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Identification of Leishmanial Antigens in the Sera of Patients with American Visceral Leishmaniasis

THOMAS G. EVANS AND RICHARD D. PEARSON*

Division of Geographic Medicine, Department of Internal Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22908

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Circulating immune complexes are present in the sera of patients with visceral leishmaniasis caused by *Leishmania donovani chagasi*. In order to determine whether these complexes contain parasite antigens, sera were collected from Brazilian patients with visceral leishmaniasis and from hospitalized control subjects with other diagnoses. High-molecular-weight complexes were precipitated from pooled sera with 2.5% polyethylene glycol. Approximately 140-fold-more protein was precipitated from patient sera than from control sera; 12% of the total patient serum protein was precipitated. Patient serum precipitates contained immunoglobulins G (525 mg/dl), M (27 mg/dl), and A (8 mg/dl). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the patient serum precipitates revealed multiple bands, including a prominent band at 70 kilodaltons, that were not seen in precipitates of control sera. The 70-kilodalton band was recognized by human and hamster sera with antileishmanial antibodies, but not by control sera. Finally, immunization of BALB/c mice with the high-molecular-weight precipitates from patients elicited antileishmanial antibodies against *L. donovani chagasi* antigens as detected by enzyme-linked immunosorbent assay and Western blot (immunoblot) assay. In summary, sera of patients with American visceral leishmaniasis were found to have high-molecular-weight complexes that contained one or more parasite antigens. These complexes may play a role in the immunology of the disease, and detection of circulating parasite antigens has potential diagnostic importance.

American visceral leishmaniasis (AVL) is caused by the protozoan *Leishmania donovani*, which resides and multiplies within mononuclear phagocytes in humans (R. D. Pearson and A. Q. Sousa, *in* G. L. Mandell, R. G. Douglas, Jr., and J. E. Bennett, ed., *Principles and Practice of Infectious Diseases*, 3rd ed., in press). Progressive disease is characterized by hepatosplenomegaly, fever, wasting, hypergammaglobulinemia, and pancytopenia (6). Massive numbers of parasites are present throughout the reticuloendothelial system. The immune response is characterized by T-cell anergy to leishmanial antigens and, paradoxically, high levels of parasite-specific and non-parasite-directed antibodies. The latter are due at least in part to polyclonal B-cell activation (8). Circulating immune complexes as measured by C1q binding assay, polyethylene glycol (PEG) precipitation with radiolabeled staphylococcal protein A, or cryoglobulinemia are present in the majority of patients (4, 5, 7, 10, 11, 17, 18, 20, 22).

Although immune complexes in AVL have not been fully characterized, it is reasonable to hypothesize that they contain parasite antigens and host immunoglobulins (12). The identification of circulating parasite antigens could have potential diagnostic and prognostic implications. Furthermore, circulating parasite antigens, corresponding antileishmanial antibodies, and possibly resulting anti-idiotype antibodies might contribute to the development of antigen-specific T-cell unresponsiveness, as is the case in *Schistosoma japonicum* infection (16). The principal objective of this study was to determine and characterize the presence of circulating leishmanial antigens in patients with AVL.

MATERIALS AND METHODS

Parasites. The strain of parasite used in this study was derived from a bone marrow aspirate of a patient with AVL in the state of Ceara, Brazil. It was identified as *L. donovani chagasi* (MHOM/BR/86/L669) by both isoenzyme analysis and kDNA hybridization. The isolate was serially passaged by intracardiac injection of amastigotes into Syrian hamsters. Promastigotes were isolated from splenic amastigotes as previously described (19) and grown in HO-minimal essential medium supplemented with fetal calf serum (FCS), glutamine, penicillin (100 U/ml), and gentamicin (50 μg/ml) (19). Promastigotes were used in the late logarithmic phase of growth.

Sera. Sera from eight Brazilian patients with parasitologically confirmed AVL were collected in the state of Ceara, stored in liquid nitrogen, and transported to the United States in liquid nitrogen. Six antibody-negative hospitalized Brazilian patients of similar ages, but with other diagnoses, were used as controls. Additional control serum was obtained from a North American with no known exposure to *L. donovani chagasi*. Convalescent-phase serum was obtained from a separate Brazilian patient with biopsy-proven AVL 2 months after he had begun clinically successful chemotherapy with meglumine antimonate (Glucantime). Sera with high-titer antileishmanial antibody were also obtained from infected hamsters with advanced *L. donovani chagasi* infections. Rheumatoid factors were measured by the standard latex agglutination assay (Behring RapiTex RF Kit).

Immune complexes. Sera from the eight patients or six Brazilian controls were pooled (15 ml total of each) and precipitated on ice in PEG (Sigma Chemical Co.; molecular weight, 8,000; final concentration, 2.5%) for 2.5 h. The precipitates were pelleted at 2,500 × *g* for 20 min and washed twice in a final concentration of 2.5% PEG. The precipitates were then dialyzed against four 2-liter changes of phosphate-buffered saline (PBS) (pH 7.2) (GIBCO Laboratories) over 24 h. The patient PEG precipitate (PEG-IC) was further purified by adsorption onto staphylococcal protein A (IgSorb; Enzyme Co.) on ice for 1.5 h. The protein

* Corresponding author.

A-bound material was pelleted at 1,000 × *g* for 10 min, and the unbound supernatant was stored. The protein A-bound pellet was washed three times in PBS, and the adsorbed material was removed by two methods: (i) elution in 0.5 M, pH 3, 0.9 M salt-acetic acid solution or (ii) boiling for 5 min in a nonreducing sodium dodecyl sulfate (SDS) solution containing 0.1 M Tris (pH 6.8). The two methods gave similar results. The protein content at each step was measured using a modification of the method of Lowry et al. (14) (Pierce BCA Reagents). The amounts of immunoglobulin G (IgG), IgM, and IgA were determined by rate nepholemetry (Beckman Array and Beckman reagents).

ELISA protocol. An enzyme-linked immunosorbent assay (ELISA) was used to detect possible leishmanial antigens in the PEG-ICs. Fifty-microliter portions of PEG-IC from patients or controls were placed in wells of a 96-well ELISA plate (Immulon II; Dynatech Laboratories, Inc.) at a protein concentration of 1 μg per well in a bicarbonate buffer (pH 9.6) and left overnight at 4°C. The plates were then aspirated and blocked for 2 h in PBS–1.5% FCS, washed three times in this solution, and incubated for 1 h at 37°C with 50 μl of serial dilutions of the immune human or hamster serum (both had antileishmanial antibody titers of greater than 1/50,000 [see below]). The plates were washed in PBS–0.05% Tween three times and then incubated for 45 min at 37°C with 50 μl of 1:1,000 goat anti-human or anti-hamster peroxidase-conjugated antibody (Sigma). After three washes, the substrate 2-azinodio-3-ethylbenzthiazoline was added in a citrate-bicarbonate-hydrogen peroxide buffer. After 30 min, the A_{414} was read on a Dynatech ELISA Multiskan Reader.

Antileishmanial antibodies in the sera of humans, hamsters, and mice were also identified and measured by ELISA. *L. donovani chagasi* promastigotes were harvested in the late logarithmic phase, washed three times in PBS, and adjusted to 2×10^7 in pH 9.6 buffer. Fifty microliters of this solution was placed in each well and incubated for 24 h. The remainder of the procedure followed the protocol outlined above. Dilutions of the first antibody were made in all cases in PBS–1.5% FCS.

Immunoblot protocol. To further characterize the precipitates, control PEG-IC, patient PEG-IC, protein A eluate, and protein A supernatants were boiled for 5 min in Laemmli reducing buffer (13), and 20 μg of each was added to lanes of a 1.5-mm SDS–10% polyacrylamide gel and run with water cooling at 30 mA per gel for approximately 4 h. The gels were then transferred onto 0.1-μm nitrocellulose filters at 150 mA per gel for 6 h. The efficiency of transfer was documented by Coomassie blue staining of the gels. Nonspecific staining of the transferred protein was accomplished with amido black. The filters were blocked in 4% dry milk, incubated with 1:1,000 dilutions of control or convalescent-phase human or hamster sera for 2 h at 37°C, washed three times in PBS, incubated with a 1:1,000 dilution of peroxidase-labeled goat anti-human or goat anti-hamster IgG antibody (Sigma), and developed with 3,3′-diaminobenzidine in 0.05 M Tris buffer (pH 7.2).

To characterize antileishmanial antibody responses in immunized mice, promastigotes were prepared by being washed three times in Hanks balanced salt solution at 4°C. Antipain and leupeptin (Sigma, 20 μg/ml each) were added to 2×10^7 promastigotes per ml. The organisms were then boiled in Laemmli reducing buffer, electrophoresed at 30 μg per lane on SDS–10% polyacrylamide gels, and blotted onto 0.1-μm nitrocellulose filters. The filters were cut into 5-mm strips, blocked in 5% dry milk for 3 h, incubated with 1:50 dilutions of the mouse serum to be tested, washed three times in PBS, and then processed in the manner described above. The molecular weights of major staining bands were calculated by semilogarithmic graphing with molecular weight standards (Sigma) as references.

TABLE 1. PEG precipitation of sera

Sera	Initial protein (g/dl)	PEG-IC (mg/ml)	Total protein (%)
Patient	9.5	11.5	12
Control	7.3	0.08	0.11

Immunization of BALB/c mice. BALB/c mice (Charles River Breeding Laboratories) were immunized with patient PEG-IC or with the protein A eluate of patient PEG-IC as follows. Protein (1 mg) from each preparation was emulsified in complete Freund adjuvant and injected into mice on day zero; one-half was injected subcutaneously into the nape of the neck, and one-half was given intraperitoneally. At 4 and 8 weeks, mice were boosted with injections of the same antigen prepared in incomplete Freund adjuvant. ELISA of sera from the PEG-IC-immunized mice at 11 weeks revealed low titers of antileishmanial antibodies. The animals were then boosted at 15 and 18 weeks with the antigen in complete Freund adjuvant and at 21 weeks with antigen in incomplete adjuvant. At 23 weeks, antibody responses were assessed by ELISA and Western blot (immunoblot) analysis as described above. Antibodies against FCS, Freund adjuvant, and boiled protein A were also measured by ELISA. Each of these antigens was allowed to bind to ELISA plates at 1 μg per well and then was exposed to serum from the immunized mice to determine whether detectable antibodies to them had arisen.

RESULTS

PEG precipitation of high-molecular-weight complexes resulted in precipitation of 140-fold-more protein from patient sera than from control sera; approximately 12% of the total patient sera was precipitated (Table 1). Analysis of the immunoglobulins in the patient PEG-IC revealed large amounts of IgG, IgM, and IgA in percentages similar to those found in nonprecipitated sera (Table 2).

Hamster serum containing high-titer antileishmanial antibody reacted strongly with the patient PEG-IC but not with controls; the geometric mean titer by ELISA against patient PEG-IC was approximately 1/50,000, compared with a titer of 1/500 against control PEG-IC (Fig. 1). The titer by ELISA against the PEG-IC of serum from a patient 21 days after initiation of therapy gave an intermediate titer. These findings could not be attributed to hamster rheumatoid factors, as the latex agglutination test was negative at dilutions greater than 1:10. The possibility that patient PEG-IC contained rheumatoid factors that bound hamster immunoglobulins could not be ruled out.

TABLE 2. Immunoglobulin concentrations in patient sera and PEG-IC

Immunoglobulin	Concn[a] (%) in:	
	Sera	PEG-IC
IgG	5,280 (55)	525 (46)
IgM	260 (2.7)	27.5 (2.4)
IgA	161 (1.7)	8.08 (0.7)

[a] Milligrams per deciliter.

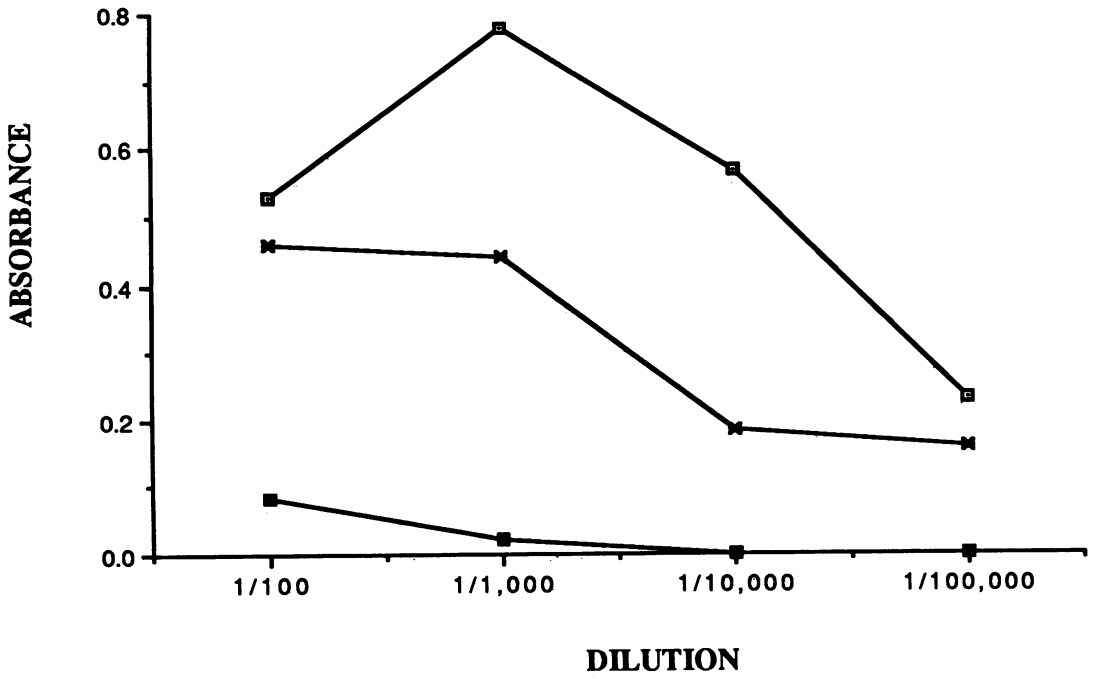

FIG. 1. ELISA of hamster immune sera versus control and patient PEG-ICs. In each instance antigen was bound to the wells at a concentration of 1 μg per well. PEG-ICs were from pooled patient sera (⊡), serum from a single patient after 21 days of therapy with pentavalent antimony (×), and control sera (■).

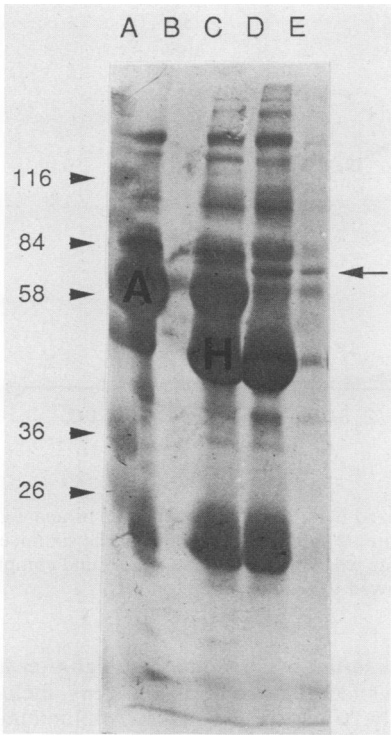

FIG. 2. SDS–10% polyacrylamide gel electrophoresis of PEG-ICs from patient and control sera transferred to nitrocellulose and stained with amido black. Lanes: A, control sera; B, PEG-IC of control sera; C, AVL sera; D, PEG-IC of AVL sera; and E, nonadsorbed supernatant of protein A. A, Albumin; H, immunoglobulin heavy chain; arrow, ~70-kDA protein of interest. Numbers indicate molecular weight standards (in thousands).

Proteins contained in the patient and control PEG-IC as identified by either Coomassie blue staining of the gel or amido black staining of the transferred material are presented in Fig. 2. Control sera (Fig. 2, lane A) had a typical

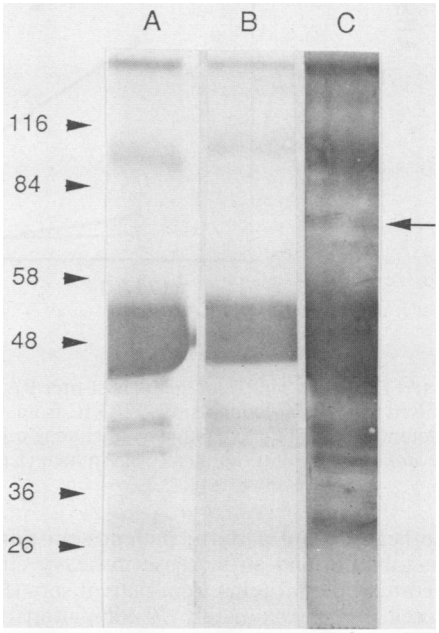

FIG. 3. SDS-polyacrylamide gel electrophoresis of PEG-ICs of patient sera transferred to nitrocellulose and developed with second antibody alone (lane A), with control human sera (lane B), or with convalescent human sera (lane C). Arrow, ~70-kDa band unique to convalescent human sera. Numbers indicate molecular weight standards (in thousands).

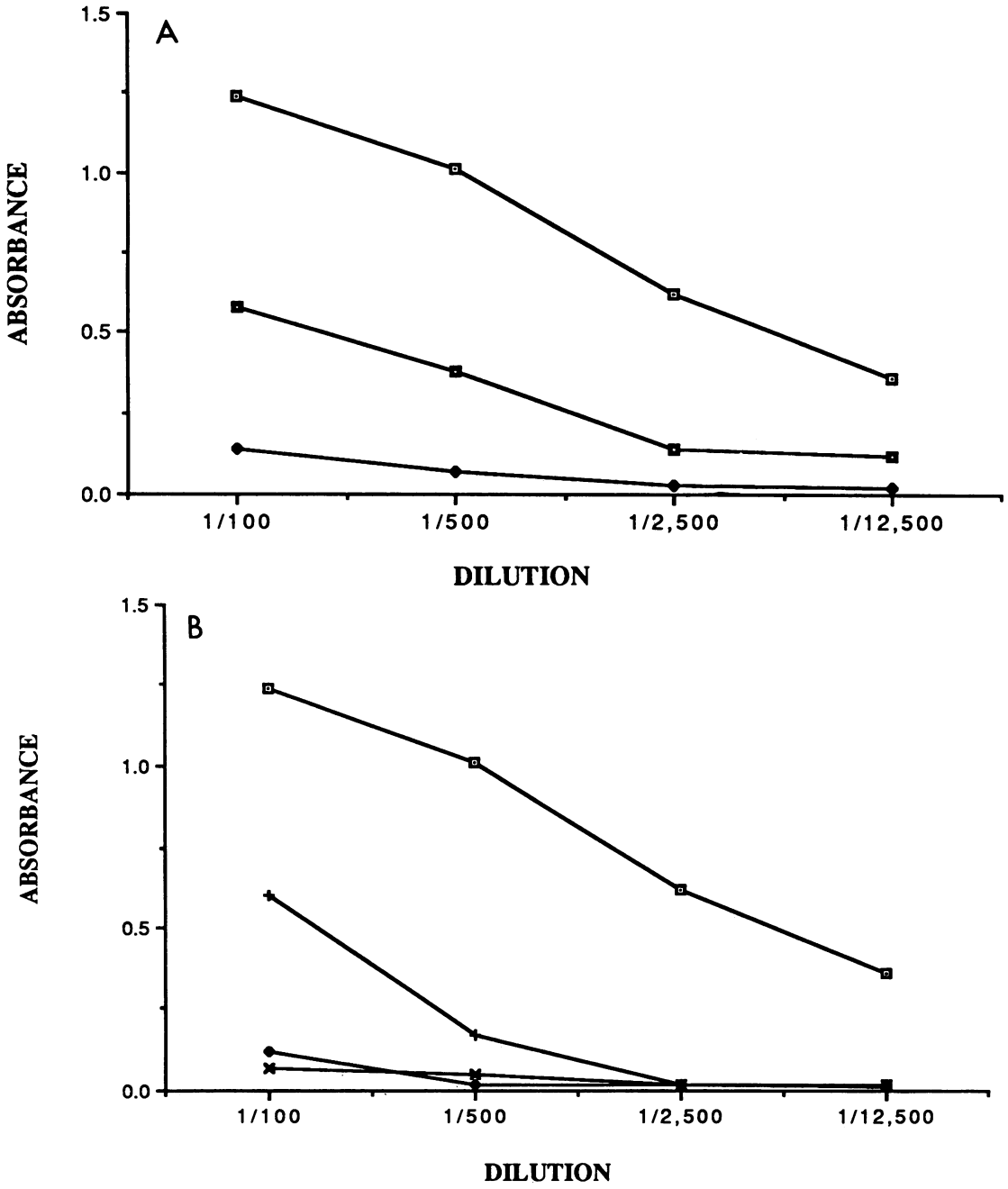

FIG. 4. (A) ELISA of sera from the highest-titer BALB/c mouse repeatedly immunized with PEG-IC. Leishmania promastigotes (10^6 per well) were used as antigen. Symbols: ⊡, PEG-IC from AVL patients; □, protein A eluate; ●, preimmune sera from the six mice. (B) ELISA assessing potential cross-reactive antigens, including protein A (●) from which the eluate was obtained, FCS (×), Freund complete adjuvant (+), and *L. donovani chagasi* antigen (⊡). Antigen (1 μg of protein per well) was allowed to bind overnight at 4°C.

albumin-to-heavy-chain pattern; patient sera (lane C) contained less albumin and an increase in heavy chains. PEG precipitated multiple proteins from patient sera (Fig. 2, lane D). A protein of approximately 70 kilodaltons (kDa) was accentuated in comparison with other proteins. When the material was adsorbed with staphylococcal protein A, the 70-kDa antigen remained in the supernatant (Fig. 2, lane E), whereas the majority of immunoglobulin remained bound to protein A.

Immunoblots of the patient PEG-IC with convalescent-phase human serum which contained high-titer antileishmanial antibodies identified multiple antigens, including one of approximately 70 kDa (Fig. 3, lane C). Nonimmune human serum did not recognize this band (Fig. 3, lane B). The bands recognized by nonimmune sera reflect merely the binding of the serum antibody to immunoglobulin in the electrophoresed complexes (Fig. 3, lane A). The same was true for hamster immune and control sera (data not shown).

BALB/c mice were then immunized with the PEG-IC (two mice) and protein A eluate (two mice) from patients to

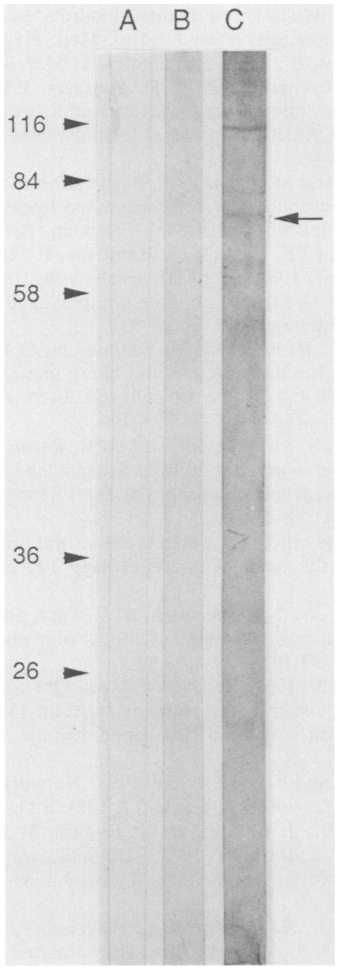

FIG. 5. Immunoblot of sera from mice immunized with PEG-IC against *L. donovani chagasi* antigen. Lanes: A, no first antibody; B, preimmunization; and C, immunization with patient PEG-IC.

determine whether they would elicit antileishmanial antibodies. At 24 weeks, mice immunized with patient PEG-IC or the protein A eluate had evidence of antileishmanial antibodies by ELISA (Fig. 4). The response to the PEG-IC was greater than that to the protein A eluate. The antibody responses of immunized mice to FCS, which is in the promastigote medium, to protein A boiled in SDS, and to Freund adjuvant are shown in Fig. 4B. Only against Freund adjuvant was there a detectable antibody response, and it was of lower magnitude than that to *L. donovani chagasi* antigen. Immunoblots of sera from mice immunized with patient PEG-IC against electrophoresed *L. donovani chagasi* identified multiple parasite antigens, including one of 70 kDa (Fig. 5).

DISCUSSION

Circulating immune complexes are present in most, if not all, persons with AVL. The complexes have been identified in previous studies by C1q binding assay, by measurement of cryoglobulins, and recently by binding of radiolabeled staphylococcal protein A to PEG-ICs. Although clinically significant immune complex glomerulonephropathy is rarely observed in AVL, immune complexes have been found in the kidneys of patients (3, 23) as well as in animals experimentally infected with *L. donovani*. Immunofluorescence studies of human biopsies have indicated that they contain IgG, IgM, IgA, complement, and fibronectin. In only one study of experimentally infected hamsters (21) have parasite antigens been identified in glomerular lesions, and then deposits of fluorescein-labeled polyclonal antileishmanial antibodies were detected only after prolonged incubation with kidney sections.

The goal of the current study was to determine whether parasite antigens circulate in patients with AVL. Attention was directed toward identifying parasite antigens in complexes precipitated by 2.5% PEG. More than 140-fold-more protein was precipitated with 2.5% PEG from patient sera than from control sera. IgG, IgM, and IgA were identified in the patient PEG-IC. Sera from leishmania-infected hamsters bound to the patient PEG-IC as measured by ELISA. Many proteins precipitated by PEG from patient sera were not seen in precipitates of controls, including one protein of approximately 70 kDa which was not adsorbed to protein A. Sera from previously infected humans recognized multiple proteins in immunoblots of the patient PEG-IC, including one protein of 70 kDa, that were not recognized by nonimmune serum.

BALB/c mice were subsequently immunized with patient high-molecular-weight complexes to determine whether antigens in the precipitate were capable of eliciting antileishmanial antibodies. The patient precipitate elicited antileishmanial antibodies as measured by ELISA. Multiple parasite antigens were identified by sera from immunized mice on immunoblots, including one antigen of approximately 70 kDa. There was no evidence of cross-reacting antibodies to protein A or FCS. However, there were antibodies to Freund adjuvant, but they were of lower magnitude and were identified only at lower dilutions of serum. Previous reports have suggested that *Leishmania* and *Mycobacterium* species share some cross-reacting epitopes (15).

The fact that antibodies that recognize *L. donovani chagasi* arise from immunization with the complexes does not in and of itself prove the existence of leishmanial antigens in the complexes. Instead, immunoglubulins in the complexes could be of an anti-idiotypic nature that share antigenic similarity with immunizing leishmanial antigens. Such antibodies, when injected into mice, could give rise to anti-antidiotypic antibodies which could bind to leishmanial antigens.

The finding of a parasite antigen(s) of approximately 70 kDa in the sera of patients with AVL was particularly intriguing. We have observed that sera from 19 of 20 patients with AVL bound an *L. donovani chagasi* antigen(s) of approximately 70 kDa that was not recognized by sera from patients with Chagas' disease or cutaneous leishmaniasis (T. G. Evans et al., submitted for publication). Jaffe and Zalis (9) produced monoclonal antibodies to *L. donovani* and then screened them to find if they were useful in identifying antigens also recognized by human sera. By this independent method they found that species-specific *L. donovani* antibodies in humans strongly recognized a 70-kDa protein, as well as an antigen of 72 kDa. It is possible that the 70-kDa antigen identified by both methods is the same.

The finding that the patient precipitate was immunogenic in BALB/c mice suggests that relevant monoclonal antibodies can be isolated and used to immunoprecipitate circulating antigens. In preliminary studies, we have isolated 32 hybridomas from the spleen of a mouse immunized with patient PEG-IC that identify leishmanial antigens as assessed by ELISA.

The identification of circulating parasite antigens may have diagnostic and prognostic potential. Current serological tests (e.g., ELISA, indirect immunofluorescence assay, etc.) are designed to detect antileishmanial antibodies (Pearson and Sousa, in press). Although these tests are in general sensitive and relatively specific, cross-reacting antibodies may be encountered in other settings such as mucocutaneous leishmaniasis or Chagas' disease. Many individuals who are infected with *L. donovani* in endemic areas have antibody responses but experience asymptomatic, self-resolving infections (1, 2). Identification of circulating parasite antigens might point to ongoing parasite replication and thus be useful in predicting the subset of seropositive individuals who warrant treatment. Further work is needed to develop and evaluate diagnostic tests based on the detection of circulating antigens.

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LITERATURE CITED

1. **Badro, R., T. C. Jones, E. M. Carvalho, D. Sampaio, S. G. Reed, A. Barral, R. Teixeira, and W. D. Johnson, Jr.** 1986. New perspectives on a subclinical form of visceral leishmaniasis. J. Infect. Dis. **154**:1003–1011.
2. **Badaro, R., T. C. Jones, R. Lorenco, B. J. Cerf, D. Sampaio, E. M. Carvalho, R. Teixeira, and W. D. Johnson, Jr.** 1986. A prospective study of visceral leishmaniasis in an endemic area of Brazil. J. Infect. Dis. **154**:639–649.
3. **Brito, T., S. Hoshino-Shimizu, V. A. Neto, I. S. Duarte, and D. O. Penna.** 1975. Glomerular involvement in human kala-azar. Am. J. Trop. Med. Hyg. **24**:9–18.
4. **Carvalho, E. M., B. S. Andrews, R. Martinelli, M. Dutra, and H. Rocha.** 1983. Circulating immune complexes and rheumatoid factor in schistosomiasis and visceral leishmaniasis. Am. J. Trop. Med. Hyg. **32**:61–68.
5. **Casali, P., and P. H. Lambert.** 1979. Purification of soluble immune complexes from serum using polymethylmetacrylate beads coated with conglutinin or C1q. Application to the analysis of the components of *in vitro* formed immune complexes and of immune complexes occurring *in vivo* during leishmaniasis. Clin. Exp. Immunol. **37**:295–309.
6. **Evans, T., F. E. Reis, J. E. Alencar, T. G. Naidu, J. A. Laceroa, J. F. McAuliffe, and R. D. Pearson.** 1985. American visceral leishmaniasis (kala-azar). West. J. Med. **124**:777–781.
7. **Flemmings, B. J., C. M. Pappas, C. M. Keenan, and W. T. Hockmeyer.** 1984. Immune complex decomplementation of canine sera for use in a complement-fixation test for diagnosis of visceral leishmaniasis. Am. J. Trop. Med. Hyg. **32**:553–559.
8. **Galvao-Castro, B., J. A. Sa-Ferreira, I. Marzochi, M. C. Marzochi, S. G. Coutinho, and P. H. Lambert.** 1984. Polyclonal B cell activation, circulating immune complexes and autoimmunity in human American visceral leishmaniasis. Clin. Exp. Immunol. **56**:58–66.
9. **Jaffe, C. L., and M. Zalis.** 1988. Purification of two *Leishmania donovani* membrane proteins recognized by sera from patients with visceral leishmaniasis. Mol. Biochem. Parasitol. **27**:53–62.
10. **Kager, P. A., C. E. Hack, A. J. Hannema, P. H. Rees, and A. E. von dem Borne.** 1982. High C1q levels, low C1s/C1q ratios, and high levels of circulating immune complexes in kala-azar. Clin. Immunol. Immunopathol. **23**:86–93.
11. **Kharazmi, A., H. R. Rezai, M. Fani and N. C. Behforouz.** 1982. Evidence for the presence of circulating immune complexes in serum and C3b and C3d on red cells of kala-azar patients. Trans. R. Soc. Trop. Med. Hyg. **76**:793–796.
12. **Kohanteb, J., S. M. Ardehali, and H. R. Rezai.** 1987. Detection of *Leishmania donovani* soluble antigen and antibody in the urine of visceral leishmaniasis patients. Trans. R. Soc. Trop. Med. Hyg. **81**:578–580.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. **Nussenzweig, V.** 1957. Reacao de fixacao do complemento para leishmaniose visceral com antigens extraido do bacilo da tuberculose. Tecnica, sensibilidade e especificidade. Hospital (Rio de Janeiro) **51**:217–26.
16. **Olds, G. R., and T. F. Kresina.** 1985. Network interactions in *Schistosoma japonicum* infection. J. Clin. Invest. **76**:2338–2347.
17. **Pearson, R. D., J. E. Alencar, R. Romito, T. G. Naidu, A. C. Young, and J. S. Davis IV.** 1983. Circulating immune complexes and rheumatoid factors in visceral leishmaniasis. J. Infect. Dis. **147**:1102.
18. **Pearson, R. D., T. Evans, D. A. Wheeler, T. G. Naidu, J. E. Alencar, and J. S. Davis IV.** 1986. Humoral factors during South American visceral leishmaniasis. Ann. Trop. Med. Hyg. **80**:465–468.
19. **Pearson, R. D., and R. T. Steigbigel.** 1980. Mechanism of lethal effect of human serum upon *Leishmania donovani*. J. Immunol. **125**:2195–2201.
20. **Pugin, P., and R. A. Miescher.** 1979. Le kala-azar. Etude clinique et physiopathologique a propos d'un nouveau cas observe en Suisse. Schweiz. Med. Wochenschr. **109**:265–269.
21. **Sartori, A., A. V. Oliveira, M. C. Roque-Barreira, M. A. Rossi, and A. Campos-Neto.** 1987. Immune complex glomerulonephritis in experimental leishmaniasis. Parasite Immunol. **9**:93–103.
22. **Sehgal, S., B. R. Aikat, and A. G. Pathania.** 1982. Immune complexes in Indian kala-azar. Bull. W.H.O. **60**:945–949.
23. **Weisinger, J. R., A. Pinto, G. A. Velazquez, I. Bronstein, J. J. Dessene, J. F. Duque, J. Montenegro, F. Tapanes, and A. R. de Rousse.** 1978. Clinical and histological kidney involvement in human kala-azar. Am. J. Trop. Med. Hyg. **27**:357–359.