher.** 1994. Parasite-induced IL-12 stimulates early IFN-gamma synthesis and resistance during acute infection with *Toxoplasma gondii*. J. Immunol. **153:**2533–2543.
18. **Gould, J. R., H. W. Carloss, and W. L. Skinner.** 1993. Groshong catheter-associated subclavian venous thrombosis. Am. J. Med. **95:**419–423.
19. **Hartikka, J., M. Sawdey, F. Cornefert-Jensen, M. Margalith, M. Marquet, P. Hobart, J. Norman, and M. Manthorpe.** 1996. An improved plasmid DNA expression vector for direct injection into skeletal muscle. Hum. Gene Ther. **7:**1205–1217.
20. **Hsieh, C., S. E. Macatonia, A. O'Garra, and K. M. Murphy.** 1995. T cell genetic background determines default T helper phenotype development *in vitro*. J. Exp. Med. **181:**713–721.

21. **Huygen, K., J. Content, O. Denis, D. L. Montgomery, A. M. Yawman, R. R. Deck, C. M. DeWitt, I. M. Orme, S. Baldwin, C. D'Souza, A. Drowart, E. Lozes, P. Vandenbussche, J. P. Van Vooren, M. A. Liu, and J. B. Ulmer.** 1996. Immunogenicity and protective efficacy of a tuberculosis DNA vaccine. Nat. Med. **2:**893–898.
22. **Iizawa, Y., R. D. Wagner, and C. J. Czuprynski.** 1993. Analysis of cytokine mRNA expression in *Listeria*-resistant C57BL/6 and *Listeria*-susceptible A/J mice during *Listeria monocytogenes* infection. Infect. Immun. **61:**3739–3744.
23. **Jiang, C., D. M. Magee, T. N. Quitugua, and R. A. Cox.** 1999. Genetic vaccination against *Coccidioides immitis*: comparison of vaccine efficacy of recombinant antigen 2 and antigen 2 cDNA. Infect. Immun. **67:**630–635.
24. **Kaposzta, R., P. Tree, L. Marodi, and S. Gordon.** 1998. Characteristics of invasive candidiasis in gamma interferon- and interleukin-4-deficient mice: role of macrophages in host defense against *Candida albicans*. Infect. Immun. **66:**1708–1717.
25. **Kirkland, T. N., and J. Fierer.** 1983. Inbred mouse strains differ in resistance to lethal *Coccidioides immitis* infection. Infect. Immun. **40:**912–916.
26. **Kirkland, T. N., F. Finley, K. I. Orsborn, and J. N. Galgiani.** 1998. Evaluation of the proline-rich antigen of *Coccidioides immitis* as a vaccine candidate in mice. Infect. Immun. **66:**3519–3522.
27. **Kirkland, T. N., P. W. Thomas, F. Finley, and G. T. Cole.** 1998. Immunogenicity of a 48-kilodalton recombinant T-cell-reactive protein of *Coccidioides immitis*. Infect. Immun. **66:**424–431.
28. **Kuhober, A., H. P. Pudollek, K. Reifenberg, F. V. Chisari, H. J. Schlicht, J. Reimann, and R. Schirmbeck.** 1996. DNA immunization induces antibody and cytotoxic T cell response to hepatitis B core antigen in H-2b mice. J. Immunol. **156:**3687–3695.
29. **Landy, M.** 1956. Increase in resistance following administration of bacterial lipopolysaccharides. Ann. N. Y. Acad. Sci. **66:**292–308.
30. **Leroux-Roels, G., E. Moreau, B. Verhasselt, S. Biernaux, V. Brulein, M. Francotte, P. Pala, M. Slaopui, and P. Vandepapeliere.** 1993. Immunogenicity and reactogenicity of a recombinant HSV-2 glycoprotein D vaccine with or without mono-phosphoryl lipid A in HSV seronegative and seropositive subjects, abstr. 1209, p. 341. *In* Program and abstracts of the 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
31. **Levine, H. B., J. M. Cobb, and C. E. Smith.** 1960. Immunity to coccidioidomycosis induced in mice by purified spherule, arthrospore, and mycelial vaccines. Trans. N. Y. Acad. Sci. **22:**436–447.
32. **Levine, H. B., Y.-C. Kong, and C. E. Smith.** 1965. Immunization of mice to *Coccidioides immitis*: dose, regimen and spherulation stage of killed spherule vaccines. J. Immunol. **94:**132–142.
33. **Louria, D. B.** 1960. Specific and non-specific immunity in experimental cryptococcosis in mice. J. Exp. Med. **111:**643–665.
34. **Luke, C. J., K. Carner, X. Liang, and A. G. Barbour.** 1996. An OspA-based DNA vaccine protects mice against infection with *Borrelia burgdorferi*. J. Infect. Dis. **175:**91–97.
35. **Morris, L., A. B. Troutt, K. S. McLeod, A. Kelso, E. Handman, and T. Aebischer.** 1993. Interleukin-4 but not gamma interferon production correlates with the severity of murine cutaneous leishmaniasis. Infect. Immun. **61:**3459–3465.
36. **Mosmann, T. R., and S. Sad.** 1996. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol. Today **17:**138–146.
37. **Orme, I. M., A. D. Roberts, J. P. Griffin, and J. S. Abrams.** 1993. Cytokine secretion by CD4 T lymphocytes acquired in response to *Mycobacterium tuberculosis* infection. J. Immunol. **151:**518–525.
38. **Orsborn, K. I., and J. N. Galgiani.** 1998. Detecting serum antibodies in patients with coccidioidomycosis to a purified recombinant proline-rich antigen from *Coccidioides immitis*. Clin. Infect. Dis. **27:**1475–1478.
39. **Pang, A.** 1994. Production of antibodies against *Bacillus thuringiensis* delta-endotoxin by injecting its plasmids. Biochem. Biophys. Res. Commun. **202:**1227–1234.
40. **Pappagianis, D., and H. B. Levine.** 1975. The present status of vaccination against coccidioidomycosis in man. Am. J. Epidemiol. **102:**30–41.
41. **Pappagianis, D., and The Valley Fever Vaccine Study Group.** 1993. Evaluation of the protective efficacy of the killed *Coccidioides immitis* spherule vaccine in humans. Am. Rev. Respir. Dis. **148:**656–660.
42. **Rudbach, J. A., D. A. Johnson, and J. T. Ulrich.** 1995. Ribi adjuvants: chemistry biology and utility in vaccines for human and veterinary medicine, p. 287–313. *In* D. E. S. Stewart-Tull (ed.), The theory and practical application of adjuvants. John Wiley & Sons Ltd., Hamilton, Mont.
43. **Salkowski, C. A., and E. Balish.** 1991. A monoclonal antibody to gamma interferon blocks augmentation of natural killer cell activity induced during systemic cryptococcosis. Infect. Immun. **59:**486–493.
44. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
45. **Schirmbeck, R., W. Bohm, K. Ando, R. V. Chisari, and J. Reimann.** 1995. Nucleic acid vaccination primes hepatitis B virus surface antigen-specific cytotoxic T lymphocytes in nonresponder mice. J. Virol. **69:**5929–5934.
46. **Stevens, D. A.** 1995. Current concepts: coccidioidomycosis. N. Engl. J. Med. **332:**1077–1082.
47. **Stoute, J. A., M. Slaoui, D. G. Heppner, P. Momin, K. E. Kester, P. Desmons, B. T. Wellde, N. Garçon, U. Krzych, M. Marchand, W. R. Ballou, and J. D. Cohen.** 1997. A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmodium falciparum* malaria. N. Engl. J. Med. **336:**86–91.
48. **Thoelen, S., P. van Damme, C. Mathei, G. Leroux-Roels, I. Desombere, A. Safary, P. Vandepapeliere, M. Slaoui, and A. Meheus.** 1998. Safety and immunogenicity of a hepatitis B vaccine formulated with a novel adjuvant system. Vaccine **16:**708–714.
49. **Tighe, H., M. Corr, M. Roman, and E. Raz.** 1998. Gene vaccination: plasmid DNA is more than just a blueprint. Immunol. Today **19:**89–97.
50. **Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, A. Friedman, L. A. Hawe, K. R. Leander, D. Martinez, H. C. Perry, J. W. Shiver, D. L. Montgomery, and M. Liu.** 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science **259:**1745–1749.
51. **Ulmer, J. B., T. M. Fu, R. R. Deck, A. Friedman, L. Guan, C. DeWitt, X. Liu, S. Wang, M. A. Liu, J. J. Donnelly, and M. J. Caulfield.** 1998. Protective $CD4^+$ and $CD8^+$ T cells against influenza virus induced by vaccination with nucleoprotein DNA. J. Virol. **72:**5648–5653.
52. **Ulrich, J. T., and K. R. Myers.** 1995. Monophosphoryl lipid A as an adjuvant. Past experiences and new directions, p. 495–524. *In* M. F. Powell and M. J. Newman (ed.), Vaccine design: the subunit and adjuvant approach. Plenum Press, New York, N.Y.
53. **Xu, D., and F. Y. Liew.** 1995. Protection against leishmaniasis by injection of DNA encoding a major surface glycoprotein, gp63, of 1-major. Immunology **84:**173–176.
54. **Yang, W., and G. J. M. D. P. Waine.** 1995. Antibodies to *Schistosoma japonicum* (Asian bloodfluke) paramycin induced by nucleic acid vaccination. Biochem. Biophys. Res. Commun. **212:**1029–1039.
55. **Zhu, W. F., C. M. Yang, D. M. Magee, and R. A. Cox.** 1996. Molecular cloning and characterization of *Coccidioides immitis* antigen 2 cDNA. Infect. Immun. **64:**2695–2699.
56. **Zimmermann, C. R., S. M. Johnson, G. W. Martens, A. G. White, B. L. Zimmer, and D. Pappagianis.** 1998. Protection against lethal murine coccidioidomycosis by a soluble vaccine from spherules. Infect. Immun. **66:**2342–2345.

Editor: T. R. Kozel