INFECTION AND IMMUNITY, Apr. 1988, p. 993–997
0019-9567/88/040993-05$02.00/0

Vol. 56, No. 4

Antiheart Antibody-Dependent Cytotoxicity in the Sera of Mice Chronically Infected with *Trypanosoma cruzi*

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Received 6 August 1987/Accepted 11 December 1987

Sera of mice chronically infected with *Trypanosoma cruzi* contain antibodies that bind to the surface of living adult syngeneic heart muscle cells. In a syngeneic system, with nonadherent spleen mononuclear cells as effector cells and cardiocytes as targets, antibody-dependent cytotoxicity (ADCC), revealed by the liberation of creatine phosphokinase from damaged cardiocytes, was observed after incorporation of serum samples from infected mice. Target damage was decreased after absorption with syngeneic myocardium, but absorption with *T. cruzi* epimastigotes or trypomastigotes or with syngeneic skeletal muscle had no effect on ADCC. No complement-dependent lysis against heart muscle cells was detected in the same serum samples. These observations indicate that serum from chronically chagasic mice contain antibodies that bind to the surface of living adult syngeneic cardiocytes and are able to exert ADCC, suggesting that they could play a role in the pathogenesis of the heart damage that occurs in Chagas' disease.

South American trypanosomiasis, or Chagas' disease, affects several million people in Latin America. About 10 to 15% of humans infected with *Trypanosoma cruzi* develop a chronic cardiopathy 10 to 20 years after infection, characterized by abnormalities of the electrocardiogram and/or cardiomegaly and heart failure (17). Recently, by the use of more sophisticated diagnostic tools, it has been demonstrated that most of the 85 to 90% apparently healthy individuals display some kind of subclinical heart abnormality, indicating that the heart is almost always damaged in chronic infection with *T. cruzi* (2). In the symptomatic stage of chagasic cardiopathy, a progressive loss of the heart muscle cells (HMC), scarring, and mild mononuclear interstitial infiltration constitute the morphologic hallmarks of the disease (1); usually, no parasites are seen at the site of the lesions (15). The latter fact leads to the postulation of indirect mechanisms as being responsible for the tissue damage. During the last decade, evidence has accumulated to suggest that cell-mediated immune mechanisms could be operating in the production of lesions (20).

Although antiheart antibodies have been observed in human (4) and experimental (14) Chagas' disease, their importance as inducers of tissue damage is unknown.

In the present report we show that serum samples from mice chronically infected with *T. cruzi* contain antibodies able to induce antibody-dependent cytotoxicity of nonadherent spleen mononuclear cells against syngeneic adult cardiocytes and suggest that antibody-dependent cytotoxicity (ADCC) can be a mechanism of heart damage in chronic Chagas' disease.

MATERIALS AND METHODS

Induction of chronic Chagas' disease. Chronic Chagas' disease was induced as previously described (12). In brief, 3-month-old female BALB/c mice were each infected with 25 trypomastigotes of the Tulahuen strain of *T. cruzi*. Parasitemia was determined weekly, and surviving animals which showed circulating parasites during the first month postinfection (p.i.) were used for the experiments. At 1, 2, 3, and 4 months p.i., animals were killed by exsanguination, sera were collected and kept at −20°C, and the hearts were removed from the animals for histological study. Control serum samples and hearts were obtained from noninfected mice of matching age and sex.

Preparation of heart cell suspension. Hearts of noninfected 3-month-old female BALB/c mice were removed aseptically, cut into 1-mm^3 fragments in Hanks balanced salt solution, washed once in Dulbecco phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} (Sigma Chemical Co., St. Louis, Mo.), and incubated in PBS containing 1 mg of trypsin (Sigma) per ml and 1 mg of collagenase (Sigma) per ml at 37°C with constant agitation. The supernatant containing released cells was removed every 10 min into an equal volume of cold RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) until most of the myocardium was disrupted. The pooled cell suspension was sedimented at 1 × *g* for 30 min at 4°C, and the supernatants were washed three times with cold RPMI 1640 medium by centrifugation for 5 min at 100 × *g*. Phase-contrast microscopy of the collected cells showed that they were isolated single cardiocytes with more than 95% viability according to the Trypan blue dye exclusion test.

Preparation of splenocyte suspension. The spleens of noninfected BALB/c mice were removed aseptically into Hanks balanced salt solution and passed gently through a 100-mesh steel screen. The cells were centrifuged at 150 × *g* for 15 min, and the pellet was suspended in a buffered ammonium chloride solution for 1 min at room temperature to lyse erythrocytes. The suspension was diluted in RPMI 1640 medium and centrifuged in a Ficoll-Hypaque gradient. The mononuclear fraction was collected in RPMI 1640 medium containing 5% fetal calf serum and incubated for 2 h in 10-cm-diameter plastic petri dishes to remove adherent cells. The supernatant, containing the nonadherent cells, was washed three times in RPMI 1640 medium, cells were counted, and viability was assessed by the Trypan blue exclusion test.

Immunohistochemistry. Isolated syngeneic HMC were suspended for 1 h at room temperature in serum from noninfected or chagasic mice diluted 1/20 in PBS (pH 7.2). After three washings in PBS, cells were centrifuged at 150 ×

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g, suspended in small drops of PBS, extended on glass slides, dried at room temperature, and fixed in cold (4°C) acetone. Smears were incubated for 1 h at room temperature with anti-mouse immunoglobulin G (IgG) goat antiserum (Dako, Copenhagen, Denmark), thoroughly washed in PBS, and posttreated for 30 min with mouse peroxidase-antiperoxidase complex (Dako). Enzyme activity was demonstrated by incubation in 0.1% diaminobenzidine chlorhydrate in 0.01% hydrogen peroxide.

ADCC assay. Isolated HMC (10^5) were incubated with syngeneic nonadherent spleen cells in a 1/30 target/effector cell ratio in RPMI 1640 tissue culture medium containing 10% fetal calf serum. For determination of ADCC, heat-inactivated (56°C for 60 min) serum from either chagasic or noninfected mice was added to obtain a final dilution of 1:40. The samples were then incubated for 16 h at 37°C in a 5% CO_2 atmosphere. At the end of the incubation, cells were spun down by centrifugation at 400 × *g* for 15 min, and cell damage was estimated by the liberation of creatine phosphokinase (CK) to the supernatant. Triplicate measurements were made with serum from each mouse. As a control, tubes with the same number of HMC were incubated in parallel under the same conditions with tissue culture medium alone, with chagasic serum without mononuclear spleen cells, or with splenocytes alone. Enzyme activity was determined by a kinetic enzymatic method, using a commercial kit (CK-NAC; Boehringer Mannheim Biochemicals). ADCC was estimated as the percentage of the total activity of a similar number of HMC lysed with 1% Triton X-100 which was liberated to the supernatant, by using the following equation: ADCC (%) = [(CK released with test serum − CK released without serum)/(Total CK)] × 100. For the cytotoxicity assay, the liberation of CK was used instead of the widely used ^{51}Cr assay to determine cell damage, because in previous experiments a marked liberation of the isotope was noticed after 16 h of incubation. This handicap was not present when shorter periods were used. For that reason, we used that assay to study complement-dependent lysis. CK is an enzyme present in high concentration in striated muscle, and its determination in serum is currently used for the diagnosis of myocardium infarction or skeletal muscle destruction in humans. Since CK is practically absent in mononuclear spleen cells, it can be safely assumed that the liberated enzyme originated almost exclusively from the damaged cardiocytes. In experiments done with serum samples from nonchagasic mice, spontaneous release of the enzyme was minimal during the 16-h assay. The possibility that CK was present in the serum samples from chagasic mice could be ruled out, since the serum samples were diluted 1:40 and were preheated at 56°C for 30 min, which inactivates the enzyme.

Absorptions. Test serum samples used for the ADCC assay were absorbed into *T. cruzi* epimastigotes and trypomastigotes and BALB/c heart and skeletal muscle. Epimastigotes were grown in diphasic medium. Parasites were washed three times in PBS and kept at −70°C. Trypomastigotes were grown in Vero cell cultures, collected from the supernatant, concentrated by centrifugation at 1,500 × *g* for 30 min, and washed three times in PBS. Epimastigotes and trypomastigotes were disrupted by successive freezing and thawing (five times) and sonication. The material obtained from approximately 10^8 parasites was used to absorb 0.2 ml of serum. Heart, masseter, and quadriceps were disrupted in a homogenizer (Potter-Elvehem) equipped with a tightly adjusted Teflon pestle and suspended 1:40 (vol/vol) in minimal essential medium. Serum (1 ml) was absorbed into 500 mg of protein (by the Lowry method [14a]). All absorptions were done for 24 h at 4°C, and serum samples were cleared by centrifugation at 10,000 × *g* for 1 h at 4°C.

For each animal, determinations were made in triplicate. The number of experiments is detailed in Tables 1 to 4.

Preparation of allogeneic antiheart antiserum. Outbred female Swiss albino mice were immunized with a homogenate of whole BALB/c mouse heart. The whole heart was dissected, minced with scissors into small pieces, thoroughly washed in PBS, suspended 1:10 (wt/vol) in PBS, homogenized in a homogenizer (Potter-Elvehem) with a tightly adjusted Teflon pestle, and kept at −70°C. Each mouse received three weekly injections of 0.2 ml of the homogenate containing 500 μg of protein incorporated in complete Freund adjuvant and administered in multiple subcutaneous sites; a booster of the same amount of homogenate was given by the intraperitoneal route. At 1 week later, animals were exsanguinated, and the collected serum was kept at −20°C. The antiheart activity of the antiserum was assessed by immunofluorescence by using anti-mouse IgG rabbit antiserum labeled with fluorescein isothiocyanate (Cappel Laboratories, Cochranville, Pa.) and isolated adult cardiocytes from BALB/c mice as targets.

Assay of complement-dependent lysis. Isolated HMC suspended in RPMI 1640 medium plus 5% fetal calf serum were incubated with ^{51}Cr (sodium chromate, 100 to 200 mCi/mg; Comisión Nacional de Energía Atómica, Buenos Aires, Argentina) for 1 h at 37°C at 3 μCi of ^{51}Cr per 10^5 cells. HMC were washed three times in cold RPMI 1640 medium, and 10^4 cells were incubated in RPMI 1640 medium containing preheated (56°C for 1 h) test sera (normal, chagasic, and allogeneic mouse antisera) diluted 1:20. After 45 min of incubation at 37°C, fresh guinea pig serum as a source of complement was incorporated into a final 1:10 dilution, and the preparation was incubated for a further 30 min. Cells were spun down by centrifugation at 150 × *g*, and the radioactivity of the supernatant was determined. The same procedure was performed with preheated (56°C for 1 h) guinea pig serum instead of the normal serum. Cell lysis was calculated as follows: % lysis = [(Experimental − Sr)/(Mr − Sr)] × 100, where Experimental is the counts per minute of cardiocytes plus serum and complement, Sr is the spontaneous release of ^{51}Cr from cardiocytes, and Mr is the maximal release of ^{51}Cr from cardiocytes.

Histological studies. The hearts were fixed in Bouin fluid, and each was divided in half and embedded in paraffin. Semiserial sections every 200 μm were stained with hematoxylin and eosin.

RESULTS

Histology. The histological study showed that the hearts of all mice chronically infected with *T. cruzi* presented a heavy mononuclear infiltrate. In animals studied at 1 and 2 months p.i., the atria were more affected than the ventricles, but at 3 and 4 months after infection the ventricles also showed interstitial infiltrates with a predominant subendocardial or subepicardial localization. In places fibrosis and scarring were present (Fig. 1).

Immunohistochemistry. The peroxidase-antiperoxidase (PAP) stain revealed autologous IgG bound to the surface of living cardiocytes incubated either with serum samples from chagasic mice or with allogeneic antiheart antisera (Fig. 2). When HMC were incubated with serum samples from noninfected mice, no binding of immunoglobulin was observed (Fig. 3).

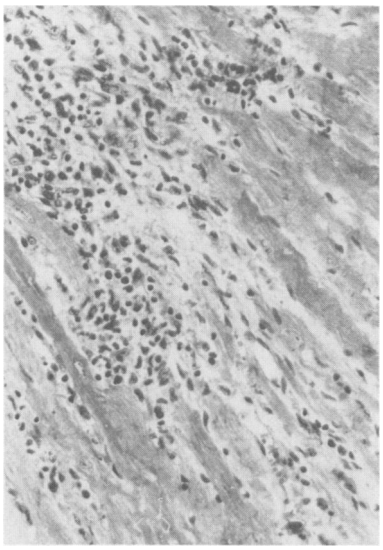

FIG. 1. Loss of myofibers, mononuclear inflammatory infiltrate, and fibrosis in the ventricle of a chagasic mouse. Staining was with hematoxylin and eosin. Magnification, ×450.

Antibody-dependent cytotoxicity. Incubation of cardiocytes with syngeneic nonadherent spleen cells and serum samples from chagasic mice led to the liberation of CK to the medium (Table 1). When serum samples from noninfected mice were incorporated into the assay instead of the chagasic serum, enzyme liberation was significantly lower. When HMC were incubated only with serum samples from chagasic mice or with syngeneic nonadherent splenocytes, liberation of CK to the supernatant was low and was similar to the release observed when cardiocytes were incubated with tissue culture medium alone (Table 2).

To find the time p.i. at which antiheart ADCC activity was present, serum samples from *T. cruzi*-infected mice were collected at 1, 2, 3, and 4 months p.i. In mice studied at 1 month p.i., no ADCC was observed (Table 3). At 2 months p.i. half the animals showed antiheart reactivity, which was present in the sera of all mice studied at 3 and 4 months p.i.

Absorption of chagasic serum by syngeneic heart homogenate significantly decreased the liberation of CK, but absorption by *T. cruzi* epimastigotes or trypomastigotes or by two skeletal muscles, masseter (rich in white fibers) and quadriceps (rich in red fibers), did not change the ADCC activity of the chagasic sera (Table 4).

Complement lytic assay. No cardiocyte damage was observed when cells were incubated with serum samples from normal or chagasic mice or with antiallogeneic heart antiserum in the presence of fresh guinea pig serum as a source of complement.

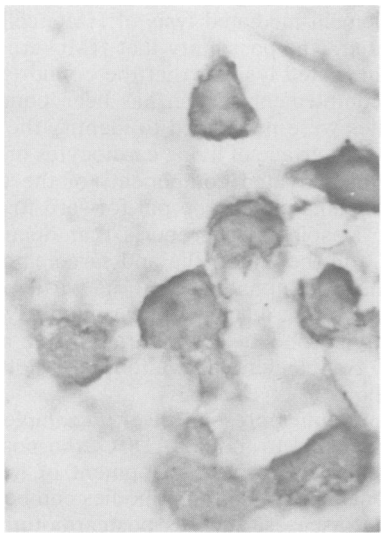

FIG. 2. IgG bound to the surface of isolated cardiocytes after incubation with serum samples from chagasic mice. This is seen as a heavily stained deposit. Staining was with PAP. Magnification, ×450.

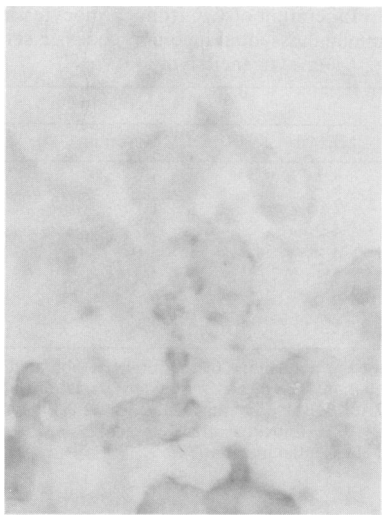

FIG. 3. Lack of bound IgG when isolated cardiocytes are incubated with serum samples from noninfected mice. Staining was with PAP. Magnification, ×450.

DISCUSSION

The pathogenesis of the slow and progressive heart damage that occurs in Chagas' disease is poorly understood.

TABLE 1. Liberation of CK from cardiocytes incubated with syngeneic nonadherent splenocytes and serum samples from chagasic or control mice

Animal no.	CK liberated in[a]:	
	Infected mice	Noninfected mice
1	17.6	2.81
2	18.1	1.9
3	16.25	2.8
4	17.25	2.16
5	13.25	4.55
6	25	3.6
7	24	3.9
8	16	3.7
9	22.2	2.2
10	10.8	6.2

[a] All data represent the percentage of the total CK liberated to the supernatant after 16 h of incubation. The means ± standard deviations are 18.04 ± 4.29 ($P < 0.01$ by the Student t test) for infected mice and 3.38 ± 1.24 ($P < 0.01$ by the Student t test) for noninfected mice.

TABLE 2. Liberation of CK from cardiocytes incubated with minimal essential medium, chagasic serum, or syngeneic lymphocytes

Expt no.	CK liberated with[a]:		
	MEM[b]	MEM + serum	Lymphocytes
1	2.1	2.8	2.6
2	2.0	1.7	1.9
3	4.2	5.2	4.6
4	4.9	5.0	4.6
5	1.7	2.4	2.2
6	2.0	2.6	2.3
7	1.8	2.8	2.4

[a] Data represent the percentage of the total CK liberated to the medium after 16 h of incubation. The means ± standard deviations are 2.6 ± 1.0 for MEM, 3.1 ± 1.2 for MEM + serum, and 2.9 ± 1.0 for lymphocytes. CK liberated from 2×10^5 cardiocytes after detergent lysis: 274 ± 7.7 IU.
[b] MEM, Minimal essential medium.

Different mechanisms have been postulated to explain the tissue alterations. Koberle et al. maintain that it is secondary to a chronic heart denervation, since it is known that *T. cruzi* infection leads to a massive destruction of autonomic neurons (10). In a different line of thinking, cell-mediated immune mechanisms have been held to be the main cause of the heart abnormalities. This assumption is based on the facts that immunization with subcellular fractions of the parasite induces a chronic myocarditis in mice (18) and heart failure in rabbits (26) and that lymphocytes from chagasic animals are cytotoxic for allogeneic embryonal HMC in in vitro assays (21). In addition, transfer of splenocytes from chagasic to normal syngeneic mice induces a transient myocarditis (13). The role that antibodies may play in the pathogenesis of heart lesions is less clear. Although several previous studies have shown that there are antigenic determinants common to mammalian cells and *T. cruzi*, such as striated muscle sarcoplasmic reticulum (19), neurons (27), and laminin (24), the possibility that antibodies raised against those components play a role in the pathogenesis of tissue damage has not been established.

Antibodies with antiheart reactivity were shown to be present in serum samples from chagasic humans early in the 1970s (3). Since their prevalence appeared to be higher in patients with cardiopathy than in asymptomatic individuals

TABLE 3. Liberation of CK from cardiocytes incubated with syngeneic nonadherent splenocytes and serum samples collected from chagasic or control mice at 1, 2, 3, and 4 months p.i.

Mice	CK liberated at following month p.i.:			
	1	2	3	4
Normal				
1	3.9	2.8	2.8	6.2
2	3.9	1.9	2.1	2.2
3	1.7	2.1	4.5	2.7
4	3.2	2.2	3.6	3.9
Mean ± SD	3.2 ± 0.9	2.2 ± 0.3	3.2 ± 0.9	3.7 ± 1.5
Chagasic				
1	4.6	4.7	14.6	25
2	5.6	17.6	16.2	15.4
3	7.3	5.2	17.2	24
4	5.1	18.1	13.2	18.7
Mean ± SD	4 ± 0.1	11.4 ± 6.4	15.3 ± 1.5	20.7 ± 3.9

[a] All data represent the percentage of the total CK liberated to the supernatant after 16 h of incubation.

TABLE 4. Effect of absorptions on ADCC against myocardiocytes

Absorbing cells	CK liberated (mean ± SEM) in[a]:	
	Noninfected mice	Chagasic mice
None	2.83 ± 0.76	16.33 ± 4.65
Myocardium	3.55 ± 0.15	3.46 ± 0.4
Masseter	3.45 ± 0.25	11.3 ± 2.03
Quadriceps	3.30 ± 0.40	13.46 ± 4.69
Epimastigotes	2.95 ± 0.05	12.2 ± 1.02
Trypomastigotes	1.70 ± 0.3	12.57 ± 2.23

[a] All data represent the percentage of CK liberated to the supernatant. Four mice were used for each experiment.

(6), it was postulated that they could exert a pathogenic effect. This interpretation was supported by the finding of autologous immunoglobulins and complement in the endothelia and sarcolemmas of heart biopsies from chagasic humans (4, 16). However, those findings have not been confirmed recently, and doubts were raised about the validity of these observations (7, 11). Since studies were performed by immunohistochemical methods and with frozen myocardium as the target, the possibility exists that antigens are destroyed during processing. Recently, in a different approach, a series of experiments have shown that serum samples from chagasic humans contain immunoglobulin that binds living atrial myocardium, where they induce an agonistic β-adrenergic effect (22, 23), indicating that living HMC can be adequate targets for the demonstration of antiheart antibodies in Chagas' disease. However, since in those studies the beating atria were from rats and the serum samples were from humans, the possibility that the antibodies operate only in a xenogeneic system was not discarded. Moreover, the same group who described antiheart reactivity by means of immunofluorescence techniques recently reported that serum samples from chagasic humans react only with murine myocardium. When human myocardium was used for the reaction, no antibody binding was observed (9).

Our results show that the sera of mice chronically infected with *T. cruzi* contain antibodies that react with the membranes of living adult syngeneic cardiocytes. In our experiments, complement-mediated lysis of HMC cells could not be demonstrated. The possibility that HMC are resistant to complement-mediated lysis or that the bound IgG does not activate the complement system has been considered; but since no studies were performed to identify the subclass of IgG bound to the surface of living cardiocytes or to establish the presence of activated components of the complement cascade, no explanation can be put forward to explain this phenomenon. Despite some reports that documented the presence of C_3 in the endothelia and sarcolemmas in heart biopsies of chagasic humans (5, 16), its participation in the pathogenesis of tissue damage appears unlikely, since vasculitis and polymorphonuclear infiltration, the morphologic hallmarks of complement activation, are not present in chagasic cardiopathy.

Since the antibodies present in serum samples from chagasic mice were able to induce ADCC, the possibility that they play some role in the development of heart damage appears reasonable. Antiheart antibodies can be detected in several human diseases, such as postcardiotomy, Dressler syndrome, acute rheumatic fever (8), and congenital complete heart block (25). Their pathogenetic role remains speculative, and it is not known whether they are an epiphenomenon secondary to the heart damage.

Apparently the antibodies including ADCC were heart specific. They could be absorbed with myocardium, but not with skeletal muscle. Cross-reactivity between *T. cruzi* and the heart appears unlikely, since reactivity could not be abolished by absorption with either epimastigotes or trypomastigotes. Moreover, ADCC antibodies were detected only after 2 months p.i., and a strong anti-*T. cruzi* immune response was already operating since day 15 p.i.

The results presented in this report lend support to the opinion that antiheart antibodies participate in the pathogenesis of the heart damage in Chagas' disease and could represent a mechanism of increase in the severity and perpetuation of the cardiopathy. Further studies, especially on humans, are necessary to ascertain this possibility.

ACKNOWLEDGMENT

This work was supported by a grant from Secretaria de Estado de Ciencia y Técnica, Argentina.

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