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Trypanosoma cruzi: Protective Immunity in Mice Immunized with Paraflagellar Rod Proteins Is Associated with a T-Helper Type 1 Response

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Miller, M. J., Wrightsman, R. A., and Manning, J. E. 1996. *Trypanosoma cruzi:* Protective immunity in mice immunized with paraflagellar rod proteins is associated with a T-helper type 1 response. *Experimental Parasitology* **84,** 156–167. We have examined the ability of mice to survive a lethal challenge with the parasitic hemoflagellate, *Trypanosoma cruzi,* following immunization with paraflagellar rod proteins (PAR) 1 and 2 either alone or in combination with the following adjuvants: Freund's, alum, QS-21, Ribi-700, or IL-12. PAR administered subcutaneously (sc) in combination with Freund's or alum provided significant protection, 100 and 83%, respectively, against a *T. cruzi* challenge. In contrast, PAR in combination with QS-21, Ribi-700, IL-12, or Freund's administered intraperitoneally (ip) or PAR alone provide no protection against a challenge. PAR-specific serum antibody titers and isotype profiles for several of the immunization regimens were determined, and no positive correlation could be seen between a protective immune response and either antibody titer or the subclass of antibody induced. We also examined the ability of PAR to stimulate T cells from the spleen and lymph nodes of mice immunized with PAR in combination with Freund's (sc), Freund's (ip), alum, or Ribi-700. Each of the adjuvants strongly enhanced the ability of enriched T cells to proliferate in a PAR-specific fashion, suggesting no obvious correlation between PAR-specific T cell activation and protection. However, examination of the cytokine profiles of the stimulated T cell groups showed that the protective groups differed from the nonprotective groups. While all four groups showed low levels of IL-10, the Freund's (sc) and alum groups had higher levels of IFN-γ and IL-2 than Freund's (ip) and Ribi-700 groups, and most strikingly, no IL-4 could be detected in either the Freund's (sc) or the alum group, in contrast to significant levels of IL-4 in both the Freund's (ip) and the Ribi-700 group. These findings indicate that protective immunity in mice immunized with PAR is associated with a Th1-type response. © 1996 Academic Press, Inc.

INTRODUCTION

The hemoflagellate protozoan *Trypanosoma cruzi* causes Chagas' disease, a major public health problem in many countries of Latin America. Chemotherapeutic agents have limited effectiveness against the parasite, and no immunoprophylactic vaccine for prevention of the disease is available. Infection with *T. cruzi* may be either acute or chronic, and the immunologic mechanisms which influence both survival of the vertebrate host and clearance of the parasite during the acute phase are not well understood. Several studies (Culbertson and Kolodny 1938; Krettli and Brener 1976; Rod-

riguez *et al.* 1981; Kierszenbaum and Howard 1976) have indicated that lytic antibodies against the parasite may play a key role in reducing parasite burden and enhancing the survival of mice infected with the parasite. These studies have been advanced, and more recent data (Takehara *et al.* 1981; Rowland *et al.* 1992; Powell and Wassom 1993) argue that antibody isotype also may be of particular importance in the clearance of blood parasites by circulating serum antibodies.

While the importance of a humoral immune response is clearly documented, it is also evident that survival of mice infected with the parasite depends upon cell-mediated immune responses (Kierszenbaum and Pienkowsky 1979; Schmunis *et al.* 1971). In particular, partial elimination of $CD4^+$ or $CD8^+$ T cells by use

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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 (Araujo 1989; Rottenberg *et al.* 1988, 1993, 1995; Russo *et al.* 1988; Tarleton 1990; Tarleton *et al.* 1992). The role of cytokines in resistance and susceptibility of the host to infection with *T. cruzi* provides some understanding of these observations. IFN-γ has been shown to play a central role in the control of parasitemia in *T. cruzi*-infected mice (Plata *et al.* 1984; Minoprio *et al.* 1986; Reed 1988; Torrico *et al.* 1991; Wirth *et al.* 1985; Joao *et al.* 1992). Along with GM-CSF (Reed *et al.* 1987) and TNF (De Titto *et al.* 1986), IFN-γ has been shown to activate macrophages *in vitro* leading to a reduction in the ability of the parasite to propagate. *In vivo,* IFN-γ has been found to be essential for parasite clearance in naturally resistant mouse strains (Minoprio *et al.* 1993), and mice challenged with an otherwise lethal inoculum of the parasite show a marked reduction in parasitemia and increased survival following treatment with IFN-γ (Reed 1988).

These findings suggest that an efficacious vaccine may exert its protective role via several immunological routes. In our previous studies (Wrightsman *et al.* 1995), we have shown that the paraflagellar rod proteins (PAR) present in the flagellum of *T. cruzi* can induce an immune response in mice capable of protecting against an otherwise lethal inoculum of *T. cruzi* trypomastigotes. In this paper, the effects of antigen dose, immunization regimen, and different adjuvants on parasite clearance and survival of immunized mice challenged with the parasite are reported. To better define the immunologic basis for the protective effect exerted by this vaccine candidate, we also have examined several immunological parameters in both protected and nonprotected mice. Specifically, the titer and isotype of PAR-specific serum antibodies were determined as well as the proliferation of splenic and lymph node T cells and production of IFN-γ, IL-4, and IL-10 by these cells following stimulation with PAR proteins. We conclude that PAR-specific antibody titer, anti-body isotype, and T cell proliferation do not correlate well with parasite reduction and host survival. In contrast, enhanced survival and parasite clearance are associated with high levels of IFN-γ and the absence of detectable levels of IL-4.

MATERIALS AND METHODS

Parasites. The Peru strain of *T. cruzi* was used in all experiments. Epimastigotes were grown in modified HM (Lanar 1979). Bloodstream trypomastigotes used for challenge inoculations of mice were obtained by cardiac puncture of female BALB/cByJ mice (Jackson Laboratories, Bar Harbor, ME) at Day 14 postinfection. An inoculum of 10^3 trypomastigotes was used for all experiments.

Antigen preparation. PAR proteins were purified as previously described (Saborio *et al.* 1989). 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-P40. 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 by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a Bio-Rad Prep Cell Model 491. Fractions containing the PAR proteins were extensively dialyzed against PBS, concentrated by centrifugation in a centricon (Amicon, Beverley, MA), and sterilized by 0.45-μm filtration.

Vaccine preparation. Vaccine preparations containing the PAR proteins were: (1) emulsified with equal volumes of Freund's adjuvant (FA), (2) adsorbed to an equal volume of alum gel (Rehsorpter aluminum hydroxide adsorptive gel; Intergen Co., Purchase, NY), (3) mixed by brief vortexing with 20 μg QS-21 (Cambridge Biotech, Cambridge, MA), (4) mixed by brief vortexing with 0.1 ml Ribi-R700 (Ribi Immunchem Research, Inc., Hamilton, MT), or (5) mixed in PBS with 1 μg of recombinant IL-12 (10^3 units/μg; Pharmingen, San Diego, CA). PAR proteins emulsified in FA were checked for the formation of a stable emulsion. Mixtures with alum were tested for adsorption by protein assay to ensure that no detectable protein was present in the supernatant.

Immunization. Six- to eight-week-old female BALB/cByJ mice were immunized by subcutaneous (sc) or intraperitoneal (ip) injection with 40 μg PAR proteins emulsified with Freund's adjuvant. Immunization of mice using adjuvants other than Freund's was always by the subcutaneous route. Mice were boosted at 2-week intervals with 20 μg protein with adjuvant. Control groups were injected with adjuvant plus PBS or PBS only. Two weeks after the last injection, mice were challenged with sc injection of 10^3 bloodstream trypomastigotes. Following challenge, mice were checked daily, and survival was recorded at days

postinfection. Parasitemias were monitored every other day from Day 12 to 28 and weekly from Week 4 to 8.

Measurement of parasitemias. Parasitemia levels were determined as previously described (Wrightsman *et al.* 1982) by removing a blood sample from the tail vein and counting the number of trypomastigotes with a Neubauer hemacytometer.

T cell proliferation assays. Mice were immunized as described above. Seven to ten days after the last injection, spleens and inguinal and axillary lymph nodes were removed and single-cell suspensions were prepared in DMEM supplemented with 25 mM Hepes buffer, 1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, 50 U/ml penicillin, and 50 μg streptomycin sulfate. Spleen cell suspensions were enriched for T cells by passage over nylon wool columns (Julius *et al.* 1973). Accessory cells for antigen presentation were prepared by irradiation (3000 rads of ^{137}Cs) of syngeneic spleen cells. Cultures consisting of 2×10^5 T cell-enriched spleen cells or lymph node cells were incubated in 96-well plates along with 4×10^5 irradiated feeder cells, supplemented as described above, plus 10% FCS. Cells were cultured with and without antigen for 3 days at 37°C in an atmosphere of 5% CO_2. After 3 days, 1 μCi [^{3}H]thymidine was added to each well. After 18 hr of incubation, cells were harvested onto filters using a cell harvester (Skatron Instruments, Inc., Sterling, VA), dried, and counted in a liquid scintillation counter. Single data points in the graphs represent the stimulation index as the mean count per minute of triplicate wells divided by the background value determined from the triplicate control wells without antigen.

Antibody titers and isotype determination. Measurements of anti-PAR antibody titer were performed by enzyme-linked immunosorbent assay (ELISA) precisely as described elsewhere (Wrightsman *et al.* 1995). Antibody isotypes were determined using the same ELISA except that the concentration of PAR antisera used was 1/100 for Freund's (sc), 1/2000 for Freund's (ip), and 1/1500 for alum, RIBI, and QS-21. Isotype-specific peroxidase-conjugated anti-mouse antibodies were used in the recommended range of dilution (Boehringer Mannheim Biochemicals, Indianapolis, IN), 1/4000 for the IgG subclasses and 1/10000 for the IgM isotype-specific antibody.

Measurement of cytokines. Culture supernatants were taken at Days 3, 5, and 7 from triplicate cultures of T cells containing 3 μg of PAR protein and assayed for the presence of IL-2, IL-4, IL-10, and IFN-γ by capture ELISA (Pharmingen, San Diego, CA). Briefly, 1–2 μg/ml cytokine-specific capture antibody was bound to 96-well microtiter plates in 0.1 M NaHCO$_3$, pH 8.2, at 4°C overnight, washed with PBST (PBS + 0.05% Tween 20), and then blocked with 10% FCS in PBS for 2 hr at room temperature. Wells were washed with PBST, 100 μl of either standards or samples was added, and the reaction was incubated at 4°C overnight. Wells were again washed with PBST and the appropriate concentration of biotinylated anti-cytokine-detecting antibody was added in a volume of 100 μl and incubated for 45 min at room temperature. The wells were thoroughly washed, 100 μl of strepavidin–peroxidase (2.5 μg/ml) was added, and the reaction was incubated for 30 min at room temperature. After extensive washes in PBST, 100 μl of 2,2′-azinodi-3-ethylbenzthiazoline sulfonate (Boehringer Mannheim Biochemicals) substrate was added and plates were read at 405 nm in an automated ELISA plate reader. Concentrations were calculated from linear regions of a titration curve of cytokine standards, values for control wells were subtracted, and final concentrations were expressed as either ng/ml or pg/ml.

RESULTS

We have previously shown that immunization of mice with PAR induces an immune response capable of protecting mice against an otherwise lethal inoculum of parasites. To determine the optimum dosage and number of injections required to induce a protective immune response, several immunization amounts and schedules were tested. As shown in Table I, mice immunized with an initial injection of 40 μg of PAR emulsified in complete FA followed by two boost injections of 20 μg PAR emulsified in incomplete FA developed low parasitemias and showed 100% survival after a challenge with 10^3 Peru strain trypomastigotes (50% lethal dose, ≤10). When the immunization schedule was reduced to one initial injection of 40 μg PAR with a single boost of 20 μg PAR, the parasitemia levels were reduced; how-

TABLE I
Protective Immunity by Vaccination with PAR Proteins + Freund's Adjuvant

Dose (μg)	No. of mice immunized	No. of survivors	Mean survival time (days)	Mean parasitemia at Day 19 (10^5) $\pm$ SD
40 + 20 + 20	10	10	>60	15 ± 5
40 + 20	6	2	>60	25 ± 13
40	6	0	19.7 ± 0.8	42 ± 42
20 + 10 + 10	3	0	18.3 ± 1.2	NA

ever, only 33% of mice survived the challenge infection. A single injection of 40 μg of PAR was ineffective in either reducing parasitemia or increasing the mean survival time of mice following challenge infection. Similarly, reducing the amount of PAR in the immunization schedule to 20 μg in the initial injection followed by two boost injections of 10 μg also failed to sufficiently reduce parasitemia or increase survival time. Based upon these results, optimal immunization conditions using Freund's adjuvant are defined as an immunization schedule of three injections with an initial injection of 40 μg PAR followed by two boost injections of 20 μg PAR.

Consistent with previous results (Wrightsman *et al.* 1995), mice receiving subcutaneous injections of PAR with Freund's adjuvant exhibited enhanced survival and lower blood parasitemias than mice immunized via the intraperitoneal route (Table II). To explore factors other than route of immunization which are also extrinsic to the antigen but likewise may influence the nature of the immune response, we compared the level of protective immunity induced by PAR in combination with various adjuvants to that observed with PAR emulsified in Freund's adjuvant. The adjuvants Ribi, QS-21, IL-12, and alum were chosen because of their known effectiveness as potent immunomodula-

tors in other experimental systems (Rawlings and Kaslow 1992; Jiménez *et al.* 1994; Afonso *et al.* 1994). Comparison of parasitemia and survival in mice immunized with PAR + Freund's (sc) to mice immunized with PAR and any of the other four adjuvants (sc) showed Freund's to be the most efficacious of the adjuvants tested. Mice immunized with PAR + Ribi, PAR + QS-21, or PAR + IL-12 showed mean survival times of 20–25 days postchallenge, similar to that observed following immunization with PBS. Immunization with PAR in the absence of adjuvant also showed no increase in mean survival time. In contrast, mice immunized with PAR + alum exhibited a significant increase in survival (83% survival) over that observed for all adjuvant/PAR combinations other than Freund's. Parasitemia in mice immunized with PAR + alum was also substantially less than that observed in PBS-immunized mice. Interestingly, parasitemia values do not necessarily correlate well with survival. Immunization with PAR + Ribi, PAR + QS-21, and PAR + IL-12 all result in peak parasitemia values that are about one-half to one-third of that observed following immunization with the adjuvant alone, and all are about the same as that observed in mice immunized with PAR + alum. Yet, the survival rates between the above three groups and the PAR + alum group are dramati-

TABLE II
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
Freunds, sc	3	0	22.3 ± 2.3	51 ± 27
Freunds + PAR, sc	10	10	>60	15 ± 5
Freunds, ip	8	0	22.9 ± 4.5	35 ± 14
Freunds + PAR, ip	9	0	20.6 ± 1.0	66 ± 23
Ribi, sc	3	0	18.7 ± 0.6	66
Ribi + PAR, sc	3	0	25 ± 6.9	37 ± 11
QS-21, sc	3	0	19	46 ± 29
QS-21 + PAR, sc	3	0	22.7 ± 0.6	27 ± 9
Alum, sc	3	0	18	NA
Alum + PAR, sc	6	5	>60	26 ± 12
IL-12, sc	3	0	19.3 ± 0.6	74
IL-12 + PAR, sc	3	0	19.7 ± 0.6	24 ± 1
PBS, sc	10	0	20.7 ± 1.7	45 ± 10
PBS + PAR, sc	3	0	17.7 ± 1.2	NA

cally different. Some differences were noted, however, between those mice immunized with PAR + alum and those immunized with PAR + Freund's. While the feeding habits and general outward appearance of the Freund's-immunized mice could not be distinguished from uninfected control mice, the alum-immunized mice showed some signs of distress. During the periods of peak parasitemia, generally Days 18–20 postchallenge, the mice were slightly scruffy in appearance and more lethargic than either the uninfected control mice or the Freund's-immunized mice. After this period, however, the habits and appearance of the alum survivors returned to that of the control mice.

In order to assess the antibody responses elicited by immunization with PAR, groups of three mice were bled from the tail vein, their sera were pooled, and antibody titers against PAR were determined by ELISA for each group. While all immunizations generated measurable PAR-specific antibody responses, the level of PAR-specific antibodies was found to be route and adjuvant dependent (Fig. 1). Consistent with our previous results (Wrightsman *et al.*

1995), a dramatic difference was observed between the titer in the Freund's (sc) group and that in the Freund's (ip) group, with the latter having a 30-fold higher titer. The titer of the Freund's (sc) group was also conspicuously lower than the titer of the alum, QS21, and Ribi groups by factors of 50, 75, and 125, respectively. Since immunization using either alum or Freund's (sc) as the immunomodulator resulted in quite different levels of circulating PAR-specific antibody, yet both provided substantial protection against a *T. cruzi* challenge, it is evident that the magnitude of the antibody response to PAR is not a positive predictor of protective immunity. To investigate the possibility that isotype-specific functional differences in the PAR-specific antibodies might provide some insight into the nature of the protective response, the relative amounts of each IgG subclass and IgM present in the pooled sera of each immunization group was measured by ELISA using isotype-specific secondary antibodies (Fig. 2). Freund's (sc) immunization elicits an IgG1-dominated response with barely detectable levels of IgG2a, IgG2b, and IgG3
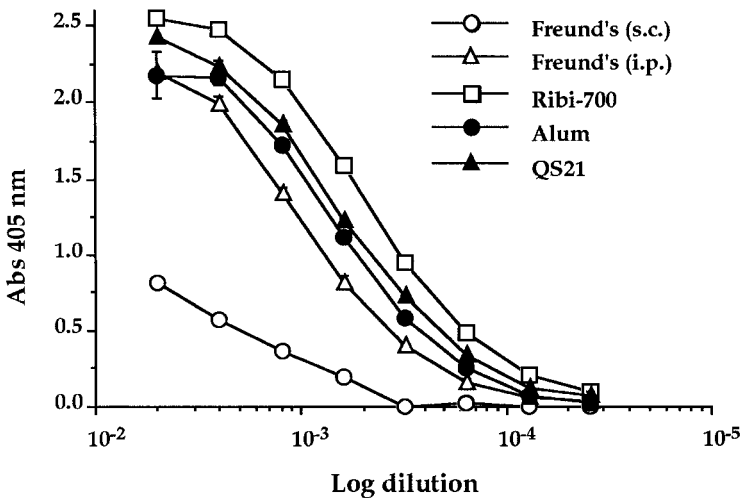

FIG. 1. Effect of different adjuvants on PAR-specific antibody titers. Groups of three BALB/c mice were immunized sc with PAR + adjuvant. Mice were immunized with an initial injection of 40 μg PAR + adjuvant, followed by two injections of 20 μg PAR + adjuvant at 2-week intervals. Sera were collected 10 days after the final injection, pooled, and tested for antibody response to PAR by ELISA. Each data point represents the mean value of duplicate samples. In immunizations with Freund's adjuvant, the initial injection contained PAR + Freund's complete adjuvant, and incomplete Freund's adjuvant was used in each of the two subsequent injections.
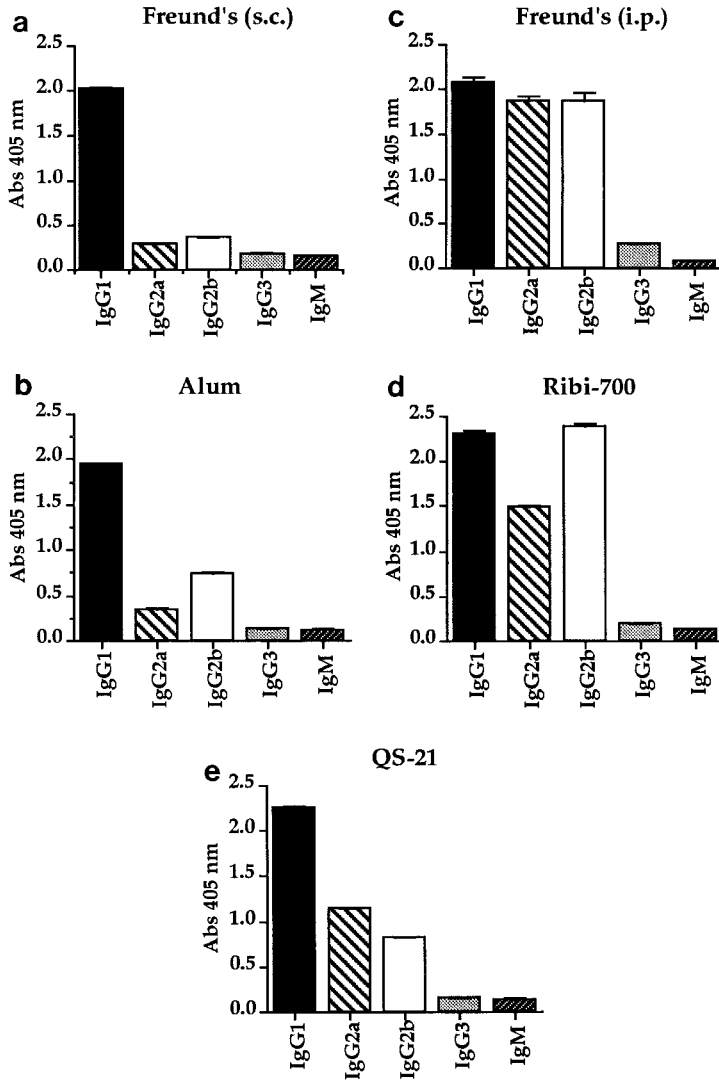
FIG. 2. Effect of different adjuvants on anti-PAR antibody isotype responses. Anti-PAR antibody isotypes of BALB/c mice, immunized as described for Fig. 1, were determined using an isotype-specific ELISA for the IgG subclasses and IgM. Dilutions of each sera pool were chosen to give an OD_{405} of approximately 2.0 for IgG_1.

being observed. In contrast, the isotype of the Freund's (ip) group clearly is switched with nearly equivalent levels of IgG1, IgG2a, and IgG2b being present along with a modest amount of IgG3. The different adjuvant groups varied in their isotype profiles with the alum group being most similar to Freund's (sc) in that IgG1 was the major isotype; however, the amount of IgG2b was measurably higher than

that observed in the Freund's (sc) group. In contrast, the Ribi and QS21 groups more closely resemble the Freund's (ip) profile with Ribi sera having high levels of IgG1, IgG2a, and IgG2b, and QS21 sera having high levels of IgG1 and IgG2a but only moderate levels of IgG2b. IgM could not be detected in any of the different groups. Based upon these results, it is difficult to ascribe any particular class of isotype as be-

ing closely identified with a protective immune response. While the alum and Freund's (sc) groups, both of which exhibit a protective response, share the distinction of having IgG1 as the dominant isotype, it is also apparent that high levels of antibodies of this class of isotype are also present in those experimental groups (i.e., Ribi, QS-21, and Freund's ip) which do not mount a protective response.

The above studies clearly show that neither antibody titer nor antibody isotype correlate with the protective immune response induced by immunization with PAR. These observations lead to the speculation that the protective response elicited by immunization with PAR is more closely associated with a cell-mediated than with a humoral immune response. To examine this possibility, the ability of T cells from PAR-immunized mice to recognize and respond to PAR proteins presented *in vitro* was investigated. T cells from spleens and lymph nodes of mice immunized with PAR in combination with various adjuvants were isolated and tested for their proliferative responses to PAR *in vitro* (Fig. 3). Interestingly, mice immunized with PAR + Freund's (ip) or PAR combined with Ribi generated T cells which demonstrated positive proliferative responses, yet both immunization regimens failed to provide protection against challenge infection. Likewise, immunization with PAR combined with alum or emulsified in Freund's (sc) also generated T cells which developed substantial T-cell-proliferative responses, yet both immunization regimens were highly effective in providing protection to challenge infections. Similar results were observed using T cells isolated from lymph nodes (data not shown).

If cell-mediated immunity is involved in the PAR-mediated protective immune response, then the observation that both protective and nonprotective immunization regimens generate positive T cell responses clearly suggests that the responses induced by these regimens may differ. To explore this possibility, the T-helper response in mice immunized with PAR using the protocols presented above was investigated by measuring the *in vitro* levels of the cytokines
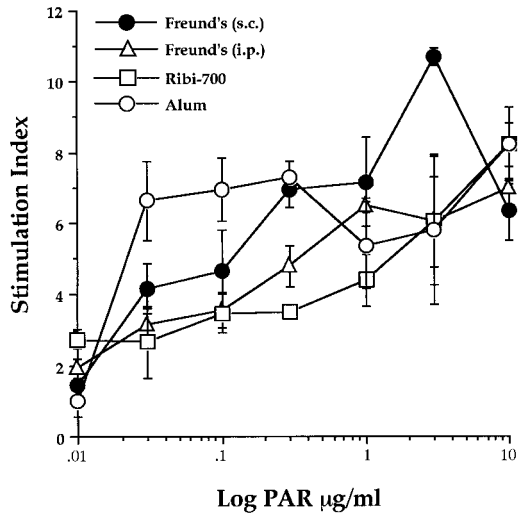

FIG. 3. Effect of immunization with PAR + different adjuvants on antigen-specific proliferative responses. BALB/c mice were immunized and boosted as described for Fig. 1. Ten days after the last boost, nylon wool nonadherent spleen cells were isolated and cultivated with various concentrations of PAR. After 3 days in culture, cells were grown an additional 18 hr in the presence of [^{3}H]thymidine. Each data point represents the stimulation index measured as the mean cpm of triplicate wells divided by the mean value of triplicate control wells without antigen.

IL-2, IFN-γ, IL-4, and IL-10 by capture ELISA in the culture supernatants of PAR-primed T cells stimulated *in vitro* with PAR protein (Fig. 4). Of the four groups tested, T cells from Freund's (sc)-immunized mice produced the highest levels of IFN-γ in response to PAR antigen. T cells from the other three groups also produced IFN-γ; however, the amounts were substantially less than that observed for the Freund's (sc) group. The next highest level of IFN-γ was produced by the alum group, about 25% of that observed for the Freund's (sc) group, and the lowest level of IFN-γ was observed in the Freund's (ip) group, about 10% of the Freund's (sc) group. IL-2 was detected in all groups with the resistant Freund's (sc) and alum groups having higher levels of IL-2 then the susceptible Freund's (ip) and Ribi groups (Fig. 4b). T cells from both groups of susceptible mice, Freund's (ip) and Ribi, responded to PAR stimulation by producing IL-4, contrasting with the complete absence of detectable IL-4 produc-
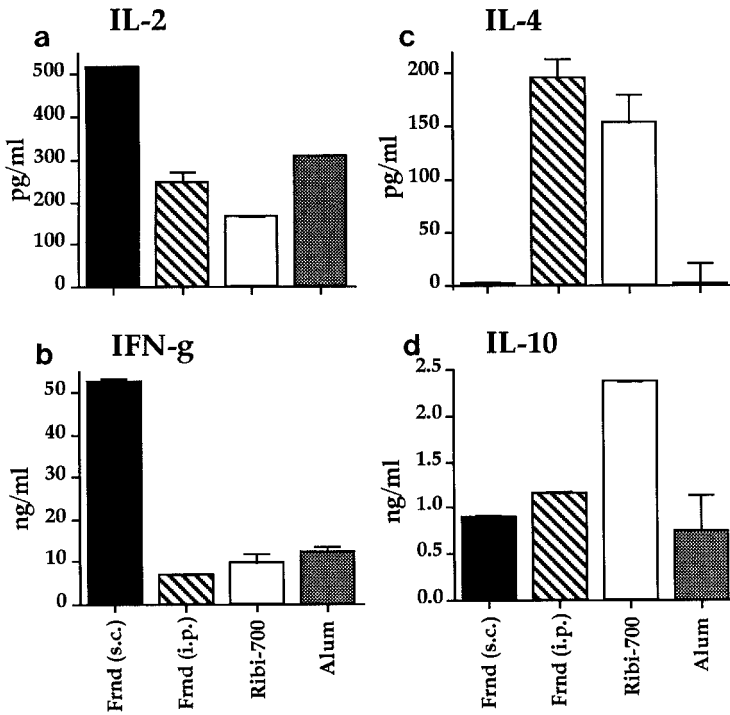
FIG. 4. Effect of immunization with PAR + different adjuvants on cytokine responses. Nylon wool nonadherent spleen cells from mice immunized and boosted as described for Fig. 1 were cultured in the presence of 3 μg/ml PAR. Supernatants were collected on Days 3, 5, and 7 following restimulation and were assayed by capture ELISA for IL-2, IL-4, IL-10, and IFN-γ as described under Materials and Methods. Maximum values for IL-2, IL-4, IL-10, and IFN-γ were observed on Days 3, 5, 5, and 7, respectively.

tion by T cells in the Freund's (sc) and alum mice (Fig. 4c). The presence of IL-10 could be detected in all of the groups with Ribi producing the highest levels of IL-10 and alum the lowest, while Freund's (sc) and Freund's (ip) both produced moderate amounts of this cytokine (Fig. 4d).

DISCUSSION

One of the main objectives of this study was to examine the ability of different classes of adjuvants + PAR to induce a protective immune response in mice against an otherwise lethal challenge with *T. cruzi*. Freund's complete adjuvant has been shown to be highly effective in inducing a protective response with PAR; however, this adjuvant is not suitable for human use. Therefore, we chose to evaluate several other adjuvants which are either licensed for use in

humans (i.e., aluminum hydroxide) or are being extensively tested for use in humans (i.e., Ribi MPL + TDM, QS-21, IL-12). Of the four adjuvants tested, only immunization with alum + PAR was found to provide significant survival of mice against a lethal *T. cruzi* challenge. Although the extent of protection (i.e., 83%) was not as high as that observed with Freund's (i.e., 100%), the fact that significant protection was observed encourages additional testing with alum using different immunization regimens, possibly in combination with other immunomodulators.

At present, the nature of immune responses which provide protection against a *T. cruzi* challenge is not well defined. Thus, a second objective was to identify those immune responses which are uniquely associated with PAR-mediated protection by comparing the immunostimulatory properties of protective and nonpro-

tective PAR immunization regimens. The immune responses which were chosen for examination are those which have been shown in previous studies to influence the severity of a *T. cruzi* infection in mice. Several studies have indicated that antibody titer, isotype, and specificity (Takahara *et al.* 1981, 1989) or a combination of these factors are important in resistance to *T. cruzi* infection. In examining the anti-PAR serum antibody titers in the different groups, it is obvious that the level of circulating antibody does not correlate with protection. The titers of anti-PAR serum antibodies in the Freund's sc and alum groups differ by a factor of 50, yet mice in both groups show significant survival. In contrast, mice in the Freund's ip, QS-21, and Ribi groups have anti-PAR titers that are very similar to that of the alum group, yet no survival was observed in any of these three groups. It is possible, however, that antibody isotype rather than quantity may be important for host survival. Previous studies have suggested that anti-parasite IgG2b may be important in resistance to *T. cruzi* infection in mice (Takehara *et al.* 1981; Rowland *et al.* 1992). However, our studies show that anti-PAR IgG2b levels are highest in those groups which show no survival. It has also been observed that anti-*T. cruzi* IgG1 levels are higher in some susceptible strains of inbred mice than in some resistant strains (Powell and Wassom, 1994), and it has been proposed that anti-parasite IgG1 actually may be beneficial to the parasite (Rowland *et al.* 1992). The studies presented in Fig. 2 show that all of the groups contain high levels of anti-PAR IgG1, indicating that this isotype does not selectively play an important role in the PAR-mediated protective response. The only correlation that can be seen between isotype level and protection is that with IgG2a. In the two protected groups, the level of IgG2a is substantially less than that seen in any of the three nonprotected groups. Interestingly, Powell and Wassom 1994 have reported that mice which share the susceptible C3H background produce significantly higher levels of anti-*T. cruzi* class IgG2a antibodies than mice which express the resistant B10 background. Taken together, these results suggest that high levels of parasite specific IgG2a may be a signature for predicting the severity of a *T. cruzi* infection in mice.

The lack of correlation between a PAR-mediated protective immune response and either antibodies titer or isotype, taken together with our previous observation that anti-PAR antibodies do not bind live parasites (Wrightsman *et al.* 1994), strongly suggests that the nature of the protective immune response is cell mediated rather than humoral. That this is the case has been confirmed by the observation that genetically altered mice which lack the heavy chain of IgM, and thus are generally antibody deficient, show 100% survival against a *T. cruzi* challenge when immunized with Freund's (sc) + PAR (Miller and Manning, manuscript in preparation). In view of these results, and since the involvement of T cell function in resistance to *T. cruzi* has been demonstrated by several laboratories, we examined whether splenic and lymph node T lymphocytes from Freund's sc-immunized mice respond to purified PAR *in vitro* (Fig. 3). The observation that all of the immunization regimens with PAR were capable of producing a PAR-specific T cell response (Fig. 3) indicates that the ability to induce a T cell response is not sufficient to elicit protection, thus leading to the suggestion that the nature of the T cells response may be important. We, therefore, examined the production of cytokines associated with Th1-type (i.e., IFN-γ and IL-2) and Th2-type (i.e., IL-4 and IL-10) responses in T cells from mice immunized with either a protective regimen or a nonprotective regimen. T cells from all of the immunization regimens examined produced IL-2, IFN-γ, and IL-10. T cells from the nonprotected groups responded to PAR *in vitro* by producing IL-4, the signature cytokine in the Th2-type response, while IL-4 was undetectable in the protected groups. The group which showed the highest level of protection, Freund's sc, also exhibited the highest levels of IFN-γ and IL-2, cytokines which are characteristic of a Th1 response. IL-10 was produced by all groups; however, IL-10 levels were highest in the nonprotected groups.

This observation also is consistent with the relatively low levels of IFN-γ observed in the nonprotected groups, since IL-10 has been shown to be an antagonist of IFN-γ (Silva *et al.* 1992). This correlation between cytokine profiles and level of infection is in keeping with previous reports that the Th2-associated cytokines IL-4 and IL-10 are associated with a higher susceptibility to infection (Silva *et al.* 1991; Gazzinelli *et al.* 1992; Rottenberg *et al.* 1995), while the Th1-associated cytokine IFN-γ plays a positive role in resistance to *T. cruzi,* possibly by activation of macrophages (Petray *et al.* 1993; McCabe *et al.* 1991; Silva *et al.* 1992) and release of NO (Munoz-Fernandez *et al.* 1992; Gazzinelli *et al.* 1992). While these results indicate that the nonprotected groups show a mixed Th1/Th2 response, and the protected groups primarily exhibit a Th1-type response, it is surprising that the high level of IFN-γ observed with PAR + Freund's (sc) is associated with an isotype profile that shows low levels of IgG2a and high levels of IgG1. These results are exactly opposite of what might be anticipated, since IL-4 primes mouse B lymphocytes for switching to IgG1 while IFN-γ induces IgG2a switching. It is not clear why our observations are not in agreement with this general paradigm. However, since cytokines can have different effects on the regulation of IgG class switching and expression in the presence of different costimuli, it is possible that PAR may be acting as, or influencing the activity of, such costimuli. It is also important to recognize that while cytokines can influence the control of class switching, alternative mechanisms exist for stimulating class switching. In the case of IgG1, class switching to IgG1 can clearly occur in the absence of IL-4, since genetically altered mice which lack a functional IL-4 gene can generate an acute IgG1 response (Kuhn *et al.* 1991).

A Th1-type immune response has been associated with protection against several infectious agents. The cytokine IL-12 is known to be a potent inducer of Th1-type responses, and in several disease models, both protective responses and Th1 signature cytokines have been enhanced by administration of IL-12 + antigen (Miller *et al.* 1995; Wynn *et al.* 1994; Afonso *et al.* 1994). It is interesting to note that while our results indicate that a Th1 response is associated with PAR-mediated protective immunization against *T. cruzi* infection, use of IL-12 as an adjuvant did not ensure survival of PAR + IL-12-immunized mice. In our studies, the mouse strain, route of immunization, and quantity of IL-12 used in the immunization protocol was similar to that used in other studies (Afonso *et al.* 1994), yet no protection against a *T. cruzi* challenge was observed. Therefore, it seems likely that while a Th1-type response might be associated with survival, it is not sufficient to ensure survival and other immune mechanisms also must be activated.

In summary, immunization of mice with PAR using the adjuvants Freund's (sc) or alum (sc) induces an immune response that provides significant protection against an otherwise lethal challenge with *T. cruzi*. The nature of the immune response appears to be cell mediated, with PAR-specific antibody playing no detectable role in the protective response. The protective response is more closely associated with a Th1 than with a Th2 cytokine profile.

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