EVALUATION OF ANTIBODY RESPONSES IN AMERICAN VISCERAL LEISHMANIASIS BY ELISA AND IMMUNOBLOT

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American visceral leishmaniasis (AVL) is an important disease among children of northeast Brazil. In order to characterize antibody responses during AVL, sera of hospitalized patients were analyzed by ELISA and Western blot using a Leishmania chagasi *antigen preparation. The ELISA was positive (absorbance* $\geqslant$ *0.196) at a serum dilution of 1:1024 in all patients at presentation, and fell to ward control levels over the following year. Only one of 72 control subjects tested positive, and that donor had a sibling with AVL. Immunoblots of the patients' sera recognized multiple bands, the most frequent of which were at approximately 116 kDa, 70 kDa, and 26 kDa. Less frequently observed were bands at approximately 93 kDa, 74 kDa, 62 kDa, 46 kDa and 32 kDa. The ELISA responses and patterns of banding were distinctive for AVL, and could be used to differentiate patients with AVL from those with Chagas' disease or cutaneous leishmaniasis. Sera from six AVL patients followed for up to six weeks after treatment identified no new bands. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of surface iodinated parasite proteins showed one major band and four minor bands, whereas SDS-PAGE of biotinylated parasite proteins revealed a banding pattern similar to those of patient sera. AVL appears to produce characteristic immunoblot patterns which can be used along with a sensitive screening ELISA to diagnose AVL.*

Key words: Visceral leishmaniasis – *Leishmania donovani chagasi* – ELISA – immunoblot

American visceral leishmaniasis (AVL), caused by *Leishmania donovani chagasi*, is a common cause of morbidity and mortality among children and young adults in Northeastern Brazil (Alencar, 1983; Evans et al., 1985). In the state of Ceará approximately 500 new cases are reported each year, and many more asymptomatic or oligosymptomatic cases probably go unrecognized.

The immune response in AVL is characterized by a lack of lymphoproliferative and delayed type hypersensitivity responses to leishmanial antigen (Carvalho et al., 1981; Pearson et al., 1983) high titers of both non-specific and specific antibodies, and the presence of immune complexes and rheumatoid factors (Galvão-Castro et al., 1984; Pearson et al., 1986). Lymphocyte responses to leishmanial antigens develop only after drug cure in classical kala-azar patients or coincident with spontaneous cure in asymptomatic individuals (Badaro et al., 1986). In animal models protective immunity is mediated by helper T cell populations, which may also help antibody production (Pearson & Wilson, 1988).

We have assessed antileishmanial antibodies in patient sera before, during, and after treatment of AVL to characterize parasite antigens that elicit an antibody response and to identify antigens which might correlate with cure and protection. Parasite antigens recognized by sera were also compared to surface antigens

labelled by the techniques of surface iodination and biotinylation.

In addition to furthering our understanding of immune responses during AVL, the characterization of specific antigens recognized by patient sera could lead to improved diagnostic capability. The diagnosis of AVL is usually straightforward in patients presenting with the characteristic hepatosplenomegaly, fever, wasting, and pancytopenia of kala-azar. However the ability to detect asymptomatic infection, or disease at an early subclinical stage is needed to improve intervention, to direct potentially toxic chemotherapy to the appropriate individuals, and to better understand the population at risk and the determinants of disease.

MATERIALS AND METHODS

Subjects — One or more sera specimens from 44 different patients with acute or treated AVL and from 72 controls were obtained in 1983 and 1986 in the state of Ceará in northeastern Brazil. The diagnosis of AVL was based on the identification of amastigotes in bone marrow aspirates in patients at the Hospital das Clinicas in the capital, Fortaleza, or at the Santa Casa Misericórdia in the city of Sobral. Controls consisted of 21 hospitalized pediatric patients with diagnoses other than AVL in Fortaleza, 49 family members and neighbors of patients in Sobral, and two project workers whose blood was used to assess the effects of freezing and transportation on serum factors. The median age of both patients and controls was seven years. Blood was drawn from all subjects, allowed to coagulate at ambient temperature, and serum stored at $-20\,^{\circ}C$.

Parasites — The leishmanial isolate used in all experiments was derived from the bone marrow aspirate of one of the patients and was identified as *Leishmania donovani chagasi* (MHOM/BR/00/L669) by both isoenzyme analysis and kDNA probes. In addition a Sudanese strain of *Leishmania donovani* (MHOM/SU/00/S3) was used for comparison in experiments with surface iodination and biotinylation. Amastigotes were harvested from the spleens of Syrian hamsters and maintained by intracardiac inoculation into new hamsters. Promastigotes were grown in a modified Eagle's Minimal Essential Medium supplemented with 10% fetal calf serum (FCS), glutamine, penicillin (100/U/ml), and gentamicin (50 $\mu g/ml$). Promasti-

gotes were harvested at late log phase for all antigen preparations.

ELISA — An IgG ELISA was developed using *L. chagasi* antigen. Promastigotes were washed three times in PBS (Gibco Laboratories, Grand Island, NY, USA) pH 7.2 and adjusted to $2 \times 10^7/ml$ in a pH 9.6 bicarbonate buffer with the protease inhibitors antipain (20 $\mu g/ml$) and leupeptin (20 $\mu g/ml$). Fifty microliters of this preparation were then placed in all wells except the control lane of 96 well Immulon II plates (Dynatech Laboratories, Chantilly, VA, USA) and allowed to adhere at 37 °C for three hours. The plates were then washed with PBS/0.05% Tween 20, dried, and either used immediately or stored at $-20\,^{\circ}C$. The plates were blocked by three washings in PBS/1.5% FCS, incubated with duplicate 50 μl serum samples diluted 1/250 in PBS/1.5% FCS for 40 minutes, washed three times in PBS/0.05% Tween 20, and incubated 40 minutes with 50 μl of a 1:1000 dilution of peroxidase-conjugated, affinity purified goat anti-human IgG (Sigma Chemical Co., St. Louis, USA). The plates were then washed an additional three times in PBS/Tween and to each well was added 200 μl of developing substrate consisting of 2-azinodio-3-ethylbenzthiazoline in a citrate-bicarbonate-hydrogen peroxide buffer. Absorbance was read after 30 minutes at 414 nm on a Dynatech ELISA Multiskan Reader (Dynatech Laboratories) after blanking on the row of substrate alone without antigen. The final absorbance was the average of duplicate values. Positive values were defined to be those greater than 2 standard deviations above the mean of the 72 control sera at a dilution of 1/256. A defined positive control serum (from a patient with confirmed AVL) and defined negative control serum (from the project laboratory worker) were used in each experimental run. All ELISA positive samples were repeated at the screening dilution and titered by serial fourfold dilutions to 1/16,384. ELISA positive sera and 21 Brazilian controls were also immunoblotted.

Immunoblot protocol — Promastigotes or amastigotes were washed three times in Hanks balanced salt solution (HBSS) at 4 °C. Antipain and leupeptin were added (each 20 μg per 2 × 10^7 promastigotes) after the last wash. Amastigotes were then separated from splenic debris by passage over a CF-11 column (Sigma Chemical Co.), followed by addition of protease inhibitors. The organisms were boiled in

Laemmli reducing buffer containing 2.5% B-mercaptoethanol for 5 minutes (Laemmli, 1970), placed at 30-50 μg/lane on 1.5 mm thick 10% SDS-polyacrylamide separating gel with a 4% stacking gel, electrophoresed at 30 mA/gel with water cooling for approximately 4 hours, and blotted onto 0.1 micron nitrocellulose at 150 mA for 8 hours. The filters were cut into 5 mm strips, blocked in 5% dry milk, incubated for 2 hours at 37 °C with 1/50-1/400 dilutions of serum, washed three times in PBS, incubated one hour with a 1:1000 dilution of peroxidase-labeled goat antihuman IgG or IgM, and developed with hydrogen peroxide/2,2'diaminobenzidine (DAB) in a pH 7.2, 0.05M Tris buffer. The standards and one lane of each blot were stained with amido black to assess the density of protein transfer, and all gels were stained with Commassie blue after blotting to ensure complete transfer. The molecular weight of the major staining bands was calculated by semilogarithmic graphing against prestained molecular weight standards (Sigma Chemical Co.) run with each gel. The defined positive and negative control sera were tested with each set of strips.

Surface iodination and biotinylation — In order to compare the immunoblot pattern with identifiable surface antigens, promastigotes were surface iodinated (Markwell & Fox, 1978) or biotinylated (Hofmann et al., 1978). *Leishmania* promastigotes were washed three times in HBSS at 4 °C, suspended in 1 ml HBSS, and simultaneously biotinylated or surface iodinated. For biotinylation 10^8 promastigotes were labelled with 100 μl of a NHS-biotin (N-hydroxysuccinimide iminobiotin hydrochloride, Pierre Chemical Co., Rockford, USA) solution (10 mg/ml in dimethylformamide, Sigma Chemical Co.). The suspension was rotated for ten minutes at room temperature and washed twice in PBS. Antipain and leupeptin were then added, and the suspension was boiled in the reducing buffer for five minutes. This sample was applied at a 10^7 parasite density per lane on a 10% SDS-PAGE gel, transferred to nitrocellulose, blocked with 0.1% Tween-20 in PBS, incubated for one hour with peroxidase-labelled avidin, and developed in DAB as described above.

For surface iodination the parasites were labelled with 0.5 mCi of ^{125}I per 10^8 promastigotes. Iodobeads (Pierce Chemical Co.) were washed thoroughly, activated with the ^{125}I for

five minutes in glass tubes, and then incubated with the promastigotes for 15 minutes on ice. These promastigotes were washed twice in cold HBSS, protease inhibitors were added as above, and then parasites were solubilized in one ml 1% Triton X-100 in PBS. The solution was centrifuged at 10,000 g. for ten minutes, and the supernatant collected and aliquoted in 100 μl samples for electrophoresis. An aliquot was boiled for five minutes in an equal volume of reducing buffer, and 30 μl was added per lane on a 10% SDS-PAGE gel along with the biotinylated promastigotes. Autoradiography was performed at — 70 °C for 24 hours with Kodak X-OMAT AR film.

RESULTS

ELISA — The ELISA was positive for all parasitologically confirmed patients using an absorbance value of 0.196 (mean + 2 SD of controls) at a dilution of 1/256 (Fig. 1) as the cutoff. At presentation 100% of patients (14/14) had a titer of $\geq$ 1/1024, and 85% (11/13) were still 2 standard deviations above the positive controls at a dilution of $\geq$ 1/16,384. During treatment (samples drawn from 3 to 21 days) 96% of sera (27/28) were positive at $\geq$ 1/1024; the titer was $\geq$ 1/16,384 in 79% (22/28). After treatment and up to three months later, 82% (9/11) of patients had a titer $\geq$ 1/256, 73% (8/11) were $\geq$ 1/1024, and 36% (4/11) were $\geq$ 1/16,384. From three until nine months after treatment 64% (7/11), 45% (5/11) and 36% (4/11) of patient sera were positive at the same respective dilutions. Patients examined more than nine months after treatment (range 9 months to 17 years) were positive at $\geq$ 1/256 in only 27% (3/11) of cases, and the titers were quite low, with only one serum obtained at nine months being $\geq$ 1/1024.

Among the 72 control sera, there was one with a high absorbance and four with absorbances between 0.196 and 0.210 on initial screening at a serum dilution of 1/256. The four sera with values near the cut-off were all negative on repeat ELISA, as well as having no bands by immunoblotting. The high titer control remained positive on repeat (absorbance 0.272).

Serum from patients with cutaneous leishmaniasis were also screened; 3/7 were positive at $\geq$ 1/256 (Fig. 1). However, all of these sera were negative at a 1/1024 dilution. Sera from patients with Chagas' disease were
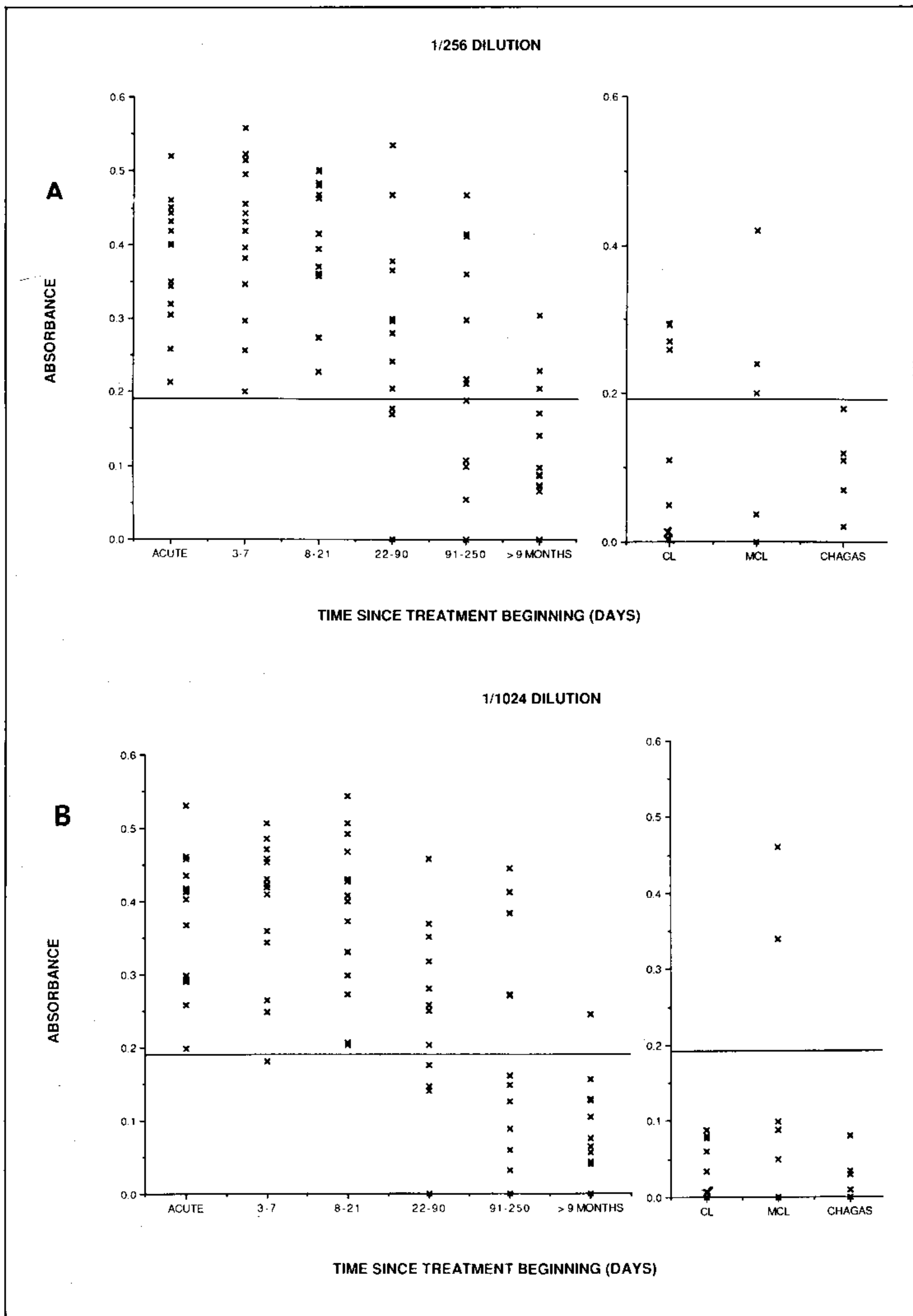
Fig. 1: ELISA values for patients with visceral leishmaniasis (left graph), cutaneous leishmaniasis (CL), muco-cutaneous leishmaniasis (MCL), and chronic Chagas' disease at serum dilutions of 1/256 (A) and 1/1024 (B). The horizontal line represents two standard deviations above the mean absorbance of 72 control sera.

negative at our screening value, although many were more than one standard deviation above the mean of the controls. Sera from two of five patients with mucocutaneous leishmaniasis had a positive value at a dilution of $\geqslant 1/1024$.

Immunoblots — Immunoblots were performed on 19 patients with acute visceral leishmaniasis (within one week of the onset of treatment). The amido black staining pattern of the electrophoresed promastigotes preparation revealed multiple bands with the most intense bands at approximately 47, 70, and 83 kDa. Simultaneous staining of transferred and non-transferred gels revealed that complete transfer of protein above a molecular weight of approximately 130 kDa was variable, and we therefore excluded bands above that molecular weight from further examination in this study.

Although the IgG banding patterns of patient sera were complex and heterogenous, they had several common features (Fig. 2, lanes D-U; Fig. 3, lane A). These characteristics included bands in regions at 116-120 kDa (18/19), 70-73 kDa (17/19), and 26-29 kDa (18/19).

Other frequent bands were seen at 93-95 kDa (11/19), 74-77 kDa (8/19), 62-65 kDa (10/19), 46-49 kDa (7/19) and 32 or 33 kDa (10/19). These results were similar using amastigote or promastigote preparations (data not shown).

In 21 hospitalized Brazilian controls with negative ELISA values, the bands mentioned above were rarely visualized (Fig. 2, lanes B, C). None of four North American ELISA negative controls had any IgG bands. The one Brazilian control, the eight-year-old brother of a patient with AVL, who had a high ELISA value also had an immunoblot which was indistinguishable from those of AVL patients with prominent bands at approximately 116, 92, 71, 48, 33, and 26 kDa.

In addition, serum from four patients with cutaneous leishmaniasis were studied (Fig. 3, lanes H-K). Antibodies were directed against different parasite antigens, except those at 116-120 kDa, and were distinguishable both by direct inspection and by the need to use lower serum dilutions (1/50) in order to visualize these bands.
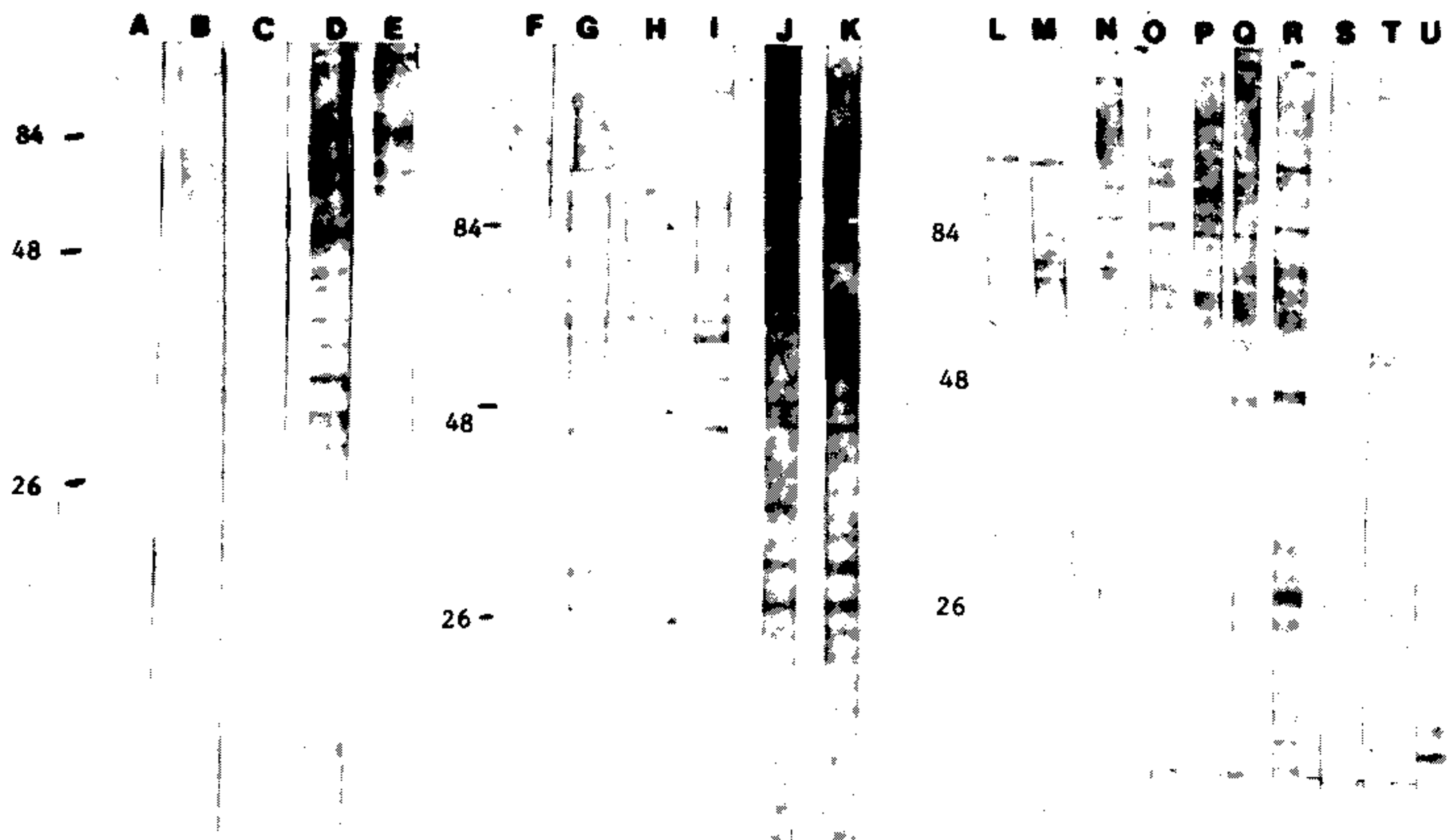

Fig. 2: immunoblots using a serum dilution of 1/200. Lane A, no first antibody; lane B and C, hospitalized controls; lanes D-U, patients with AVL.
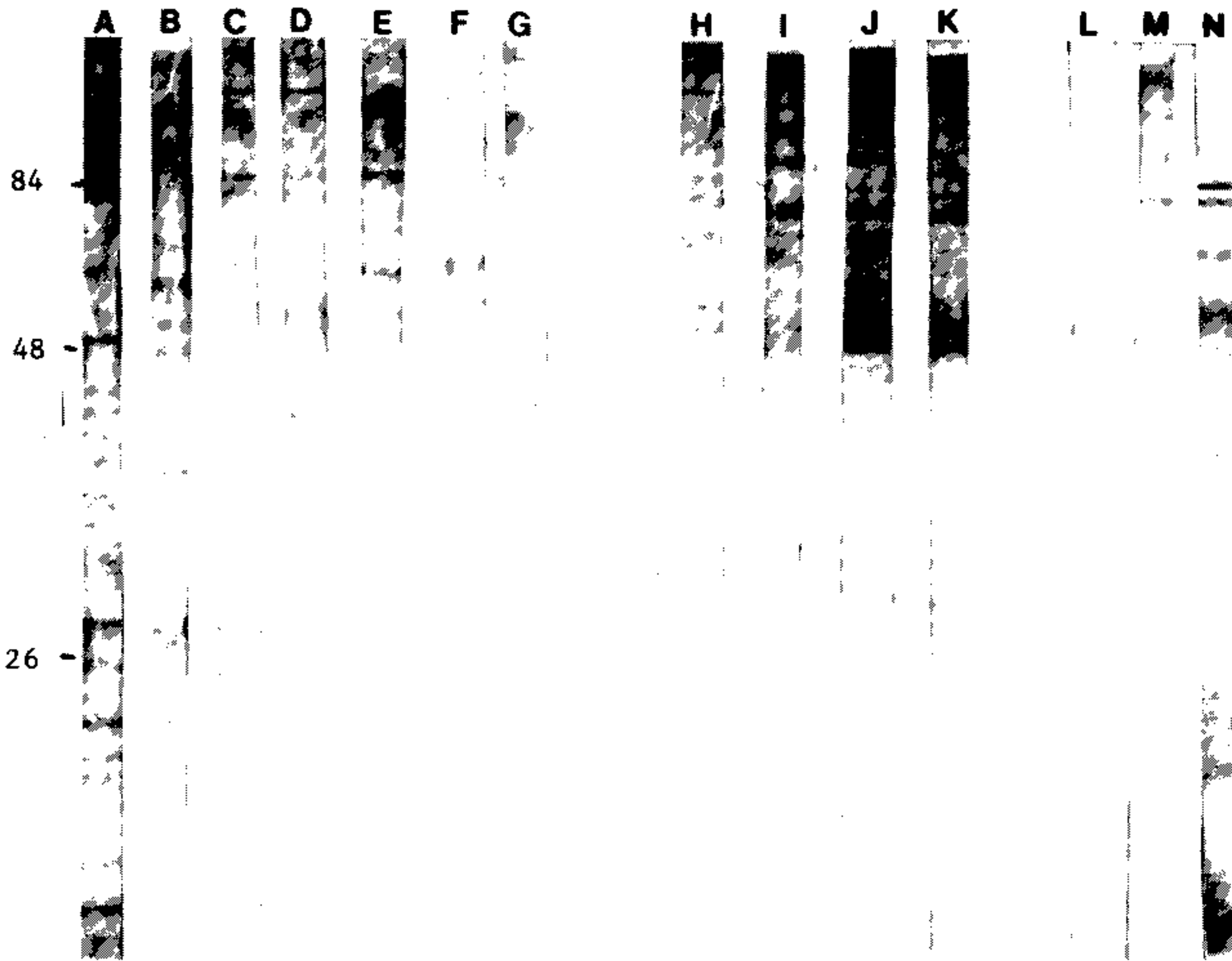
Fig. 3: immunoblots from different patients with AVL at presentation (A) and at two and one-half months (B and C), four months (D), six months (E), eight months (F), and ten months (G) after therapy at a dilution of 1/200. Cutaneous leishmaniasis (lane H-K) at a dilution of 1/50 and Chagas' disease (lane L-N) at a dilution of 1/50.

Since *Leishmania* species are in the same family as *Trypanosoma cruzi*, we examined the immunoblot pattern of patients with chronic Chagas' disease against *L. chagasi* antigen even through the ELISA with these sera was negative. A 116-120 kDa band was present at low serum dilutions (1/50), and there were other cross-reactive low molecular weight bands. The antigen recognized in the 70 kDa region by AVL sera was not recognized by the low dilution Chagas' sera (Fig. 3, lanes L-N).

To examine the possibility that detectable antibodies arise after treatment, serum from six patients, who were followed for six weeks after initiation of treatment, were blotted. After two weeks of treatment, all of the patients had resolution of fever and reduction in spleen size. As can be seen in representative patient sera in Fig. 4, no new antigens were identified by sera from these patients. Twelve additional sera from patients three months to a year after therapy were also examined. No consistent new banding patterns were seen in these blots (Fig. 3, lanes B-G).

Surface labelling – The majority of surface iodinated promastigote proteins migrated as a fairly wide band centered at 54 kDa in the *L. donovani* promastigote preparation, and at 56 kDa in the *L. donovani chagasi* preparation (Fig. 5, lanes A and C). Doublets were seen in each species in the 41-45 kDa region and at less than 20 kDa. The number of surface antigens identified by iodination was fewer than parasite antigens identified by immunoblots suggesting that only a minority of antibodies were directed against parasite surface antigens. In contrast, biotinylation revealed a complex, reproducible staining pattern which was remarkably similar to that seen after immunoblotting of AVL sera. Strongly staining bands were seen at 120, 95, 58, 46, 32-22, and 25-26 kDa (Fig. 5, B and D). Diffuse staining was seen throughout the 76-70 and 66-60 kDa regions. In addition, control promastigotes that were not biotinylated had a strong avidin binding epitope at approximately 71 kDa (not shown). There was no discernible difference in biotinylation patterns between *L. donovani* and *L. donovani chagasi.*
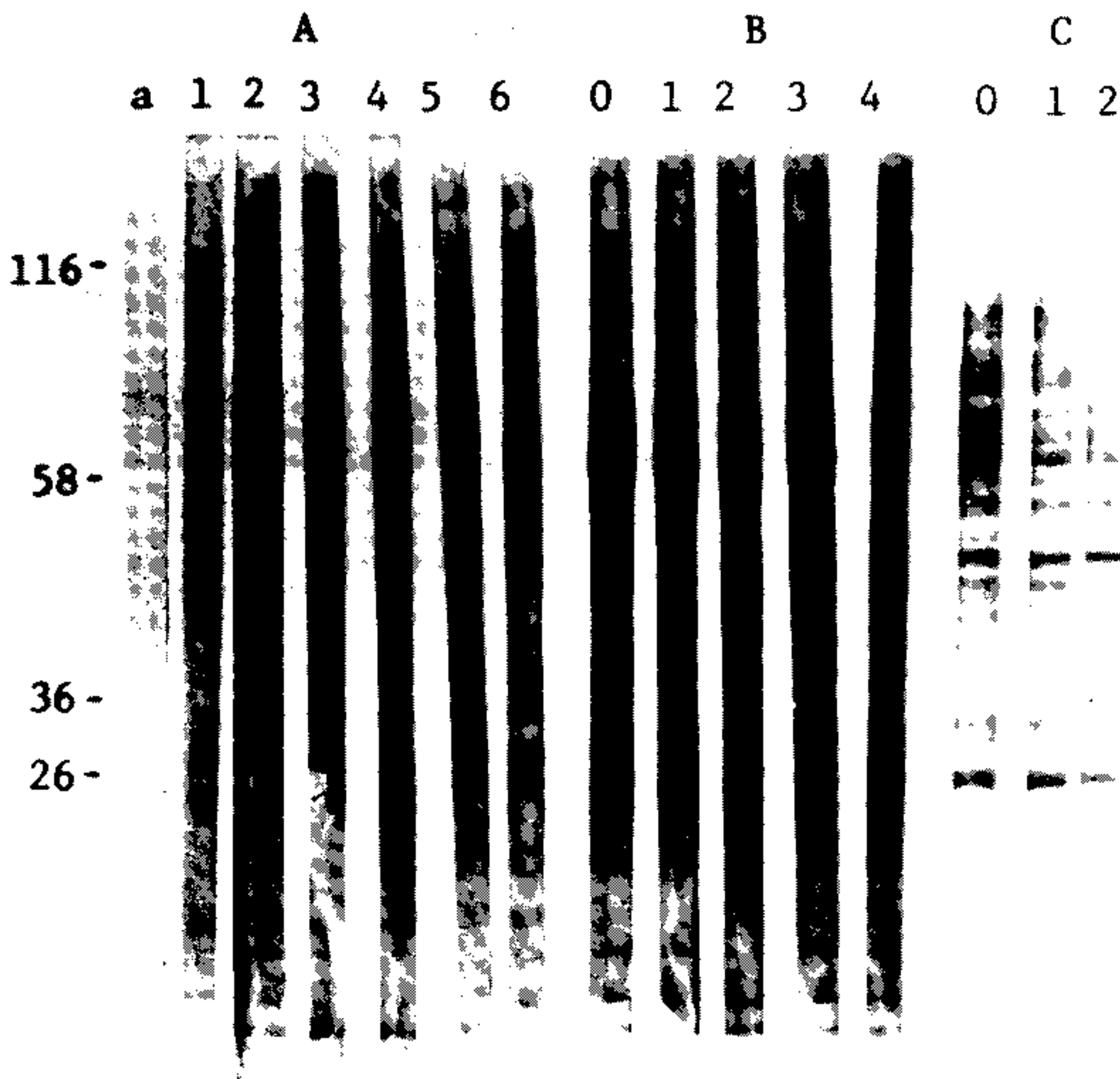
Fig. 4: lane a, no first antibody. Lanes A (1-6), B (0-4), and C (0-2) represent successive serum samples from three separate AVL patients obtained at the initiation of treatment and up to six weeks thereafter.

DISCUSSION

Visceral leishmaniasis is characterized by the production of anti-leishmanial antibodies that can be easily detected by ELISA using whole, protease-inhibitor treated promastigotes (Mohammed et al., 1985). The ELISA is a highly sensitive screening assay in symptomatic individuals (Hommel et al., 1978; Badaro et al., 1986). The use of sonicated, ultracentrifuged promastigote fractions resulted in higher background with no increase in sensitivity or specificity (data not shown). The results presented above suggest that the ELISA can discriminate between patients presenting with AVL and those with cutaneous leishmaniasis or chronic Chagas' disease. The ELISA did not differentiate between AVL and chronic mucocutaneous leishmaniasis, but mucocutaneous disease can be easily differentiated from AVL by clinical criteria. The ability of the ELISA to detect early, subclinical or asymptomatic infections remains conjectural.

Antibodies against *L. chagasi* were identified by immunoblot. We found heterogeneous antibody responses, however, a consistent pattern occurred with frequent bands at 116, 93, 74, 70, 62, 46, 32 and 26 kDa. Antibodies recognizing all but the approximately 70 kDa antigens occurred in cutaneous leishmaniasis or Chagas' disease. The one positive immunoblot among the healthy controls probably represented an early or asymptomatic case of AVL as one of the donor's siblings had confirmed AVL.

Our results are similar to those of Reed et al. (1987), who used an alkaline phosphatase technique analogous to our peroxidase method. Reed et al. (1987) identified a 66 kDa glycoprotein that was specific for AVL when electroluted from acrylamide gel and used in an ELISA. That antigen may be the same as the antigen identified at 70-72 kDa under our electrophoresis conditions. Our findings also closely parallel those of dos Santos et al. (1987) who
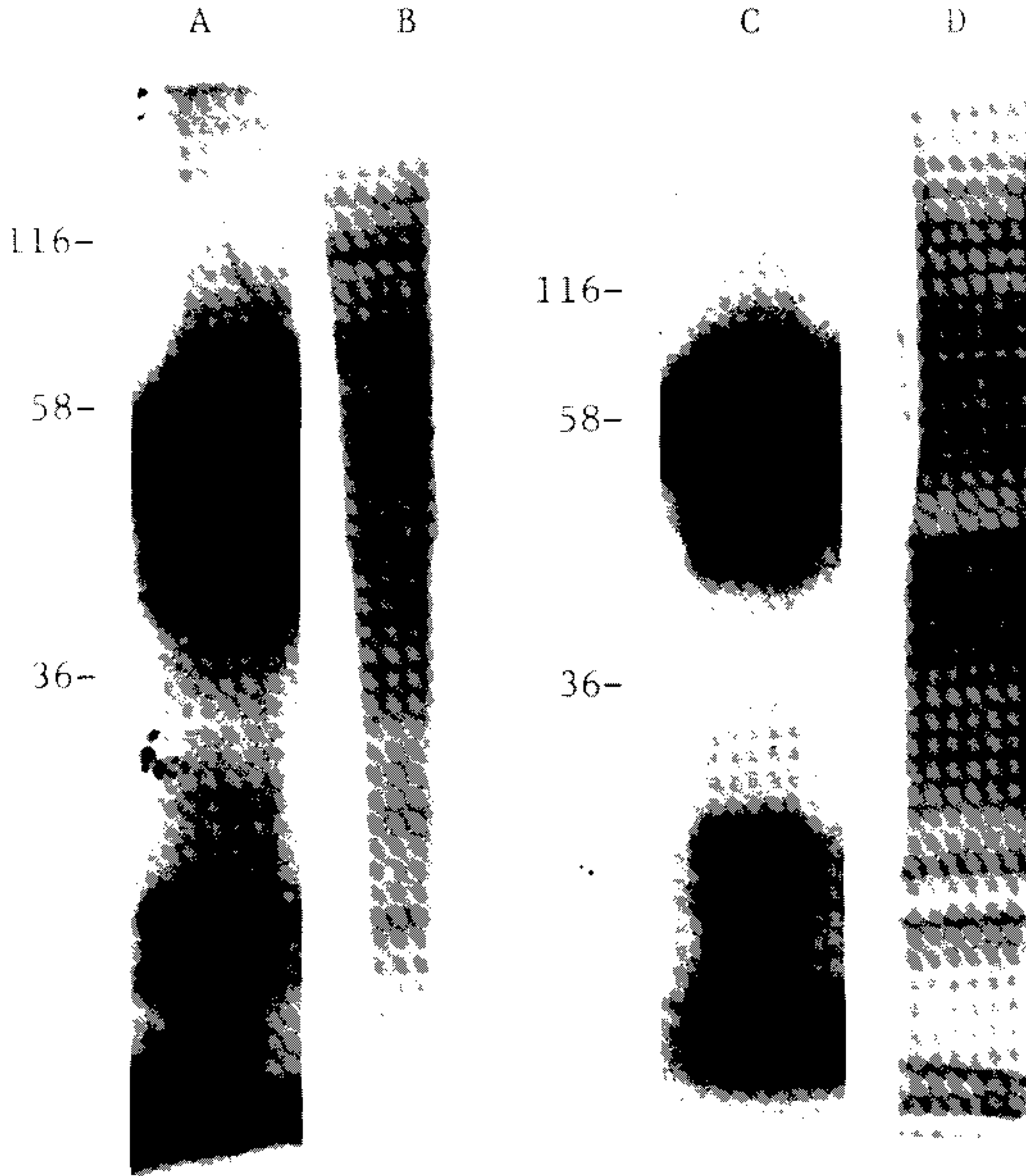
A   B   C   D

116–

58–

36–

116–

58–

36–

Fig. 5: lane A − surface iodinated *Leishmania donovani;* lane B – biotinylated *L. donovani* run on the same gel; lane C − surface iodinated *L. d. chagasi;* lane D − biotinylated *L. d. chagasi* run on the same gel.

found complex patterns. In their hands 119 and 123 kDa antigens were found to be specific.

Attempts to study leishmania-specific surface antigens have been carried out by a number of different investigators (Fong & Chang, 1983; Ramasamy et al., 1983; Etges et al., 1985; Lemestre et al., 1985). In almost all cases, the methods used involved radiolabelling with surface iodination or ^{35}S methionine incorporation. In these studies a major surface protein termed gp63 was identified at approximately 59-65 kDa under reducing conditions (Gardiner et al., 1984), and at 50 kDa under non-reducing conditions. This protein is recognized by immune sera from patients with many disparate forms of leishmaniasis. Lepay et al. (1983) and Colomer-Gould et al. (1985) attributed doublets, which they identified at approximately 46 and 23 kDa, to proteolytic

cleavage products of the larger glycoprotein. Surface iodination of our *L. chagasi* isolate yielded similar results, with a major diffuse band at 56 kDa and two lower molecular weight doublets. This band, however, was not strongly or consistently identified by human immune sera in our blots. Of interest, surface biotinylation, which requires only that a epsilon-amine group of lysine be present (Hofmann et al., 1978), gave a surface pattern similar to our AVL immunoblots. It is possible that many antibodies are directed against antigenically significant surface carbohydrate or protein epitopes not identified by standard surface radiolabelling techniques.

Finally, the antibody banding patterns remained constant in patients following therapy. The lack of newly recognized antigens is in agreement with earlier results of Reed et al.

(1987). The immunoblot patterns provide no support for the hypothesis that parasite antigens might be newly recognized after clinically successful chemotherapy.

The ELISA/immunoblot system offers a potentially sensitive and specific approach to the serodiagnosis of AVL, but the validity of this approach must still be evaluated in the field. In addition, immunoprecipitation of the 66 kDa protein of Reed or our 72 kDa protein may be a very specific marker for infection. The situation is complicated by the lack of a good marker to document subclinical or early *L. donovani* infection in which the parasites are often undetectable by traditional means. Prospective, longitudinal field trials of the above ELISA/immunoblot system are thus warranted.

RESUMO

Avaliação da resposta imune humoral na leishmaniose visceral americana pelos métodos ELISA e "immunoblot" — A leishmaniose visceral americana é uma doença importante entre crianças do nordeste do Brasil. Com o objetivo de caracterizar a resposta imune humoral durante o curso da doença, foram analisados soros de pacientes hospitalizados, pelos métodos ELISA e Western blot, usando-se preparações de antígenos de *Leishmania donovani chagasi*. O teste de ELISA foi positivo (absorbância > 0,196), numa diluição de 1:1024, em todos os pacientes no início da doença, diminuindo no decorrer do ano, para os níveis dos indivíduos controles. Apenas um dos 72 controles, que tinha um irmão com leishmaniose visceral, apresentou positividade para o teste. "Immunoblots" de soros de pacientes apresentaram bandas múltiplas, sendo as mais freqüentes de aproximadamente 116 kDa, 70 kDa e 26 kDa. Com menor freqüência foram observadas bandas de 93 kDa, 74 kDa, 62 kDa, 46 kDa e 32 kDa. As respostas, através de ELISA e dos padrões de separação por "immunoblot", foram distintas na leishmaniose visceral e podem ser usadas para diferenciar pacientes com leishmaniose visceral daqueles com doença de Chagas ou leishmaniose cutânea. As amostras sorológicas de pacientes acompanhados durante seis semanas após o tratamento, não apresentaram nenhuma banda nova. A eletroforese em gel de poliacrilamida-sulfato de dodecil de sódio (SDS-PAGE) das proteínas iodinadas da superfície do parasita mostrou uma banda principal e quatro menores, enquanto a eletroforese de proteínas

biotiniladas do parasita mostrou um padrão semelhante àquele apresentado pelos soros dos pacientes. A leishmaniose visceral parece produzir um padrão de "immunoblot" característico que pode ser usado, juntamente com um método sensível tipo ELISA, no diagnóstico de leishmaniose visceral americana.

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