INFECTION AND IMMUNITY, Jan. 1995, p. 122–125 Vol. 63, No. 1
0019-9567/95/$04.00+0
Copyright © 1995, American Society for Microbiology

Pure Paraflagellar Rod Protein Protects Mice against *Trypanosoma cruzi* Infection

RUTH A. WRIGHTSMAN, MARK J. MILLER, JOSE L. SABORIO, AND JERRY E. MANNING*

Department of Molecular Biology and Biochemistry, University of California-Irvine, Irvine, California 92717

Received 26 August 1994/Returned for modification 3 October 1994/Accepted 13 October 1994

The paraflagellar rod proteins (PAR) purified from *Trypanosoma cruzi* epimastigotes were shown to protect mice against an otherwise lethal challenge inoculum of 10^3 bloodstream-form trypomastigotes. The injection route used for immunization was shown to have a marked impact on the development of protective immunity. Mice receiving subcutaneous (s.c.) injections of PAR proteins had reduced bloodstream parasitemias and showed 100% survival following challenge. In contrast, mice immunized via the intraperitoneal (i.p.) route developed parasitemia levels equivalent to those of unimmunized controls and did not survive infection. Western blotting (immunoblotting) demonstrated that sera from both i.p. and s.c. immunized mice reacted specifically with PAR proteins; however, the antibody titer of the i.p. immunized mice was approximately 64-fold greater than that of the s.c. immunized mice, suggesting that the protective response in the s.c. immunized mice is cell mediated rather than humoral.

Trypanosoma cruzi is a parasitic hemoflagellate that causes Chagas' disease, a major human health problem in Central and South America (4). Chemotherapeutic agents have limited effectiveness against the disease, and no vaccines are available for disease prevention. Experimental vaccines consisting of attenuated forms of the parasite, crude parasite lysates, partially purified proteins, and synthetic peptides derived from the sequence of a 24-kDa excretory protein (ESA) have been tested in laboratory animals with varying degrees of success (reviewed in references 2, 5, 7, 16, and 22). Several of these studies show that susceptible hosts may be partially protected against death by vaccination prior to challenge; however, individual antigens capable of ensuring survival against an otherwise lethal challenge have yet to be identified.

In an attempt to identify and purify protective antigens, we focused on proteins present in the flagellum of *T. cruzi*. The rationale for this approach is based on studies demonstrating that vaccination with crude flagellar extracts are effective in both inducing protective immunity (i.e., 90% survival) in mice after challenge (15, 20, 21) and reducing tissue lesions in immunized mice compared with nonimmunized mice following a challenge infection (14). In contrast, immunization with a membrane-enriched fraction from the body of the parasite was less effective in inducing a protective response, suggesting that the protective antigen(s) may be unique to the flagellum. A potential candidate for such antigens is the paraflagellar rod, a prominent structure present only in the flagellum of the parasite (5). We have shown that the paraflagellar rod contains two distinct proteins, PAR 1 and PAR 2, and have determined conditions that allow these proteins to be isolated in amounts and purity sufficient for immunization trials (3, 18).

In this paper, we report the ability of the purified PAR proteins to induce an immune response in mice capable of protecting against an otherwise lethal inoculum of *T. cruzi* trypomastigotes.

* Corresponding author. Phone: (714) 824-5578. Fax: (714) 824-8551.

MATERIALS AND METHODS

Parasites. The Peru strain of *T. cruzi* was used in all experiments. Epimastigotes were grown in modified HM (9): 37 g of brain heart infusion (Difco Laboratories, Detroit, Mich.) per liter, 2.5 g of hemin per liter, and 10% heat-inactivated fetal calf serum. Bloodstream trypomastigotes used for challenge inoculations of mice were obtained by cardiac puncture of female BALB/cByJ mice at day 14 postinfection. An inoculum of 10^3 trypomastigotes was used for all experiments.

Mice. Six- to 8-week-old female BALB/cByJ mice (Jackson Laboratories, Bar Harbor, Maine) were used for all experiments.

Measurement of parasitemias. Parasitemia levels were determined as previously described (24) by removing a blood sample from the tail vein and counting the number of trypomastigotes with a Neubauer hemocytometer.

Antigen preparation. PAR proteins were purified as described previously (18). Briefly, 10^{11} Peru strain epimastigotes were harvested by centrifugation, washed in phosphate-buffered saline (PBS), and lysed in 0.1 M Tricine (pH 8.5) containing 1% Nonidet P-40. The pellet was extracted with high-salt buffer consisting of 0.1 M Tricine, 1 M NaCl, and 1% Triton X-100, using sonication. This crude flagellar pellet was successively extracted with 2.0 and 6.0 M urea in 10 mM Tricine (pH 8.5). The resulting supernatant contains approximately 50% PAR and 50% tubulin. The PAR proteins were separated from tubulin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and recovered by electroelution.

Vaccination. Female BALB/cByJ mice were immunized with 40 μg of PAR emulsified in complete Freund's adjuvant. Mice were boosted twice at 2-week intervals with 20 μg of PAR emulsified in incomplete Freund's adjuvant. Control groups were injected with Freund's adjuvant plus PBS or with PBS only. Two weeks after the last injection, mice were challenged with a subcutaneous (s.c.) injection of 10^3 bloodstream trypomastigotes. Mice were checked daily, and percent survival was recorded at days postinfection. Parasitemias were monitored every other day from days 7 to 28 and weekly from weeks 4 to 20.

Immunoblotting. Samples were separated by one-dimensional PAGE and transferred to nitrocellulose by methods previously described (18), using a transblot cell (Bio-Rad, Richmond, Calif.) overnight at 150 mA. Blots probed with mouse sera were reacted with peroxidase-linked anti-mouse immunoglobulin G (IgG) and detected with the ECL system (DuPont, Wilmington, Del.). Blots probed with human sera were reacted with ^{125}I-sheep anti-human IgG (Amersham, Arlington Heights, Ill.).

ELISA. Sera from immunized mice were obtained via the tail vein 10 days following the last injection. To measure PAR-specific antibody titers, 96-well microtiter plates were coated with 50 μl of PAR protein (10 μg/ml) in PBS and incubated overnight at room temperature. Wells were washed with distilled H_2O and blocked with PBS plus 1% bovine serum albumin; serial dilutions of anti-PAR serum were added, and plates were incubated for 2 h at room temperature. Wells were washed, reacted with peroxidase-conjugated anti-mouse IgG with distilled H_2O, and blocked with PBS plus 1% bovine serum albumin; serial dilutions of anti-PAR serum were added, and plates were incubated for 2 h at room temperature. Wells were washed, reacted with peroxidase-conjugated anti-mouse IgG (Sigma Chemical Co., St. Louis, Mo.) and 2,2′-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) (Boehringer Mannheim Biochemicals, Indianapo-

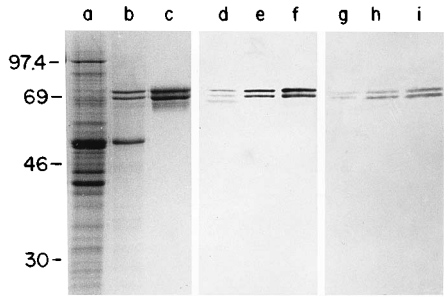

FIG. 1. Purification and immunoreactivity of PAR proteins. Lanes a to c: SDS-PAGE stained with Coomassie blue. Lanes d to f and g to i, Western blots (immunoblots) reacted with serum from BALB/c mice immunized with purified PAR via the i.p. (d to f) or s.c. (g to i) route. Lanes a, d, and g, epimastigote whole-cell lysate; lanes b, e, and h, 6 M urea extract of flagellar pellet; lanes c, f, and i, SDS-PAGE-purified and electroeluted PAR.

lis, Ind.) substrate, and read at 405 nm in an automated enzyme-linked immunosorbent assay (ELISA) plate reader.

RESULTS AND DISCUSSION

To investigate the vaccine potential of purified PAR proteins, BALB/cByJ mice, a strain highly susceptible to infection with the Peru strain of *T. cruzi* (24), were immunized with PAR proteins purified from epimastigotes (Fig. 1). Since the ability of an antigen to induce protective immunity may be dependent on the route of injection, two immunization routes, s.c. and intraperitoneal (i.p.), were tested. As shown in Fig. 1, both routes of immunization elicited an immune response as evidenced by serum antibodies against the PAR proteins. The antibody response in both groups of immunized mice was entirely restricted to the PAR 1 and PAR 2 proteins, and no reaction to other parasite proteins present in total cellular lysates was observed, indicating that the immune response was directed solely to the PAR proteins.

The efficacy of the PAR proteins as vaccine candidates was assessed by monitoring the survival and parasitemia burden of immunized mice after challenge with a lethal inoculum of the highly virulent Peru strain of *T. cruzi* (50% lethal dose, $\leq$10). As shown in Fig. 2, the route of immunization was critical for the development of protective immunity. Following a challenge with 10^3 bloodstream trypomastigotes, only mice that received s.c. injections of the PAR proteins showed a reduction in parasite burden and survived infection. In contrast, immunization via the i.p. route was completely ineffective in either reducing parasitemia burden or increasing the mean survival time (Table 1).

TABLE 1. Protective immunity by vaccination with PAR proteins

Vaccination	No. of mice immunized	No. of survivors	Mean survival time (days)	Mean parasitemia at day 19 (10^5) ± SD
PAR, s.c.	8	8	>120	13.0 ± 5.2
PAR, i.p.	9	0	20.8	66.6 ± 22.6
Adjuvant	8	0	21.6	51.3 ± 27.2
No adjuvant	8	0	21.1	44.8 ± 9.8

As shown in Fig. 1, sera from mice immunized i.p. reacted with a much higher intensity with the PAR proteins than sera from mice immunized s.c., suggesting that differences existed in the antibody titers of the two groups. Measurement of anti-PAR antibody titers by direct ELISA shows that levels of anti-PAR antibodies from mice immunized i.p. were 64-fold greater than those observed in mice immunized s.c. (Fig. 3). The reduction in antibody titer observed in the s.c. immunized mice was accompanied by a change in the major isotype subclasses of anti-PAR antibodies. In i.p. immunized mice, approximately equivalent levels of IgG1, IgG2a, and IgG2b subclasses were observed, while in s.c. immunized mice, IgG1 was the only detectable subclass (data not shown).

To determine whether the PAR proteins generate an immune response in humans, serum samples from 16 Chagasic patients were tested for anti-PAR antibodies by immunoblot analysis. All of the sera tested reacted positively with the PAR proteins (Fig. 4). Patient sera tested represent infection by four genetically distinct zymodemes, indicating that the PAR protein may be sufficiently conserved among *T. cruzi* strains to allow immunological recognition across different strains. This possibility is strengthened by previous results which show that the sequence of the PAR 2 protein is highly conserved (90%) even between *T. cruzi* and its distant relative, *T. brucei*, the causative agent of African sleeping sickness (3). Recognition of

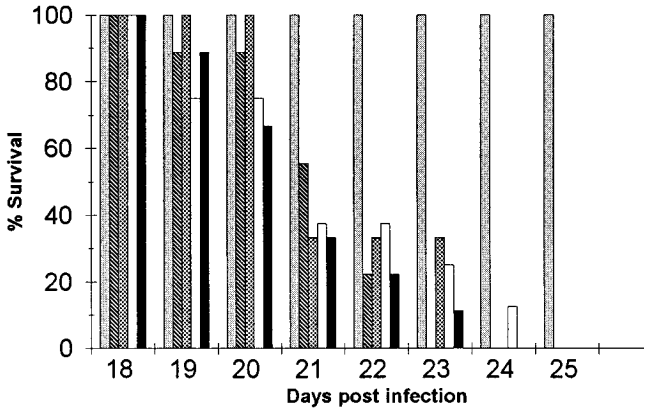

FIG. 2. Survival of PAR-immunized mice after challenge with *T. cruzi*. Immunization groups shown are as follows: PAR plus Freund's injected s.c. (▤); PAR plus Freund's injected i.p. (▧); Freund's adjuvant plus PBS injected s.c. (▨); Freund's adjuvant plus PBS injected i.p. (□); PBS only (■).

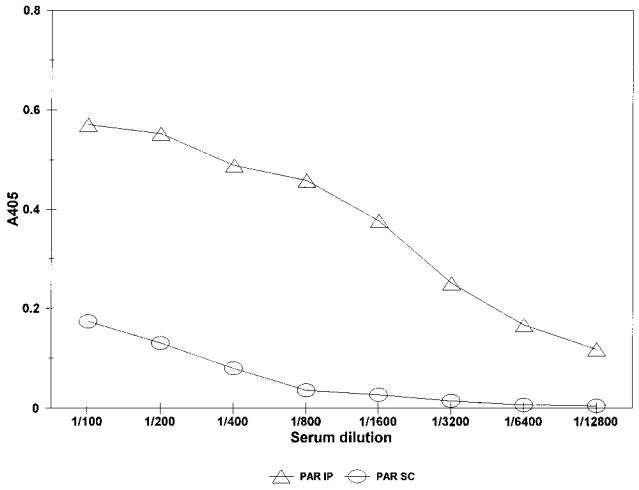

FIG. 3. Measurement of anti-PAR antibody titer. The anti-PAR antibody titers of sera from mice immunized with PAR proteins via the i.p. (△) or s.c. (○) route of injection were measured by ELISA. Each point represents the mean absorbance values of duplicate wells.

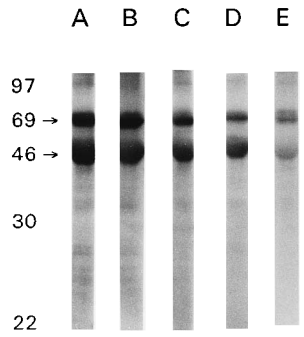

FIG. 4. Reaction of human Chagasic sera with PAR proteins. Western blots of 4.0 M urea extract of flagellar pellet were reacted with sera from human Chagasic patients and then with ^{125}I-anti-human IgG. Representative reactions of samples tested are shown. Lanes were reacted with the following serum samples: A, A210; B, C181; C, B151; D, B274; E, D143. Arrows indicate the positions of PAR (top arrow) and tubulin (bottom arrow).

the PAR proteins by the human Chagasic sera tested also suggests that the vaccine potential of the PAR proteins may not be severely compromised by immunological restriction in the human population.

The results of these studies clearly show that immunization of mice with the PAR proteins provides significant protection (100% survival) against an otherwise lethal challenge of the parasite. Although these studies do not identify the immunological responses necessary for survival, they do provide some insight into possible protective mechanisms. The observation that the anti-PAR antibody titer in the s.c. immunized mice is substantially lower than that found in the i.p. immunized mice suggests that the protective response that develops following s.c. immunization with PAR might not be antibody mediated. This interpretation is consistent with the localization of PAR proteins to an internal structure of the parasite and the observation that anti-PAR antibodies do not bind live parasites, making it unlikely that anti-PAR antibodies directly affect parasitemia levels or host survival by either complement-mediated lysis of parasites or blocking of parasite penetration of host cells. Also, given the observation that the isotype subclass of anti-PAR antibodies in s.c. immunized mice is also present in i.p. immunized mice, there is no indication that destruction of parasites in infected host cells is occurring by antibody-dependent cell-mediated cytotoxicity.

The paucity of evidence for a protective humoral response leads to the suggestion that survival of mice immunized with PAR proteins involves cell-mediated immune responses. In considering this possibility, it should be pointed out that immunization of mice with the schistosome protein paramyosin, also a nonsurface protein, induces protective cell-mediated immunity against challenge with *Schistosoma mansoni* (11). The suggested involvement of cell-mediated immunity also is consistent with results of other studies which show T cells to be one of the major cell populations involved in providing resistance to *T. cruzi* (8, 10, 19). In particular, partial elimination of $CD4^+$ or $CD8^+$ T cells by use of monoclonal antibodies, or use of mice which lack CD4 or CD8 molecules as a result of specific genomic deletions, results in mice which are strikingly susceptible to *T. cruzi* infections (1, 12, 13, 17, 23). Also consistent with the possible involvement of T cells in the protective response elicited by immunization with the PAR proteins is the observation that splenic T lymphocytes from s.c. immunized mice respond to purified PAR protein in vitro (25). The availability of purified PAR proteins and subregions of this protein generated from the cloned PAR genes (3, 10a) will aid further investigations of the relative contributions of lymphocyte subpopulations in the survival of PAR-immunized mice.

ACKNOWLEDGMENTS

We acknowledge Barbara Granger for technical assistance and Steve Reed and Donna Russo for helpful discussions.

This work was supported by grant AI1887 from the National Institutes of Health.

REFERENCES

1. **Araujo, F. G.** 1989. Development of resistance to *Trypanosoma cruzi* in mice depends on a viable population of $L3T4^+$ ($CD4^+$) lymphocytes. Infect. Immun. **57:**2246–2248.
2. **Araujo, F. G., and B. Morein.** 1991. Immunization with *Trypanosoma cruzi* epimastigote antigens incorporated into iscoms protects mice against lethal challenge in mice. Infect. Immun. **59:**2909–2914.
3. **Beard, C. A., J. L. Saborio, D. Tewari, K. G. Krieglstein, A. H. Henschen, and J. E. Manning.** 1992. Evidence for two distinct major protein components, PAR 1 and PAR 2, in the paraflagellar rod of *Trypanosoma cruzi*. J. Biol. Chem. **267:**21656–21662.
4. **Brener, Z.** 1973. Biology of *Trypanosoma cruzi*. Annu. Rev. Microbiol. **27:** 347–382.
5. **De Souza, W., and T. Souro-Padron.** 1980. The paraxial structure of the flagellum of trypanosomatidae. J. Parasitol. **66:**229–236.
6. **Gonzalez, J., M. F. Araguth, and N. Yoshida.** 1991. Resistance to acute *Trypanosoma cruzi* infection resulting from immunization of mice with a 90-kilodalton antigen from metacyclic trypomastigotes. Infect. Immun. **59:** 863–867.
7. **Kierszenbaum, F.** 1989. Chagas' disease, p. 173–195. *In* F. Y. Liew (ed.), Vaccination strategies of tropical diseases. CRC Press, Boca Raton, Fla.
8. **Kierszenbaum, F., and M. Pienkowsky.** 1979. Thymus-dependent control of host defense mechanisms against *Trypanosoma cruzi*. Infect. Immun. **24:**117–120.
9. **Lanar, D.** 1979. Growth and isolation of *Trypanosoma cruzi* cultivated with *Triatoma infestans* embryo cell line. J. Protozol. **26:**457–462.
10. **McCabe, R., J. Remington, and F. Araujo.** 1985. In vivo and in vitro effects of cyclosporin A on *Trypanosoma cruzi*. Am. J. Trop. Med. Hyg. **34:**861–865.

10a. **Miller, M. J., and J. E. Manning.** Unpublished observations.

11. **Pearce, E. J., S. L. James, S. Hieny, D. E. Lanar, and A. Sher.** 1988. Induction of protective immunity against *Schistosoma mansoni* by vaccination with schistosome paramyosin (SM97), a nonsurface parasite antigen. Proc. Natl. Acad. Sci. USA **85:**5678–5682.
12. **Rottenberg, M. E., M. Bakhiet, T. Olsson, K. Kristensson, T. Mak, H. Wigzell, and A. Orn.** 1993. Differential susceptibility of mice genomically deleted of CD4 or CD8 to infections with *Trypanosoma cruzi* or *Trypanosoma brucei*. Infect. Immun. **61:**5129–5133.
13. **Rottenberg, M., R. Cardoni, R. Andersson, E. Segura, and A. Orn.** 1988. Role of T helper/inducer cells as well as natural killer cells in resistance to *Trypanosoma cruzi* infection. Scand. J. Immunol. **28:**573–582.
14. **Ruiz, A. M. M. Esteva, P. Cabeza-Meckart, R. P. Laguens, and E. L. Segura 1985**. Protective immunity and pathology induced by inoculation of mice with different subcellular fractions of *Trypanosoma cruzi*. Acta Trop. **42:**299–309.
15. **Ruiz, A. M., M. Esteva, A. Riarte, E. Subias, and E. L. Segura.** 1986. Immunoprotection of mice against *Trypanosoma cruzi* with a lyophilized flagellar fraction of the parasite plus adjuvent. Immunol. Lett. **12:**1–4.
16. **Ruiz, A. M., M. Esteva, E. Subias, M. Moreno, A. Rosenstein deCampanini, E. Valazquez, and E. L. Sequra.** 1990. Monoclonal antibodies against the flagellar fraction of epimastigotes of *Trypanosoma cruzi*: immunoprotection against metacyclic trypomastigotes obtained by immunization of mice with an affinity-purified antigen. Mol. Biochem. Parasitol. **39:**117–125.
17. **Russo, M., N. Starobinas, P. Minoprio, A. Coutinho, and M. Joskowicz.** 1988. Parasitic load increases and myocardial inflammation decreases in *Trypanosoma cruzi*-infected mice after inactivation of T helper cells. Ann. Inst. Pasteur Immunol. **139:**225–236.
18. **Saborio, J. L., J. M. Hernandez, S. Narayanswami, R. Wrightsman, E. Palmer, and J. E. Manning.** 1989. Isolation and characterization of paraflagellar proteins from *Trypanosoma cruzi*. J. Biol. Chem. **264:**4071–4075.
19. **Schmunis, G. A., S. Gonzalez Cappa, O. Traversa, and J. Janovsky.** 1971. The effect of immunodepression due to neonatal thymectomy on infections with *Trypanosoma cruzi*. Trans. R. Soc. Trop. Med. Hyg. **65:**89–94.
20. **Segura, E. L., I. Paulone, J. Cerisola, and S. M. Gonzalez Cappa.** 1976. Experimental Chagas' disease: protective activity in relation with subcellular fractions of the parasite. J. Parasitol. **62:**131–133.
21. **Segura, E. L., C. Vasquez, A. Bronzina, J. M. Campos, J. Cerisola, and S. M. Gonzalez Cappa.** 1977. Antigens of the subcellular fractions of *Trypanosoma cruzi*. II. Flagellar and membrane fraction. J. Protozool. **24:**540–543.
22. **Taibi, A., B. Plumas-Marty, A. Guevara-Espinoza, R. Schoneck, H. Pessoa,**

M. Loyens, R. Piras, T. Aguirre, H. Gras-Masse, M. Bossus, A. Tartar, A. Capron, and A. Ouaissi. 1993. *Trypanosoma cruzi*: immunity induced in mice and rats by trypomastigote excretory-secretory antigens and identification of a peptide sequence containing a T cell epitope with protective activity. J. Immunol. **151:**2676–2689.

23. **Tarleton, R.** 1990. Depletion of CD8+ T cells increases susceptibility and reverses vaccine-induced immunity in mice infected with *Trypanosoma cruzi*. J. Immunol. **144:**717–724.
24. **Wrightsman, R., S. Krassner, and J. Watson.** 1982. Genetic control of responses to *Trypanosoma cruzi* in mice: multiple genes influencing parasitemia and survival. Infect. Immun. **36:**637–644.
25. **Wrightsman, R., and J. E. Manning.** Unpublished observations.