ANTIBODIES TO LAMININ IN CHAGAS' DISEASE*

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Chagas' disease occurs in Central and South America in individuals infected with the protozoan *Trypanosoma cruzi.* In the acute stage of the disease, subcutaneous swelling around the eye (chagoma), myocarditis, encephalitis, and fever are observed. These conditions are related to the invasion of the tissues by the parasite (1). Years later, in the chronic stage of Chagas' disease, when few, if any, parasites are present in the tissues, inflammatory, degenerative, and fibrotic changes are observed, particularly in the endocardium, myocardium, esophagus, and colon (1, 2). Such lesions can be life threatening (1).

Immunological causes are thought to underlie the chronic lesions in Chagas' disase (3). Sera from both acute and chronic cases of Chagas' disease show reactivity with the parasite as well as with normal endocardium, blood vessels, and the interstitium of striated muscle (the so-called EVI[1] pattern), and Schwann sheaths of peripheral nerve (4–6). Studies on skeletal muscle biopsies of individuals infected with *T. cruzi* indicate the existence of immunoglobulins (Ig) with a similar distribution (4). The extent of the clinical alterations found in individuals with chronic Chagas' disease was correlated with these tissue-reacting antibodies in one (4) but not in other geographical areas (7). These antibodies were not found to occur in various other heart diseases examined (4). Here, we demonstrate that these antibodies react with connective tissue structures. We also have assayed purified components of connective tissues for their reaction with the antibodies present in humans and in Rhesus monkeys infected with *T. cruzi.* Our studies indicate that these antibodies are specific for laminin (8), a basement membrane glycoprotein, which mediates the attachment

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[1] Abbreviations used in this paper: ANA, antinuclear antibodies; CHO, chinese hamster ovary; EHS tumor, Engelbreth-Holm-Swarm tumor; ELISA, enzyme-linked immunoabsorbent assay; EVI, endocardium, blood vessels and interstitium; FN, fibronectin; IF, immunofluorescence; LIT, ox liver-infusion tryptose; PBS, phosphate-buffered saline; pepsin fragments of laminin: Pl, Mr = 285,000; Pli, Mr = 272,000; Pb, Mr = 70,000; Pc, Mr = 52,000.

of epithelial and endothelial cells to type IV collagen (9).

Materials and Methods

Patients. Sera were examined from nine patients, ages 18–40, with chronic Chagas' disease (five with cardiomegaly and three with megacolon) in whom the infection had been present for at least 5 yr (mean 7 yr). In addition, sera were studied from six patients between the ages of 3 mo and 4 yr in whom acute Chagas' disease was present. Each child had unilateral chagoma of the eye and acute myocarditis within the last 2 mo, and the diagnosis was confirmed by identifying parasites in blood smears. All patients had a positive serology (titers ≥1:64) for *T. cruzi* by the indirect immunofluorescence (IF) test using cultured epimastigote forms of the parasite (Tulahuen strain) (10). Sera of 20 normal individuals and 10 patients with inflammatory diseases other than Chagas' disease (3 with osteomyelitis, 4 with paracoccidioidomycosis, and 4 with bacterial meningitis) from the same geographical areas (Argentina and Brazil), all showed a negative reaction for *T. cruzi* (10). Additional negative controls included sera from 10 patients with surgical wounds and 10 with invasive neoplasms of the breast and digestive system, 10 with toxoplasmosis, 10 with rheumatoid arthritis, and 10 with malaria, all from the United States.

Infected Monkeys. The insect *Dipetalogaster maximus,* infected 3 mo earlier with blood from an acute case of Chagas' disease, was kindly supplied by Dr. P. Marsden from the University of Brasilia, Brasilia, Brazil. Two Rhesus monkeys were infected under the eyelid with 1,000 metacyclic trypomastigotes obtained from the feces of the insect. After infection of the monkey, *T. cruzi* trypomastigotes were observed in blood smears, and 1 mo after infection the animals were found to have developed antibodies against the parasite (titer ≥1:64) (10).

Parasites. Human foreskin fibroblasts strain CRL 1475 (kindly provided by Dr. W. Gleiber, National Institutes of Health), which do not synthesize laminin, were grown in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) with 4% fetal calf serum (Gibco Laboratories). When confluent, cells were infected with blood trypomastigote forms of *T. cruzi,* Tulahuen strain (kindly provided by Dr. T. Mercado, National Institutes of Health). The amastigote and trypomastigote stages were obtained from the supernatant fluids from cultures 5 d after infection. Epimastigotes were cultured in ox liver-infusion tryptose (LIT) medium pH 7.2 as described (11). Blood trypomastigotes were purified as described (12). The parasites were fixed with 0.5% paraformaldehyde, allowed to dry on slides, and used as antigens in an indirect IF test (10). The purified antibodies were used at a concentration of 50 μg/ml. The unbound fraction was reconstituted to the original serum volume and used diluted 1:10.

Laminin. Laminin was extracted from the Engelbreth-Holm-Swarm (EHS) tumor grown in C57 Bl mice and was isolated as previously described (8). For additional purification, 10 mg of laminin, dissolved in 1 ml of 8 M urea, 0.05 M Tris-HCl, pH 8.6, was applied to a column of DEAE-cellulose (2.5 × 25 cm) and eluted with a gradient of 0–0.3 M NaCl. Portions of the collected fractions were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis or tested as antigen in an enzyme-linked immunoabsorbent assay (ELISA) (sera were diluted 1:50, and purified antibodies were used at 50 μg/ml) (see below). Various fragments of mouse laminin (Pl from soluble laminin and Pli, Pb, and Pc from insoluble laminin), obtained from laminin by digestion with pepsin (Boehringer, Federal Republic of Germany) (20 mg/g laminin), were a gift from Dr. Rupert Timpl, Max Planck Institut für Biochemie, Martinsried, Federal Republic of Germany (13). Laminin was also tested after reaction with an equal weight of trypsin and *Streptomyces griseus* protease (Sigma Chemical Co., St. Louis, MO). Each of these antigens were tested at a concentration of 500 ng of antigen per well.

Gel electrophoresis of the protein precipitated by serum from either control or infected monkeys, or of the affinity-purified antibodies, was carried out as described (8). Rabbits and sheep were immunized with laminin as previously described (8). The sera obtained from these animals, as well as sera from monkeys infected with *T. cruzi,* were chromatographed on a column of laminin-Sepharose (8). Unbound material was washed from the column with 0.02 M phosphate-buffered saline (PBS), pH 7.4, and the bound material containing the affinity-purified antibodies was eluted with 0.5 M NaCl, 0.5 M acetic acid, pH 3. The fractions

containing protein were adjusted to pH 7, dialyzed against PBS, and concentrated by ultrafiltration.

Immunoassays. Indirect IF (4) was used to test the reaction of antibodies obtained from humans and monkeys with freshly prepared cryostat sections of mouse peripheral nerve, skin, kidney, heart, and the EHS mouse tumor. The protease inhibitors *p*-hydroxymercuribenzoate (50 μg/ml) and phenylmethylsulfonyl fluoride (50 μg/ml) (Sigma Chemical Co.) were present during all steps of the reaction. In some experiments, the same section was exposed first to antibodies from infected monkeys purified by laminin-Sepharose affinity chromatography (50 μg/ml) and then to antilaminin antibodies purified from rabbit serum (50 μg/ml). The tissue distribution of these antibodies was established with species-specific secondary fluorochromes (rhodamine conjugated goat anti-human IgG and fluorescein conjugated goat antirabbit IgG) (N. L. Cappel Laboratories, Inc., Cochranville, PA).

Attempts were made to identify masked antigens by treating the tissue sections for 3 h with chondroitinase ABC (50 U/ml) (Miles Laboratories, Elkhart, IN) (14), testicular hyaluronidase (40 mg/ml) (Type IV, Sigma Chemical Co.), or purified bacterial collagenase form III (500 U/ml), Advance Biofactures, Lynbrook, NY) (15) before reaction with antibodies.

The blocking reaction with collagen types I, III, IV, or V, fibronectin (FN), BM-1 proteoglycan, chondronectin, laminin, or bovine serum albumin was performed at antigen concentrations of 150 μg/ml for 1 h at 37°C and overnight at 4°C. The antigens used were obtained from the following sources: type I collagen from lathyritic rat skin (16), type III collagen from a pepsin digest of fetal calf serum (17), type IV collagen from the EHS tumor (18), type V (α-AB) from calf skin (19), FN from mouse serum (20), BM-1 proteoglycan from the EHS tumor (21), and chondronecin from chicken serum (22). When one of these materials inhibited fluorescence, a titer was obtained by absorbing the sera with different concentrations of antigen. The reactivity of sera from humans or Rhesus monkeys infected with *T. cruzi* with these components of connective tissue and laminin fragments (see above) were also tested by ELISA, using a concentration of 500 ng of antigen per well (23).

The class of antibodies reacting with parasites tissue sections or laminin was assessed by immunofluorescence or by ELISA using fluorescein or peroxidase-conjugated goat antihuman IgG or IgM (N. L. Cappel Laboratories, Inc.). Radial immunodiffusion of antibodies with laminin was examined by Ouchterlony immunodiffusion at 4°C using 1% agarose by standard procedures.

To demonstrate that laminin does not bind nonspecifically to other IgG molecules, we preincubated two sera containing antinuclear antibodies (ANA) with 150 μg/ml of laminin for 1 h at 37°C and overnight at 4°C, before performing the immunofluorescent reaction. Nuclei of hepatocytes were used as antigen.

Attachment Assays. The attachment to a type IV collagen substrate of endothelial cells from sheep aorta, or of fibroblastic cells of the Chinese hamster ovary (CHO) line to plastic dishes, was measured in the presence and absence of sera or antibodies from infected primates as previously described (9).

Results

Distribution of the Antigen. In preliminary studies, a careful analysis was made of the histological structures that were stained by the tissue-reacting antibodies in Chagas' patients and infected monkey sera. In general, they reacted in those areas of tissue where basement membranes were present. The pattern resembled that obtained when antibodies to isolated basement membrane components, such as laminin, were used (8). As discussed below, the tissue-reacting antibodies could be isolated by laminin Sepharose affinity chromatography.

The reaction of antibodies from infected monkeys purified by laminin-Sepharose affinity chromatography (Fig. 1, first column) was compared with the reaction of rabbit antilaminin antibodies (Fig. 1, second column). Both antibodies showed an identical and intense staining of the extracellular matrix of the EHS tumor (not shown), and of peripheral nerve in the perineurium, epineurium, and capillaries, and

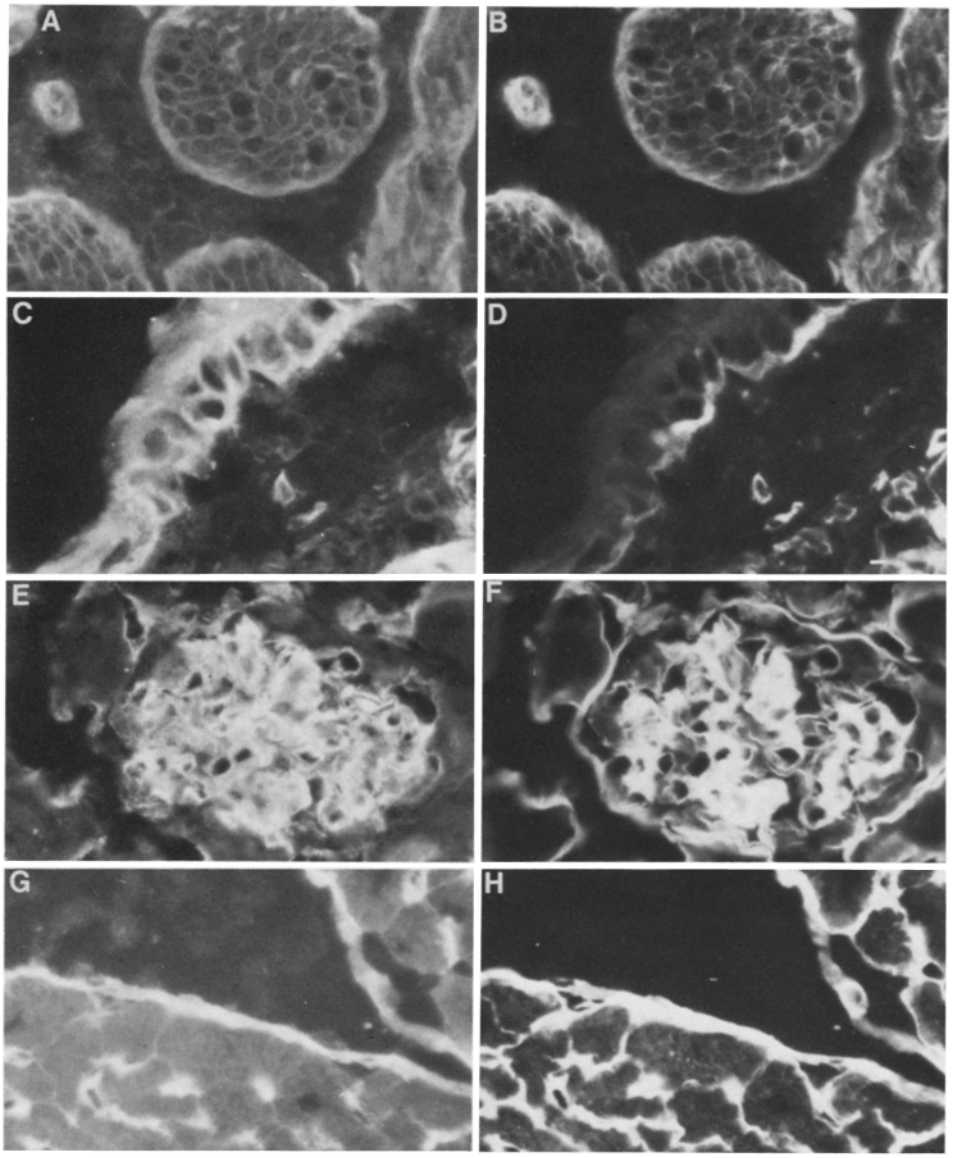

FIG. 1. Comparison by double immunofluorescence histochemistry of the tissue distribution of purified antibodies from monkeys infected with *T. cruzi* (first column) or from rabbits immunized with laminin (second column). Sections of mouse peripheral nerve (A and B), skin (C and D), kidney (E and F), and heart (G and H) are shown.

a less intense staining of myelin sheaths (Fig. 1 A and B). Differences in IF were observed with other tissues. The antibodies from infected monkeys showed a broad distribution along the epidermal-dermal junction of skin, including the epidermis (Fig. 1 C), whereas the antilaminin antibodies from rabbits showed a linear distribution along the epidermal-dermal junction (Fig. 1 D). In the kidney, the antibodies from infected monkeys strongly stained peritubular capillaries, glomerular endothelium, and mesangial matrix; but reacted only very weakly with the glomerular and

tubular basement membranes and did not stain Bowman's capsule (Fig. 1 E), whereas rabbit antilaminin antibodies strongly stained all kidney basement membranes (Fig. 1 F). In the heart, the antibodies from infected monkeys localized in the endocardium, vessels, and interstitium in a diffuse distribution (Fig. 1 G) compared with the fine double line in certain regions with rabbit antilaminin antibodies (Fig. 1 H).

Prior treatment of tissue sections with chondroitinase ABC, hyaluronidase, and bacterial collagenase did not change the distribution or the intensity of the staining with antibodies from infected primates. The whole sera from monkeys and humans infected with *T. cruzi* reacted with the same histological structures as the purified antibodies from infected monkeys. Laminin (25 μg/ml) completely blocked the reaction while other purified connective tissue components (i.e., collagen types I, III, IV, and V, FN, BM-1 proteoglycan, or chondronectin) had no effect.

Immunochemical Characterization. ELISA assays indicated that sera from both acute and chronic patients contained antibodies to laminin and that the highest titers were present in the acute phase (Table I). No significant reaction of sera from patients infected with *T. cruzi* was observed with type I, III, IV, or V collagen, FN, BM-1 proteoglycan, or chondronectin. Sera from the various other healthy and diseased humans or from uninfected monkeys did not react with tissues as judged by immunofluorescence and did not contain antibodies to laminin as judged by ELISA tests (titers ≤1:64).

Monkeys and humans with acute *T. cruzi* infection had high titers (titers ≥1:6000 and ≥1:1024, respectively) of IgM and IgG antibodies, which reacted with tissues and with laminin. The sera at the chronic stage of the infection had no reacting IgM (≤1:2) and lower titers of IgG (1:500). The shift from IgM and IgG antibodies present

TABLE I

*Immunofluorescence (IF) and ELISA Titers for Antibodies to Connective Tissue Components in Sera from Patients Infected with Trypanosoma cruzi**

	Clinical stage	IF‡	ELISA							
			Laminin	Collagen type				FN	BM-1 Proteoglycan	Chondronectin
				I	III	IV	V			
Chagas' patients	Acute $n = 6$	≥1024 *(1024–8192)*§	≥4096 *(4096–16384)*	≤64	≤32	≤64	≤32	≤32	≤32	≤32
	Chronic $n = 9$	≥512 *(512–2048)*	≥512 *(512–2048)*	≤64	≤32	≤64	≤32	≤32	≤32	≤32
Controls from endemic area	Normal $n = 20$	≤32‖	≤32‖	≤64	≤32	≤32	≤32	≤32	≤32	≤32
	With other inflammatory diseases $n = 10$	≤64	≤64	≤32	≤64	≤32	≤32	≤32	≤32	≤32

Statistically significant values are italicized.

* Goat anti-human IgG was used as a second antibody.

‡ Indirect IF test using heart, skin, and EHS tumor cryostat sections as antigens. Each serum sample showed the same endpoint titer with all of the tissues studied.

§ The numbers in parenthesis represent the range of values.

‖ Two sera had antibody titers of 128.

at 3 mo of infection to only IgG antibodies at 12 mo after infection in monkeys was verified with the purified antibodies by immunoelectrophoresis and radial immunodiffusion.

Laminin was precipitated from solution by sheep antilaminin antibodies or by serum or antibodies purified from infected monkey serum. The presence of laminin in the precipitate was established by electrophoresis, based on the presence of two chains with M_r = 400,000 and 200,000 (Fig. 2). In addition, purified antibodies obtained from infected monkeys were found to precipitate laminin in a single line of identity with sheep antilaminin antibodies (data not shown).

When laminin was chromatographed on DEAE-cellulose under dissociating conditions (8 M urea), a single peak was eluted (Fig. 3). Electrophoresis of the fractions corresponding to this peak revealed the characteristic chains of laminin. The same peak contained material that reacted in ELISA tests with the serum from infected monkeys and sheep antilaminin antibodies (Fig. 3), the purified antibodies from infected monkeys, or serum from infected humans (not shown). No reaction was

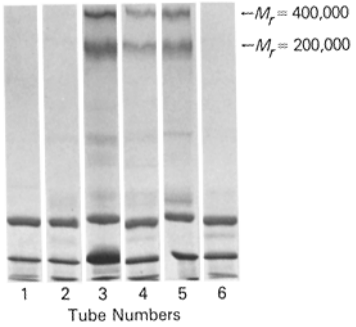

FIG. 2. Gel electrophoresis of laminin immunoprecipitated with normal sheep serum (lane 1), normal monkey serum (lane 2), sheep antilaminin antiserum (lane 3), serum from a monkey infected with *T. cruzi* (lane 4), antibodies purified by laminin affinity chromatography of 4 (lane 5), and unbound fraction after laminin affinity chromatography of 4 (lane 6). Bands of M_r = 200,000 and 400,000 correspond to laminin.

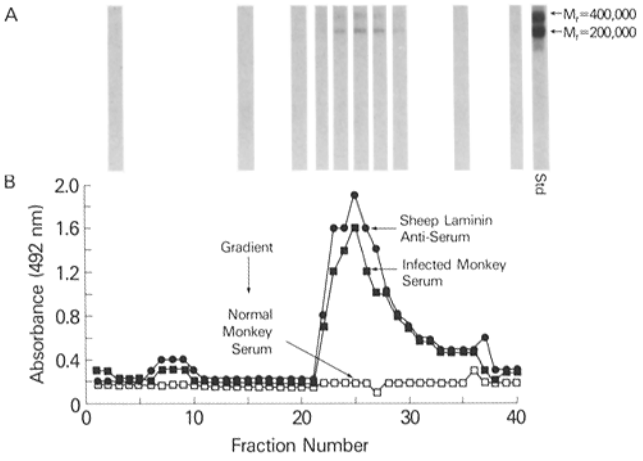

FIG. 3. Identification of laminin after purification by DEAE cellulose chromatography. Each fraction was subjected to sodium dodecyl sulfate gel electrophoresis (A) and was assayed for laminin by ELISA (B). Std., laminin standard.

observed with normal monkey sera (Fig. 3) or with the fraction of infected monkey serum not bound to laminin-Sepharose.

The specific antigenic site that reacted with antibodies from the infected monkeys and sheep antilaminin antibodies was further characterized by testing various protease-derived fragments of laminin in the ELISA assay. The antigenic activity of both antibodies was retained in the Pl and Pli fragments of laminin (M_r = 280,000) produced by digestion with pepsin; however, when laminin was treated with *Streptomyces griseus* protease or trypsin, its reactivity was lost (Table II). Laminin did not bind nonspecifically to other IgG molecules, because laminin did not block antinuclear antibodies from binding to the nuclei of hepatocytes.

Reaction with Parasites. Antibodies (both the IgM and IgG types) from infected monkeys, purified by laminin-affinity chromatography, produced a very strong reaction with trypomastigotes and amastigotes, but showed only a weak reaction with epimastigotes when used in the indirect IF technique. Laminin (25 μg/ml) completely blocked this reaction. In contrast, the antibodies present in the infected serum, which did not bind to the laminin affinity column and which did not stain tissues, intensely stained the epimastigotes and amastigotes, but only weakly stained the trypomastigotes.

Functional Studies. Laminin is the attachment protein that binds endothelial cells to type IV collagen (9). Serum from infected monkeys (Fig. 4), as well as serum from Chagas' patients, similarly inhibit the attachment of endothelial cells to type IV collagen. In addition, purified antibodies from infected monkeys (50 μg/ml) inhibit the attachment of these cells. This inhibition was reversed by the addition of laminin to the medium in which the cells were suspended (Fig. 4). As expected, the attachment of CHO fibroblasts, which is mediated by FN, was not inhibited by these antibodies (Fig. 5).

Discussion

Humans and monkeys infected with *T. cruzi* produce antibodies that react with extracellular structures in some areas where basement membranes are present. Their

TABLE II

Reaction of Laminin and Laminin Fragments with Antibodies Using the ELISA Technique

Substrate	Antibodies purified by affinity chromatography to laminin		Normal monkey serum‡	Normal sheep serum‡
	Laminin-immunized sheep*	*T. cruzi*-infected monkey*		
Laminin	*1.2*	*1.4*	0.1	0.1
Laminin plus trypsin	0.1	0.1	0.1	0.1
Laminin plus *S. griseus* protease	0.1	0.1	0.1	0.1
Laminin pepsin-derived fragments				
Pb	0.2	0.2	0.1	0.1
Pc	0.1	0.2	0.1	0.1
Pl	*0.8*	*0.4*	0.1	0.1
Pli	*0.8*	*0.8*	0.1	0.1

Detected with peroxidase-conjugated goat antihuman IgG. Absorbance was read at 492 nm. Statistically significant values are italicized.

* At a concentration of 50 μg/ml.

‡ Diluted 1:10.

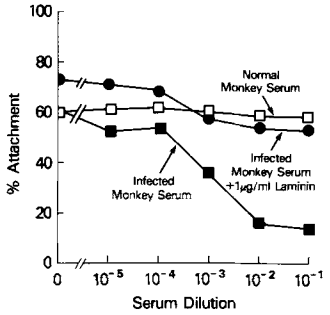

FIG. 4. Inhibition by monkey serum of attachment of endothelial cells to type IV collagen. The serum from an infected monkey inhibits the attachment to type IV collagen, and is reversed by added laminin. Normal monkey serum has no effect.

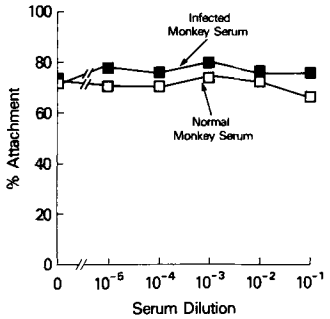

FIG. 5. Lack of inhibition by monkey serum of attachment of CHO cells. Serum from an infected monkey and normal monkey serum has no effect.

sera were found to contain high titers of antibodies to laminin but not to various other connective tissue components.

Several different tests were used to confirm the identification of laminin as the tissue antigen. These studies included immunoprecipitation of laminin, absorption of the antibodies to laminin covalently bound to Sepharose beads, reaction of laminin with the antibodies in the Ouchterlony test, binding of antibodies to laminin-coated wells (the ELISA test), and inhibition of the attachment of endothelial and epithelial cells (not shown). For preliminary studies using the ELISA technique, we have found that laminin obtained from human amnion is able to block the reactivity of laminin antibodies obtained from infected primates to laminin obtained from the EHS tumor (A. Szarfman, V. P. Terranova, and G. R. Martin, unpublished observation). The Pl and Pli fragments of laminin produced by proteolytic digestion shows a high degree of reaction with these antibodies. Furthermore, it is likely that laminin represents the major, or even the only, tissue antigen in the EVI system, because passage of sera from Chagas' patients or infected monkeys over laminin-Sepharose beads eliminates the tissue reactions.

Direct comparison of the reaction of these antibodies with rabbit antibodies to laminin in tissue sections revealed similar, but not identical, reactions. The antigen in tissues reacting with antibodies from infected monkey serum is more labile than the antigen detected by antilaminin antibodies produced in rabbits (data not shown). Furthermore, rabbit antilaminin antibodies stained all basement membranes uni-

formly, whereas the antibodies from infected primates showed a more limited distribution. However, both sera stained the matrix of the EHS tumor, which is thought to be solely basement membrane (18). It is likely that these two antibodies recognize different antigenic sites in laminin. The sheep or rabbit antibodies are probably heterogeneous and directed against a variety of antigenic sites in the laminin molecule. The antibodies arising after *T. cruzi* infection may recognize fewer and different antigenic determinants in laminin. The interaction of laminin with other components of the basement membrane, such as type IV collagen and the heparan sulfate proteoglycan, might mask some antigenic sites and make the protein unreactive with antibodies which recognize a limited number of antigenic determinants. Therefore, laminin may be a heterogeneous family of proteins or could exist in several stages of processing which could affect the reactivity and specificity of the antigen.

After infection by *T. cruzi,* antibodies are produced against laminin and not against any of the other known basement membrane or connective tissue components. Furthermore, production of antibodies against laminin is a distinctive feature of *T. cruzi* infection in primates. Such antibodies are not present in sera from normal individuals, persons who have undergone surgery, or patients with various inflammatory conditions or neoplasms, malaria, toxoplasmosis, or rheumatoid arthritis. However, we have found high titers of antibodies to laminin in Rhesus monkeys infected with *Trypanosoma rhodesiense* (A. Szarfman, K. Esser, V. P. Terranova, and G. R. Martin, unpublished observation). It is possible that the induction of antibodies to laminin is associated with trypanosomal infections.

It is not clear why *T. cruzi* infection induces antibodies to laminin. The titer of antibodies is highest during the early phase of the infection as would be expected if laminin or an immunologically related protein were produced by the parasite. In this regard, we have found that the trypomastigote and amastigote forms of the parasite have a higher reactivity with purified antibodies from infected monkeys than the epimastigote forms. Furthermore, recent studies have shown that sera from Chagas' patients increase the attachment and penetration of blood trypomastigotes into WI 38 cells (24), and that purified antibodies to laminin decrease the penetration of the parasites (Dr. G. A. Schmuñis, personal communication). Because the trypomastigote form of *T. cruzi* encounters basement membranes during its penetration into the host, the production of a laminin-like protein and or incorporation of host laminin may permit the parasite to interact with the host tissues.

The significance of the antilaminin antibodies to the pathology of Chagas' disease is unclear. As shown here, the antibodies in infected monkeys that react with laminin are able to block the attachment of endothelial cells to basement membranes, and this could damage blood vessels and endocardium leading to the endocardial fibrosis found in Chagas' disease (1) and in African Trypanosomiasis (25). The initial changes would arise during the parasitic invasion of the host, and the changes that occur later on would be part of a slow destructive process secondary to autoimmune reactions (26, 27). The study of the antilaminin antibodies that appear after infection with *T. cruzi* holds considerable promise of yielding important information of the interactions of the parasite and it's host. In addition, when immunization with *T. cruzi* is taken into consideration, these antibodies can become useful in producing antigenic preparations of *T. cruzi,* void of host tissue components.

Summary

We have found that sera from humans with Chagas' disease and Rhesus monkeys infected with *Trypanosoma cruzi* contain IgM and IgG antibodies, which react with structures in a variety of connective tissues. These antibodies react with laminin but not with various other purified connective tissue components like collagen types I, III, IV, and V, fibronectin, heparan sulfate (BM-1) proteoglycan, or chondronectin. The tissue-reacting antibodies were isolated by absorption to a laminin-Sepharose column. The bound fraction contained all the tissue-reacting antibodies. These antibodies strongly stained trypomastigotes and amastigotes, but weakly stained epimastigotes. These studies show that sera from *T. cruzi*-infected primates contain antilaminin antibodies, which may be produced by those host in response to a laminin-like molecule present in the parasite.

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