Clin. exp. Immunol. (1991) **87**, 386–392

Immune complex glomerulonephritis in experimental kala-azar II: Detection and characterization of parasite antigens and antibodies eluted from kidneys of *Leishmania donovani*-infected hamsters

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(Accepted for publication 11 September 1991)

SUMMARY

In a previous report analysing kidney sections by immunofluorescence we showed that hamsters infected with *L. donovani* develop a glomerulonephritis (GN) associated with deposition of hamster immunoglobulins and parasite antigens in the glomeruli. In this study we characterize these immune components eluted from the kidneys. The eluted immunoglobulins showed specificity for *L. donovani* antigens and hamster immunoglobulins (rheumatoid factor-like activity). The four isotypes IgG1, IgG2, IgA and IgM were detected. Several *L. donovani* antigens were detected in the renal eluates by Western blot and immuneprecipitation using ^{125}I-labelled eluates. Proteins with mol. wt of 134, 82, 52, 31, and 26 kD were detected by Western blot and proteins with 134, 110, 93, 89 and 48 kD were detected by immunoprecipitation. With the exception of the 134 kD protein which was recognized by both rabbit anti-promastigote and rabbit anti-amastigote sera all the others were recognized only by the anti-amastigote serum. The 134 kD protein was the only one isolated from the kidneys of infected hamster immunocomplexed with IgG and was the only one detected in a promastigote lysate using IgG from *L. donovani*-infected hamsters.

Keywords *Leishmania donovani* glomerulonephritis experimental kala-azar immune complexes

INTRODUCTION

Kala-azar or visceral leishmaniasis is a disease caused by *L. donovani*, an obligatory intracellular protozoan, which infects exclusively the macrophages of the reticuloendothelial system, predominantly in the spleen, liver and bone marrow. The infection is clinically characterized by fever, hepatosplenomegaly, anaemia and leucopenia [1]. The absence of any detectable cell-mediated immunity [2] and a marked hypergammaglobulinaemia [3,4] are the main immunological features of the disease. Most of the immunoglobulins are not parasite-specific antibodies, but seem to be a consequence of a polyclonal B cell activation [5]. There is also an extremely high incidence of circulating immune complexes (IC) in patients with visceral leishmaniasis [6–8]. Immune complexes isolated from patients' sera were found to contain IgG, IgA, IgM, various complement components [9] and leishmania antigens [10,11]. As is well known, IC play an important role in the modulation of the immune response [12] and in the occurrence of renal abnormalities [13,14]. Renal alterations have been observed in leishmaniasis [15–17] and in other protozoan-induced infections such as malaria [18], schistosomiasis [19] and trypanosomiasis [20]. A common trait observed in renal lesions resulting from protozoan infection is the presence of immunodeposits occurring in a granular pattern. The characterization of the molecules found in these deposits can help to understand why they accumulate in the renal structures and how they cause renal injury.

The hamster is a valuable animal model for kala-azar. In this animal the disease results in hepatosplenomegaly, anaemia [21] heightened serum globulin levels [22], immunosuppression [23], polyclonal B cell activation [24,24a] and renal involvement [25,25a].

In previous reports we described that hamsters infected with *L. donovani* developed a conspicuous immune complex glomerulonephritis (GN) defined by the presence of granular deposits of *L. donovani* antigens and hamster immunoglobulins in the mesangium and glomeruli loops [25–27].

In this study we characterize the immunoglobulins and the *L. donovani* antigens eluted from the kidneys of infected hamsters.

MATERIALS AND METHODS

Parasites

Amastigotes of *L. donovani* 1S, a courtesy of Dr Dennis Dwyer (Laboratory of Parasitology, NHI, Bethesda, MD) were main-

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tained by *in vivo* passage of the amastigotes from hamster to hamster. Promastigotes of *L. donovani* were maintained in NNN medium. *Staphylococcus aureus*, Cowan I strain, a courtesy of Dr Lucia Peralta and Dr J. M. Peralta (Instituto de Microbiologia–UFRJ, Rio de Janeiro, Brasil) was stored at −20°C in rabbit blood.

Preparation of L. donovani *promastigote antigens*
The parasite antigens were prepared as previously described [28]. *L. donovani* promastigotes grown in Schneider's Drosophila medium were washed three times with PBS 0·1 M pH 7·2. The pellet was resuspended in lysis buffer (10 mM TRIS-HCl pH 7·5; 1% v/v, NP-40; 1 mM PMSF; 2·8 U/ml aprotinin) and incubated for 10 min at 37°C. The insoluble material was pelleted at 10000 ***g*** for 15 min, at 4°C. The supernatant was immediately stored at −70°C.

Antibodies
Anti-hamster immunoglobulin antiserum and anti-hamster isotype antisera were prepared in rabbits as previously described [24,29–31].

Anti-*L. donovani* amastigote and promastigote antisera were also raised in rabbits. Rabbits received two intramuscular injections (1-week interval) with 10^8 parasites emulsified in Freund's complete adjuvant. One week after the second injection the animals received three intravenous injections (on alternate days) with 10^7 parasites. The rabbits were bled 7 days after the last injection. Both antisera were exaustively adsorbed with hamster serum, erythrocytes, kidney, liver and spleen cells.

Infection of experimental animals
Mesocricetus auratus, the golden hamster, were obtained from our colonies. The animals were 3–4 months old outbred females and males weighing approximately 120 g. The hamsters were infected by intracardiac injection of 5×10^6 amastigotes obtained by differential centrifugation from the spleen of infected animals.

Kidney eluates
The kidney eluates were obtained as previously described [32]. Normal hamsters and hamsters with 5–7 weeks of infection with *L. donovani* were used. Our previous results indicated that the immunofluorescence with anti-*L. donovani* antiserum was more pronounced by this time of infection. The hamsters were perfused *in toto* by injection of PBS 0·1 M pH 7·2 into the left ventrical until bleaching of the kidneys. Pooled kidneys were homogenized in cold PBS. The homogenate was washed five times with PBS by centrifugation at 2000 ***g***, 0°C. The pellet was suspended in 0·02 M pH 3·2 citrate buffer (1 g tissue/20 ml buffer) and stirred for 2 h at room temperature. After centrifugation for 30 min at 4000 ***g*** the sediment was discarded and the supernate adjusted to pH 7·0 with 1 M Na_2HPO_4. Eluates were concentrated to 1 ml by ultrafiltration and dialysed overnight against PBS. The eluates were aliquoted and stored at −70°C. Protein concentration was determined by the method of Lowry *et al.* [33]. The eluate obtained from the kidneys of normal hamsters was called NE and the one obtained from kidneys of infected hamsters IE.

SDS–PAGE
SDS–PAGE was performed as previously described [34,35].

Western blot
After electrophoresis the proteins were transferred to 0·45-μm pore nitrocellulose (Schleicher & Schuell, Keene, NH) in a semi-dry transfer apparatus as described [36]. The nitrocellulose sheets were placed in blocking buffer (5% non-fat dry milk in PBS 0·1 M pH 7·2) for 1 h at room temperature. To search for *L. donovani* antigens the nitrocellulose strips were incubated overnight at 4°C with rabbit anti-promastigote or anti-amastigote antisera diluted in blocking buffer. The strips were then washed three times for 15 min each with 0·05% Tween 20 in PBS (PBS/T) pH 7·2 and incubated with conjugate, sheep anti-rabbit IgG-horseradish peroxidase for 1 h at room temperature. The peroxidase activity was visualized using 3,3′-diaminobenzidine tetrahydrochloride [37].

Immuneprecipitation of ^{125}I-labelled eluates
Immuneprecipitation was carried out as reported by Kessler [38]. Eluates from kidneys of normal and infected hamsters were labelled with ^{125}I by the lactoperoxidase method [39].

Approximately 80 μl of each radiolabelled eluate were incubated overnight at 4°C with purified rabbit IgG anti-amastigote extensively adsorbed with hamster tissues and serum. The samples were then immunoprecipitated with protein A-Sepharose. The pellets were washed three times with TRIS-HCl 100 mM pH 7·4 containing NaCl 300 mM, EDTA 10 mM, NaN_3 0·04%, NP-40 0·05% and BSA 0·1%. The pellets were resuspended in sample buffer and analysed by SDS–PAGE 7·5%. The dried gel was exposed, at −70°C, for autoradiography, using QA-f films (BRAS-raios X medico-KODAK).

Immuneprecipitation of L. donovani *promastigote antigens by IgG from uninfected hamster*
Approximately 300 μl of a stock suspension of *S. aureus* (Cowan I strain) prepared as described by Kessler [38], was incubated for 30 min with 200 μg of protein A purified hamster IgG obtained from a pool of *L. donovani*-infected hamster sera. The bacteria were washed three times and the pellet was mixed with 650 μg of *L. donovani* antigens, and incubated for 2 h at room temperature. After washings the pellet was suspended in solubilizing sample buffer and submitted to SDS–PAGE 7·5%. The same procedure was adopted as a control, with the exception that no leishmania antigens were added to the reaction.

Isolation of immune complexes (parasite antigen/antibodies) from the kidneys of infected hamsters
Eluate from infected hamsters' kidneys (5·5 mg) was submitted to affinity chromatography on a Sepharose protein-A column previously equilibrated with PBS 0·1 M pH 8·0. The retained material was eluted with glycine-HCl buffer pH 2·8 and concentrated by ultrafiltration. This material was then dialysed overnight against PBS; the identification of antibodies and parasite antigens was done by SDS–PAGE.

Analysis of the immunoglobulins present in the eluate
The immunoglobulins and respective isotypes were determined by the immunodiffusion technique [40] using rabbit serum, anti-whole hamster immunoglobulins or specific hamster isotype immunoglobulin antisera.

The presence of specific antibodies for *L. donovani* antigens and for DNA was assayed by an indirect immunofluorescence reaction [41,42]. Rheumatoid-like factor was assayed according

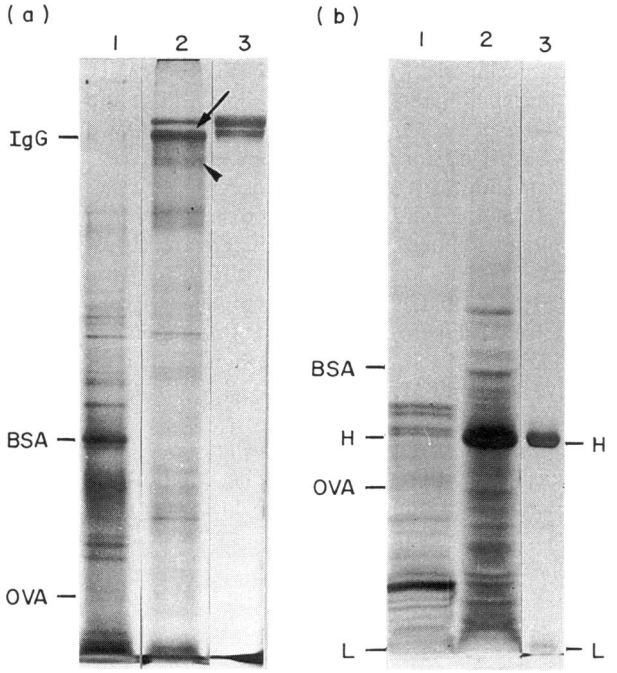

Fig. 1. SDS–PAGE (7·5%) analysis of eluates obtained from the kidneys of normal (NE) and infected (IE) hamsters (silver stained). (a) Unreduced samples; (b) reduced samples. Lane 1, NE (25 μg); lane 2, IE (25 μg); lane 3, protein A purified hamster IgG (3 μg). Arrow indicates a protein that has the same electrophoretic mobility as hamster IgG; arrowhead indicates a 134 kD protein present in the IE but absent from the NE. IgG, H, L, BSA and OVA refer to the position of the mol. wt markers, respectively hamster IgG (150 kD); hamster IgG heavy chain (50 kD); hamster IgG light chain (25 kD); bovine serum albumin (66 kD) and ovalbumin (45 kD).

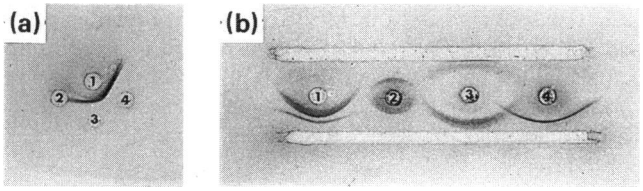

Fig. 2. Detection and identification of hamster immunoglobulins in the eluates obtained from the kidneys of normal (NE) and infected (IE) hamsters by gel diffusion analysis. (a) (1) Rabbit anti-hamster immunoglobulins; (2) NE (2 mg/ml); (3) IE (2 mg/ml); (4) protein A purified hamster IgG (2 mg/ml); (b) Upper trough-IE (4 mg/ml); lower trough-whole normal hamster serum; (1) rabbit anti-hamster gamma$_1$ heavy chain; (2) rabbit anti-hamster gamma$_2$ heavy chain; (3) rabbit anti-hamster alpha heavy chain; (4) rabbit anti-hamster mu heavy chain. The bands were stained with Amido black.

to the classical Waaler–Rose reaction [43] using subagglutinating doses of hamster IgG anti-sheep erythrocytes.

RESULTS

Electrophoretic profile of renal eluates

Renal eluates subjected to SDS–PAGE (Fig. 1a) revealed that at least three protein bands of high molecular weight are present in

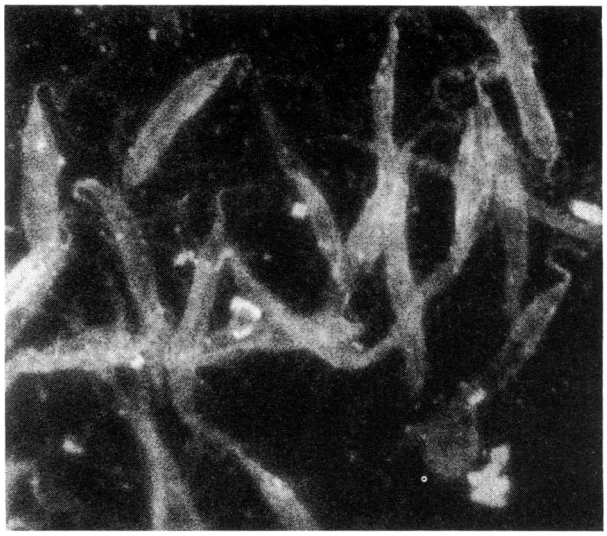

Fig. 3. Detection of anti-*Leishmania donovani* antibody in the eluate obtained from kidneys of infected hamsters (IE) by immunofluorescence. *L. donovani* promastigotes were incubated with IE (2 mg/ml), washed and then reacted with rabbit IgG anti-hamster immunoglobulins. Reaction was developed with fluoresceinated sheep anti-rabbit immunoglobulins. × 1000.

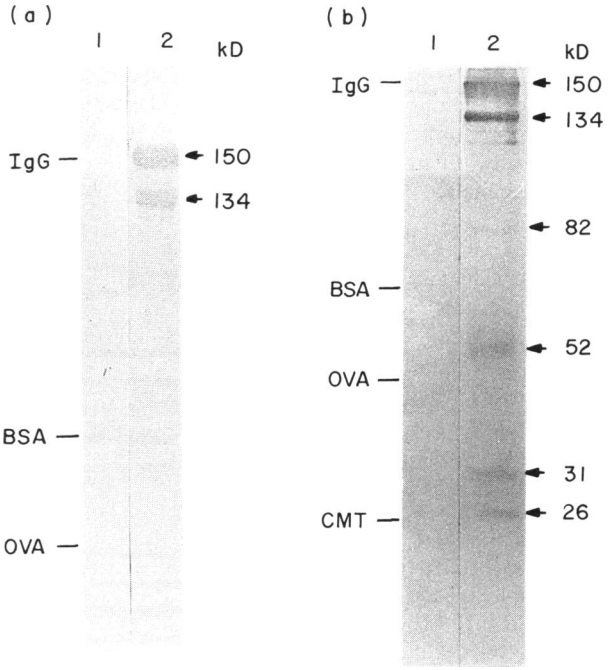

Fig. 4. Western blot analysis of eluates obtained from the kidneys of normal (NE) and infected (IE) hamsters revealed by rabbit anti-promastigote serum (a) and rabbit anti-amastigote serum (b). Lane 1, NE, 25 μg; lane 2, IE, 25 μg were submitted to SDS–PAGE ((a) 7·5%; (b) 10%) under non-reducing conditions. The mol. wt of the bands are indicated to their right. IgG, BSA, OVA and CMT refer to the position of mol. wt markers, respectively hamster IgG (150 kD); bovine serum albumin (67 kD), ovalbumin (45 kD) and chymotrypsinogen (25·7 kD).

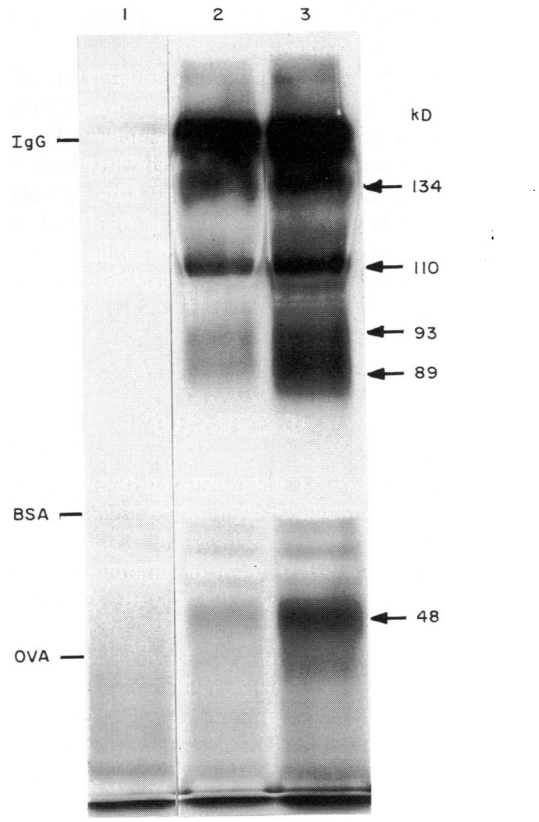

Fig. 5. Autoradiograph of SDS–PAGE (7·5%)-separated, ^{125}I-labelled kidney eluates immunoprecipitated with anti-amastigote serum. Proteins of labelled eluates were incubated with rabbit anti-*Leishmania donovani* amastigote IgG immunoprecipitated with protein A Sepharose and submitted to SDS–PAGE under non-reducing conditions. Lane 1, NE, 65 μg; lane 2, IE, 16 μg; lane 3, IE, 65 μg; these concentrations refer to the quantity of protein initially incubated with the rabbit anti-amastigote IgG. The mol. wt of the bands are indicated to their right. IgG, BSA and OVA refer to the position of mol. wt markers, respectively, hamster IgG (150 kD); bovine serum albumin (66 kD) and ovalbumin (45 kD).

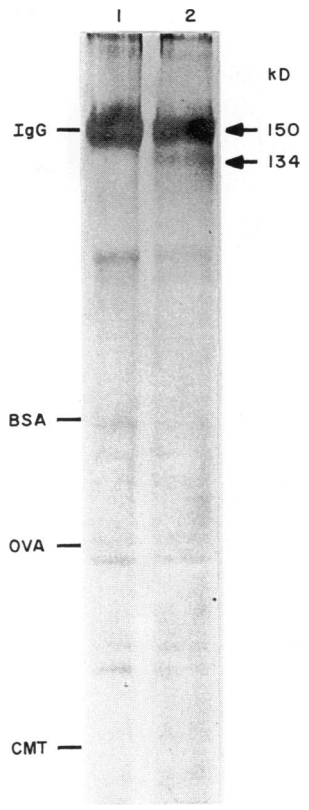

Fig. 6. SDS–PAGE (7·5%) analysis of *Leishmania donovani* promastigote antigen recognized by infected hamster IgG. *Staphylococcus aureus* (Conwan I) sensitized with IgG from infected hamster sera was mixed with promastigote lysate. After centrifugation the pellet was washed and treated with solubilizing sample buffer. The samples were analysed by SDS–PAGE under non-reducing conditions. Lane 1, *S. aureus* + IgG; lane 2, *S. aureus* + IgG + promastigote lysate. The mol. wt of hamster IgG and of the recognized *L. donovani* antigen are indicated to their right. IgG, BSA, OVA and CMT refer to the position of mol. wt markers, respectively, hamster IgG (150 kD); bovine serum albumin (66 kD); ovalbumin (45 kD) and chymotrypsinogen (25·7 kD).

the infected hamster kidney eluate (IE) but absent from the normal hamster kidney eluate (NE). Two of these high molecular weight protein bands in the IE have the same mobility as purified hamster IgG and are susceptible to reduction (Fig. 1b). These results suggest that hamster immunoglobulins are present in the IE. The third band (with an estimated molecular weight of 134 kD) was also susceptible to reduction. It is conceivable that this band is an *L. donovani* antigen.

Analysis of the immunoglobulins present in the kidney eluates

The presence of immunoglobulins in the eluates was determined using a gel diffusion technique. The result is shown in Fig. 2a. The IE showed one line of total identity with hamster IgG. No immunoglobulins were detected in the NE. The reactions with anti-gamma$_2$ and anti-alpha heavy chains antisera clearly revealed the presence of IgG2 and IgA in the IE. Anti-gamma$_1$ and anti-μ heavy chain antisera produced very weak precipitin lines, faintly visible in the photograph of the gel (Fig. 2b).

The specificity of these immunoglobulins was assayed by indirect immunofluorescence using promastigotes of *L. donovani* as antigen. A positive reaction was seen with the IE (Fig. 3), whereas no fluorescence was observed when NE was used. We also investigated the presence of rhematoid factor (RF)-like substances and anti-DNA antibodies because these autoantibodies have been detected in the sera of humans with kala-azar. RF-like activity was detected only in the IE. The presence of anti-DNA antibodies was determined by indirect immunofluorescence reaction. Imprints of normal hamster liver were used as the antigen substrate. No anti-DNA antibodies could be detected in either NE or IE.

Detection of L. donovani *antigens in the renal eluates*

The presence of leishmania antigens in the IE was initially determined using the Western blot technique. *L. donovani* antigens were detected using two different sera: a rabbit anti-*L. donovani* promastigote and a rabbit anti-*L. donovani* amastigote antisera. Both sera were extensively adsorbed with hamster cells (kidney, spleen, liver and erythrocytes) and with hamster serum. The rabbit anti-promastigote serum recognized two bands with estimated molecular weights of 150 and 134 kD (Fig. 4a). The rabbit anti-amastigote serum, in addition to the 150 and 134 kD bands, recognized four other more discrete bands with esti-

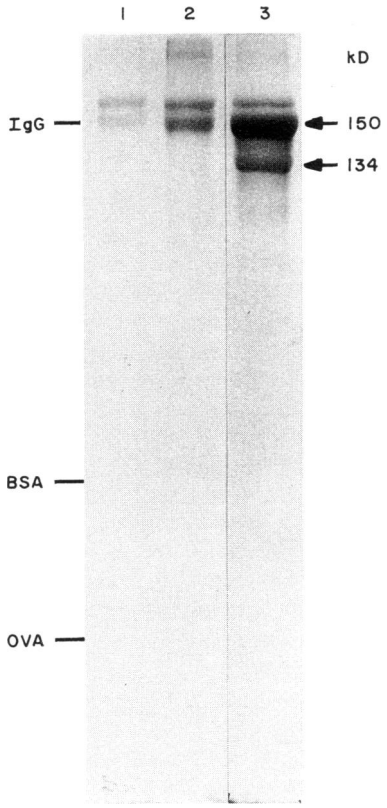

Fig. 7. SDS–PAGE (7·5%) analysis of parasite antigen-antibody immune complex isolated from the kidneys of infected hamsters. IE was incubated with protein A Sepharose and the bound material was submitted to SDS–PAGE under non-reducing conditions and stained by Coomassie blue. Lane 1, Protein A purified IgG from normal hamster serum; lane 2, protein A purified IgG from infected hamster serum; lane 3, protein A isolated immune complex from IE. The mol. wt of the protein A isolated molecules are indicated to their right. IgG, BSA and OVA refer to the position of mol. wt markers, respectively hamster IgG (150 kD); bovine serum albumin (66 kD) and ovalbumin (45 kD).

mated mol. wts of 82, 52, 31 and 26 kD (Fig. 4b). No bands were seen in the transblotted material obtained from NE. Also, no bands were seen when the strips were incubated with the sheep anti-rabbit IgG-horseradish labelled antibody alone (not shown).

The presence of *L. donovani* antigens in the IE was then determined by immuneprecipitation of radiolabelled eluates (Fig. 5). Bands of 150, 134, 110, 93 and 48 kD were revealed; the lower mol. wt proteins detected by Western blot (31 and 26 kD) were not observed.

Recognition of a 134-kD antigen in L. donovani *promastigotes by IgG from infected hamsters*

L. donovani promastigotes were treated with lysis buffer and immunoprecipitated by IgG, purified from a pool of sera from infected hamsters, bound to *S. aureus*. Precipitated proteins were submitted to SDS–PAGE. Figure 6 shows that the IgG from infected hamsters recognized one protein with an estimated mol. wt of 134 kD.

Isolation of immune complexes of parasite antigen/antibody from the kidney of infected hamsters

In order to evaluate if the antibodies and parasite antigens were present in an immune complex manner in the kidney, IE was passed over a protein A-Sepharose column. The beads were washed, the bound proteins were eluted with glycine-HCl buffer pH 2·8 and submitted to SDS–PAGE. Figure 7 clearly shows that three bands were detected. Two of the bands are hamster immunoglobulin. The other band of 134 kD corresponds to the leishmania antigen detected by Western blot and immunoprecipitation.

DISCUSSION

In a previous report we demonstrated that hamsters infected with *L. donovani* presented immunoglobulinuria caused by GN. The immunofluorescence analysis of frozen kidney sections revealed the presence of hamster immunoglobulins and *L. donovani* antigens [25], suggesting that this glomerulonephritis was caused by the deposition of IC.

In the present investigation we studied the IC eluted from the kidney of *L. donovani*-infected hamsters.

Analysis of the electrophoretic profile of eluates obtained from kidneys of NE and IE hamsters showed proteins present in the IE and absent from the NE. One of these proteins shared the same electrophoretic mobility as purified hamster IgG and was susceptible to reduction with 2-ME yielding molecules of 50 and 25 kD. The presence of immunoglobulins in the IE was further confirmed by gel immunodiffusion. By employing specific antisera we demonstrated that these immunoglobulins were of the IgG1, IgG2, IgA and IgM isotypes. In human kala-azar these isotypes have also been detected in kidney sections from human patients [16,17].

The specificity of these immunoglobulins was initially determined by immunofluorescence using *L. donovani* promastigotes as antigens. Anti-leishmania antibodies were detected in the IE and not in the NE. Other specificities, such as RF-like activity and anti-DNA antibodies, were then investigated. These antibodies were selected because they are found in immune deposits of GN associated with polyclonal activation [44]. Polyclonal activation of B cells has been demonstrated in both human [5] and experimental kala-azar [24]. In addition, these antibodies have already been described in the sera of patients with kala-azar [45]. RF-like activity was present in the IE and not in the NE. Anti-DNA antibodies were not detected in the kidney eluates. We also did not find these autoantibodies (anti-DNA and anti-immunoglobulins) in the sera of normal and infected hamsters (data not shown). The failure to detect RF-like activity in the sera of highly infected hamsters has also been reported [11].

To determine if there were molecules of parasite origin in the IE we used anti-*L. donovani* promastigote and anti-*L. donovani* amastigote hyperimmune rabbit sera adsorbed with hamster sera and cells (erythrocytes, kidney, liver and spleen). When these antisera were used in a Western blot, the rabbit anti-promastigote serum recognized only two proteins (150 and 134 kD) while the rabbit anti-amastigote serum recognized, in addition to these bands, four other proteins of 82, 52, 31 and 26 kD. The band of 150 kD, detected by both antisera, has the same

electrophoretic mobility as hamster IgG. Possible cross-reactions between *L. donovani* antigens and hamster IgG were eliminated by exhaustive adsorption of the anti-leishmania sera with hamster immunoglobulins. The origin of this 150 kD band could not be ascertained. The possibility that it is hamster IgG can not be excluded if one assumes that this molecule has RF-like activity and can react with the rabbit anti-*L. donovani* antibodies used in the Western blot. The possibility that this band is of parasite origin can not be eliminated and remains to be determined.

To further investigate the presence of leishmania antigens in the IE, immunoprecipitation of ^{125}I-labelled eluates was performed. Three bands with the same molecular weight as seen in the Western blot (150, 134 and 48 kD) and three additional bands (110, 93 and 89 kD) were detected. The lower mol. wt antigens (31 and 26 kD) observed with the Western blot were not detected by immunoprecipitation. Differences between the sensitivity of the two methodologies have been described [46]; whereas immunoblotting favours detection of sequential determinants, the immunoprecipitation allows effective detection of both sequential and conformational determinants.

Our results also indicated that at least some of the immunoglobulins and *L. donovani* antigens detected in the IE were present in the kidneys as an immune complex. This was concluded because when the IE was adsorbed on protein A-Sepharose the retained proteins were hamster immunoglobulins and one *L. donovani* antigen of 134 kD.

The mechanism by which these antigens and antibodies are retained in the kidneys remains unsolved. One possibility is that part of the IC could be formed *in situ* as a consequence of charge interactions [47–49]. This possibility is supported by the fact that sera from patients with kala-azar contain *L. donovani* antigens of 92, 52 and 30 kD with isoelectric points (pI) ranging from 8·71 to 9·16 [50]. Molecules with these pI have been shown to interact with the negatively charged glomerular basal membrane. *L. donovani* antigens with this range of molecular weights were detected in the IE when we used the rabbit anti-amastigote serum. Whether or not the *L. donovani* antigens detected in the IE have the appropriate pI and are directly involved in the development of renal injury remains to be determined. Nonetheless the GN that develops in experimental visceral leishmaniasis can be used as an interesting model to underestand the mechanisms of renal injury caused by exogenous antigen, i.e. circulating IC trapping *versus in situ* IC formation.

Among the antigens detected in the eluates, that of 134 kD mol. wt deserves special attention. This molecule was reducible by 2-ME and was the only one detected by both antisera (rabbit anti-*L. donovani* promastigote and amastigote). In addition, this antigen seems to be the only one recognized in a promastigote lysate by a pool of sera from infected hamsters and is present in the IC isolated from the IE. Interestingly, this antigen shares several similarities with *L. donovani* acid phosphatase. This enzyme has a mol. wt of 134 kD [51], is reducible by 2-ME [52], is present in the leishmania cellular compartment [53], is excreted by the parasite [54] and is detected by Western blot using both monoclonal anti-promastigote antibody and human serum from patients with kala-azar (anti-amastigote serum) [55]. These similarities strongly suggest that the 134 kD protein present in the IE and the *L. donovani* acid phosphatase are one and the same molecule. This attractive possibility is currently under investigation.

ACKNOWLEDGMENTS

We would like to thank Ms Maryellen Feeney and Dr Isabel Kinney Ferreira de Miranda-Santos for reviewing the manuscript and Mr Ronaldo Sordi Campanini for skilful secretarial assistance. This work was supported by grants from FINEP, FAPESP, CNPq and CAPES, and is based on a thesis submitted by A. Sartori in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Institute of Microbiology, UFRJ, Brazil.

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