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THE IMMUNOLOGY OF EXPERIMENTAL CHAGAS' DISEASE

III. Rejection of Allogeneic Heart Cells In Vitro*

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In previous reports it was shown that rabbits chronically infected with *Trypanosoma cruzi*, as well as rabbits immunized with subcellular fractions of *T. cruzi*, demonstrated intensive, cell-mediated immune phenomena.[1,2] Yet immune sera and sensitized lymphocytes from both the chronically infected and the actively immunized rabbits did not have any demonstrable effect on the infective trypomastigote forms of *T. cruzi* in vitro.[1,2]

It is of considerable interest that the myocarditis of chronic Chagas' disease is characterized by lymphocytic infiltrates and myocardial cell destruction in the absence of encysted parasites in situ (1). We postulate that delayed hypersensitivity plays an important role in the pathogenesis of the cardiac lesions observed in the chronic form of Chagas' disease. This paper reports on experiments which were designed to test this hypothesis by observing the cytotoxic interaction of *T. cruzi*-sensitized lymphocytes with parasitized and nonparasitized allogeneic heart cells.

Materials and Methods

Strain of Trypanosoma cruzi.—The Ernestina strain of *T. cruzi*, maintained in rabbit heart and skeletal muscle cultures, was used for these experiments. This strain of *T. cruzi* has been described previously.[1]

Cell Culture System.—Primary cell line cultures of the fetal rabbit heart were established and maintained according to techniques described previously.[1] The fetal hearts were obtained and the great vessels and atria removed. The valves and chordae tendineae were removed by careful dissection, and the ventricular myocardium was minced. The myofibers were released by trypsinization. A concentration of approximately 2×10^5 cells/ml was used to seed the culture flasks. Primary cell line cultures of the fetal rabbit kidney, established by similar methods, were used for control studies. When a monolayer of the growing cells covered the surface of the

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[2] Teixeira, A. R. L., and C. A. Santos-Buch. 1974. The immunology of experimental Chagas' disease. II. Delayed hypersensitivity to *Trypanosoma cruzi* antigens. *Immunology*. In press.

plastic vessel (usually within 5 to 7 days), the cultures were inoculated with 2×10^4 trypomastigotes of *T. cruzi.*

Rabbit T. cruzi Immune Sera.—Antiserum from a rabbit immunized many times with the initial homogenate (TH)[3] of *T. cruzi*, and with a hemagglutination titer of 1:2048, was used.[1] Another antiserum from a rabbit of the same experimental group, immunized many times with the cytosol (F7 fraction) of *T. cruzi* and with a hemagglutination titer of 1:4096, was used.[1]

Peripheral Blood Mononuclear Cells and Isolation of Lymphocytes.—The method used for collection of peripheral blood mononuclear cells is described previously.[2] Lymphocytes were separated by decantation from culture flask-adherent, peripheral blood mononuclear cells following 1-h incubation at 37°C. About 87–90% of the initial mononuclear cell suspension consisted of lymphocytes, whereas following adherence of the larger monocytes, over 99% of the cells in suspension were the smaller lymphocytes.

T. cruzi-Sensitized Mononuclear Cells.—Blood mononuclear cells and isolated lymphocytes were obtained from rabbits CIRb-C, CIRb-D, CIRb-E, and CIRb-F, inoculated with *T. cruzi* 1 yr previously and now in negative parasitemia. Similarly, blood mononuclear cells and lymphocytes were obtained from two rabbits, AIRb-F5, which had received many weekly injections of the lysosome-rich F5 subcellular fraction of *T. cruzi.*[2] When blood mononuclear cells from these rabbits were tested with particulate antigens of *T. cruzi*, a high degree of inhibition of monocyte migration was elicited.[2]

Effect of Lymphocytes on Allogeneic Heart and Kidney Cell Cultures.—6–8 days after inoculation with trypomastigotes of *T. cruzi*, allogeneic heart and kidney cells were heavily parasitized. Culture flasks containing parasitized cells and culture flasks with nonparasitized cells of the same age received lymphocytes suspended in MEM-HS. Care was taken to keep the pH of the culture medium at 7.1–7.3 during the course of the incubation, since pilot studies had shown that heart and kidney cells became detached from the monolayer at pH 6.7 and 6.5, respectively. A final concentration of 10×10^6 lymphocytes per 10 ml was used in each parasitized and nonparasitized culture flask. At the end of 1, 6, and 18 h of incubation at 37°C, the culture medium was changed once. The cultures were washed four more times to remove lymphocytes which were not attached to culture cells, and the effects were scored.

Effects of T. cruzi Immune Sera on the Interaction of Lymphocytes with Allogeneic Cells.—Immune and control sera were tested at a 10% (vol/vol) concentration in minimal essential medium (MEM). Two experiments were designed. In the first series of tests, fetal heart cells isolated in the usual manner were suspended in MEM containing the test *T. cruzi* antiserum and monolayers were established. To these allogeneic heart cells previously grown in antiserum, test lymphocytes were added and the effects were scored. In the second series, test lymphocytes isolated in the usual manner were suspended in MEM containing the test *T. cruzi* antiserum and treated for 1 h at 37°C. The treated lymphocytes were inoculated in the monolayers of allogeneic heart cells grown in MEM + 10% (vol/vol) horse serum (MEM-HS) and the effects were scored.

Scoring of Effects of Lymphocytes on Target Cells.—A double-blind method and a phase-contrast inverted microscope were used in the scoring of the effects of lymphocytes on allogeneic cells. The observers, who did not know the nature of the experiment, were able to record three distinct changes which were temporally related. At 1-h incubation, lymphocytes were adherent to and encrusted on the surfaces of the cultured cells. At 4-h incubation, more lymphocytes were adherent, and retraction of cytoplasmic processes with "plaque" formation in the mono-

[3] *Abbreviations used in this paper:* AIRb-F5, rabbit actively immunized with the subcellular homogenate; CIRb-C to F, rabbits infected with trypomastigotes of *T. cruzi* one year before experiments; F, subcellular fractions (F1 to F7) derived from the initial F5 fraction; LM, light microscopy; MEM-HS, MEM + 10% (vol/vol) horse serum; SEM, scanning electron microscopy; TH, initial homogenate.

layer was observed. From about 6 h to the end of the experiment (18 h), palisading lymphocytes, rounding of cultured cells, and detachment ("cytolysis") were observed. The degree of change was scored as follows: 3+, marked lymphocytic adherence and cytolysis; 2+, moderate degree of lymphocytic adherence and cytolysis; 1+, mild lymphocytic adherence and cytolysis; and ±, random lymphocytic adherence and no cytolysis. The degree of change was confirmed by photomicrography of Giemsa-May-Grünwald-stained cover slips which had been previously introduced into the flasks before seeding fetal culture cells.

In addition, the changes observed at 1, 6, and 18 h were studied using scanning electron microscopy. Cultured cells to be examined were grown on cover slips inserted on the bottom of plastic culture flasks. The cultures were fixed for several days in three changes of 1.5% (vol/vol) glutaraldehyde in sucrose buffered with 0.05 M imidazole-HCl, pH 7.3, isosmolar to rabbit plasma at 311 mosm/liter. The cover slips were removed, washed in several changes of isosmolar sucrose buffer, and the cells dehydrated sequentially, first in a series of graded alcohols, then in a series of mixtures of amylacetate and alcohol. After this treatment, the cells were critical point-dried in CO_2 at a temperature of 39–41°C and at a pressure of 1,300 pounds/in^2. Small 1 x 1 cm fragments of the tissue-coated cover slips were then glued onto polished aluminum stubs, coated uniformly in vacuo with about 200 Å thickness of gold and viewed with a scanning electron microscope (Autoscan, Model V-1, ETEC Corp., Hayward, Calif.).

Partition of Rabbit Heart Antigens.—The method used paralleled that utilized for the partition of homogenates of *T. cruzi.*[1] A 6-mo old, New Zealand, white, male rabbit was sacrificed by decapitation. The isolated ventricular myocardium was transferred into an ice-cold solution of 0.25 M sucrose, buffered with imidazole-HCl, pH 7.2, and minced. After three washings in sucrose solution the minced tissue was transferred to a freshly prepared, ice-cold solution of 0.25 M sucrose, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.25 mM KCl, 10 mM 2-mercaptoethanol, and 1 mM Na_2ATP buffered with 50 mM imidazole-HCl, pH 7.2. The homogenization was achieved in the cold using an Omni-Mix blender (Ivan Sorvall, Inc., Norwalk, Conn.) by stirring at 6,000 rpm for 15 sec followed by a 5-min rest period. At the end of 1 min, the homogenate was filtered through two layers of cheesecloth. The partition of the homogenate was obtained by differential centrifugation using the same centrifugation speeds described previously.[1] The particulate subcellular fractions obtained from the initial homogenate were washed in isosmolar buffered sucrose at least once. The cytosol and aliquots of the initial homogenate were dialysed against 20 vol of isosmolar-buffered sucrose overnight in the cold. The heart antigens were tested within 48 h of decapitation.

Transmission Electron Microscopy of the Particulate Heart Cell Fractions.—The particulate subcellular fractions derived from the initial homogenate of rabbit myocardium were prepared for electron microscopy as described previously.[1]

Inhibition of Monocyte Migration.—The modification of the capillary method described in a previous paper[2] was used to test the migration of blood mononuclear cells in the presence of the TH and of the subcellular fractions (F1 to F7) derived from rabbit myofibers. The results were compared with those obtained when inhibition of monocyte migration tests were run in the presence of the TH and the subcellular fractions (F1 to F7) derived from *T. cruzi* forms.[2] The TH and the subcellular fractions of the heart cells were made to equal concentrations of 150 μg of protein/ml in MEM-HS and tested.

Protein Determination.—The Lowry method using bovine albumin as a standard was utilized (2).

RESULTS

Effect of T. cruzi-Sensitized Lymphocytes on Allogeneic Heart and Kidney Cell Cultures.—The results of a typical experiment scoring the cytotoxic interaction of *T. cruzi*-sensitized lymphocytes with monolayers of parasitized and nonparasitized allogeneic heart and kidney cells are summarized in Table I. The incuba-

TABLE I

Effect of T. cruzi-Sensitized Lymphocytes on Allogeneic Heart and Kidney Cell Cultures

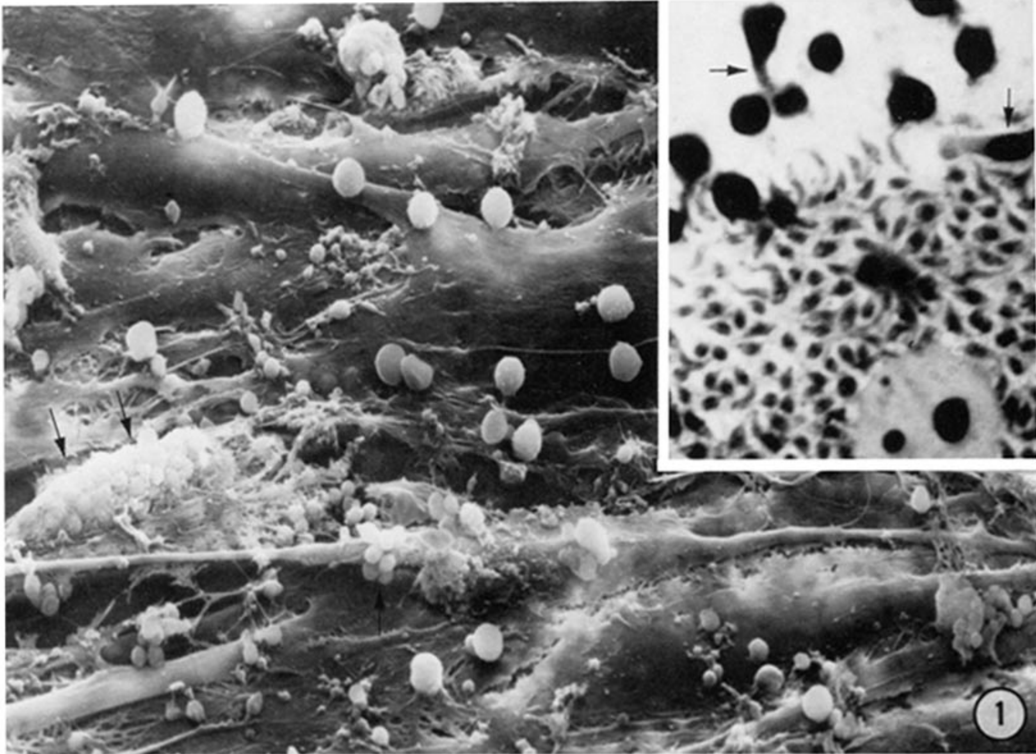
Blood lymphocytes	Degree of cytolysis and lymphocyte adherence*			
	Parasitized heart cells	Nonparasitized heart cells	Parasitized kidney cells	Nonparasitized kidney cells
CIRb-D	3+	2+	±	±
AIRb-F5	3+	2+	±	±
Control Rb-C4‡	±	±	±	±

* 3+, marked cytolysis and lymphocytic adherence; 2+, moderate degree of cytolysis and lymphocytic adherence; 1+, mild cytolysis and lymphocytic adherence; ±, random cytolysis and lymphocytic adherence.

‡ Control Rb-C4, normal rabbit.

tion of lymphocytes from a chronically infected rabbit (CIRb-D) with allogeneic heart cells parasitized by *T. cruzi* showed marked cytolysis of the target cells, and a 3+ score was tabulated after 18 h at 37°C. When lymphocytes derived from a rabbit actively immunized (AIRb-F5) with the lysosome-rich F5 fraction derived from *T. cruzi* homogenates were incubated with parasitized allogeneic heart cells, cytolysis of target cells occurred to a marked degree (3+). Moreover, when lymphocytes from both the CIRb-D and AIRb-F5 rabbits were incubated with nonparasitized heart cells, destruction of allogeneic target cells was observed (2+).

Typically, when 10×10^6 lymphocytes derived from CIRb-D and from AIRb-F5 rabbits were incubated with *T. cruzi* parasitized and nonparasitized allogeneic heart cells, encrustation of many lymphocytes on the surface of target cells was observed within 1 h. The earliest characteristic change that was recorded after scanning electron microscopy consisted of attachment of cytoplasmic filipodia of round lymphocytes to the exposed surfaces of cultured heart cells (3). At 1 h, in experimental and control tests, about 70–80% of the lymphocytes were round, small, unattached, and smooth surfaced, with relatively few cytoplasmic filipodia (Fig. 1). From 6 to 18 h incubation, remarkable changes occurred in the appearance of both the lymphocytes and the target cells. The cultured heart cells retracted their interdigitating cytoplasmic processes and surface bubbling was quite prominent. Palisading lymphocytes were enlarged and their surfaces were altered by extensive broad ruffles on all sides and cytoplasmic pods attached to the target cell surfaces (Fig. 2). In many instances, rough-surfaced lymphocytes had crawled beneath heart cells which showed early evidence of detachment (Fig. 3). Frequently, a halo surrounded adherent lymphocytes (Fig. 4). The target cells with encrusted lymphocytes became rounded shortly before detachment from the surface of the culture flask, and subsequently died. By 18 h, about 65–75% of the lymphocytes were larger, attached and rough surfaced. It appeared at this time that parasitized heart cells were destroyed more prominently by sensitized lymphocytes than were non-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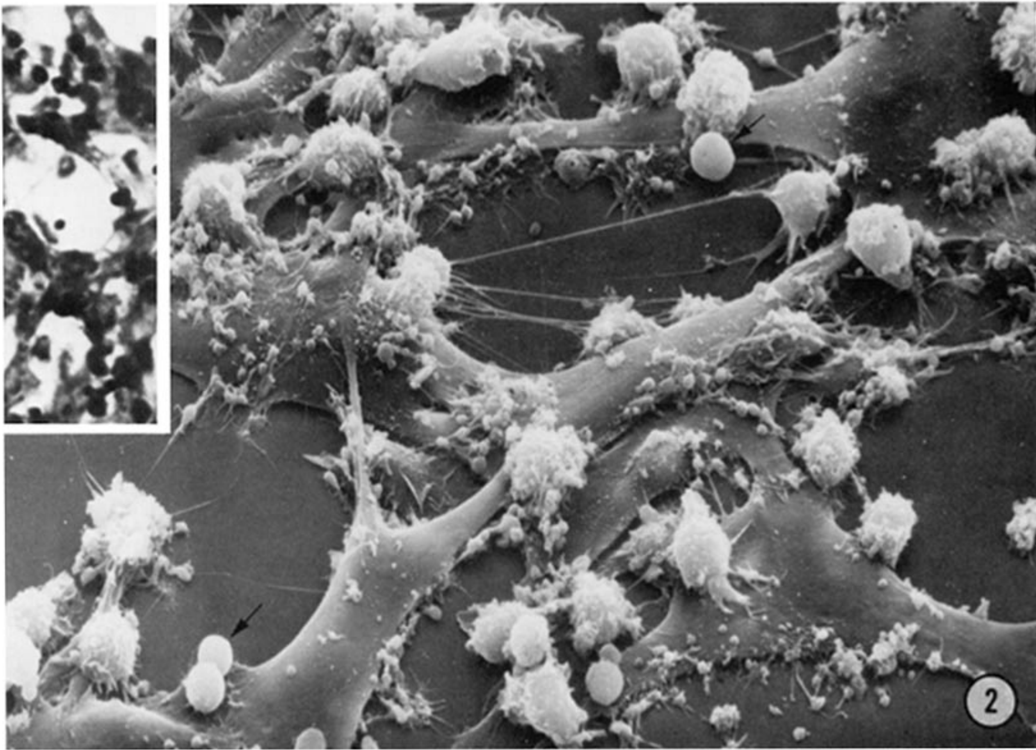
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parasitized heart cell cultures. By the end of the experimental day, there were clumps of dead lymphocytes floating in the medium and only small islands of heart cells were present on the surface of the culture flasks. The active lymphocytes involved in target cell lysis could not be removed from the cells on which they were encrusted even when the cultures were washed five consecutive times with fresh culture medium.

In similar experiments, lymphocytes from chronically infected rabbits CIRb-C and CIRb-E, which had shown evidence of delayed hypersensitivity in vitro,[2] also demonstrated cytotoxicity to both parasitized and nonparasitized heart cells. On the other hand, lymphocytes of CIRb-F, a female rabbit which had produced litters of rabbits with *T. cruzi* parasitism in tissues, failed to destroy allogeneic target heart cells. However, lymphocytes from another rabbit AIRb-F5′, which had been repeatedly immunized with the lysosome-rich F5 fraction derived from homogenates of *T. cruzi*, were cytotoxic to allogeneic heart cells in different experiments.

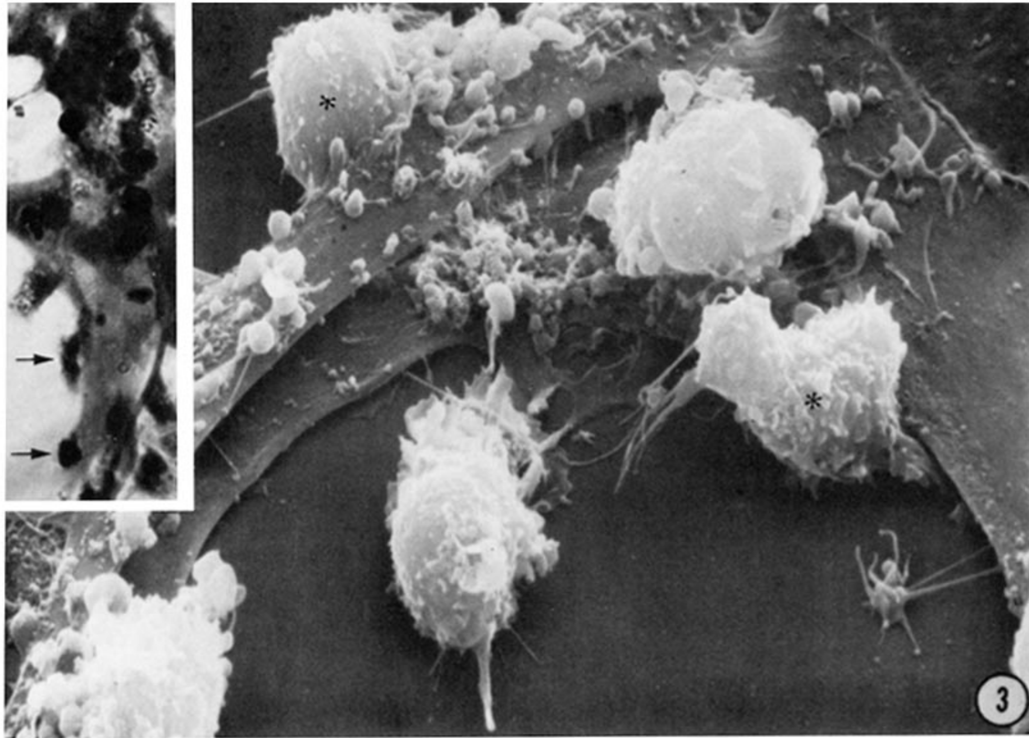
An indication of organ specificity was suggested by studies which consisted of incubation of *T. cruzi*-sensitized lymphocytes from CIRb-D and AIRb-F5 rabbits with parasitized and nonparasitized allogeneic kidney cells. At the end of 18 h, no plaque formation or cytolytic effects were observed (Table I).

Control studies, consisting of incubation of nonsensitized normal lymphocytes and parasitized and nonparasitized allogeneic heart cells, showed none of the morphologic changes described above; and not less than 80% of the few remaining trapped lymphocytes in the intact monolayer were small, unattached, and smooth surfaced.

Effect of Rabbit Immune Sera on the Cytotoxic Interaction of T. cruzi-Sensitized Lymphocytes with Parasitized and Nonparasitized Allogeneic Heart Cells.—Lymphocytes collected from chronically infected rabbits and from actively immunized rabbits might contain surface-bound immunoglobulins that may be implicated in target cell destruction. Therefore, experiments were designed to study the effect of rabbit immune sera on the cytotoxic interaction of *T. cruzi*-

FIG. 1. Inset is a light microphotograph showing the appearance of adherent *T. cruzi*-sensitized lymphocytes on the surface of a parasitized heart cell after 1-h incubation at 37°C. Note the tailed lymphocytes (arrows) and the trypomastigotes gaining access to the external milieu. The rounder amastigotes are present in greater number near the prominent nucleus with nucleoli of the heart cell. (LM, Giemsa-May-Grünwald. × 1,200.) The scanning electron-micrograph illustrates the rejection phenomenon at 1-h, 37°C incubation. Note many relatively smooth-surfaced lymphocytes (~6–8 μ) adherent to the surfaces of heart cells which show early separation of interdigitating processes and delineating boundaries. Grapelike clusters of round amastigotes (~2–4 μ) are present (arrows). (SEM, gold, 45° angle. × 1,200.)

FIG. 2. Inset and scanning electron micrograph are of nonparasitized heart cells incubated for 18 h at 37°C with *T. cruzi*-sensitized lymphocytes derived from CIRb-D (see text). Note the increased plaque formation, the attached ruffled effector cells and the bubbling of the surfaces of the target heart cells. Now, only a few smooth-surfaced lymphocytes are present (arrows). Long, thin cytoplasmic filaments form tangles across plaques stemming from effector cells. (LM, Giemsa-May-Grünwald. × 850. SEM, gold, 45° angle. × 2,900.)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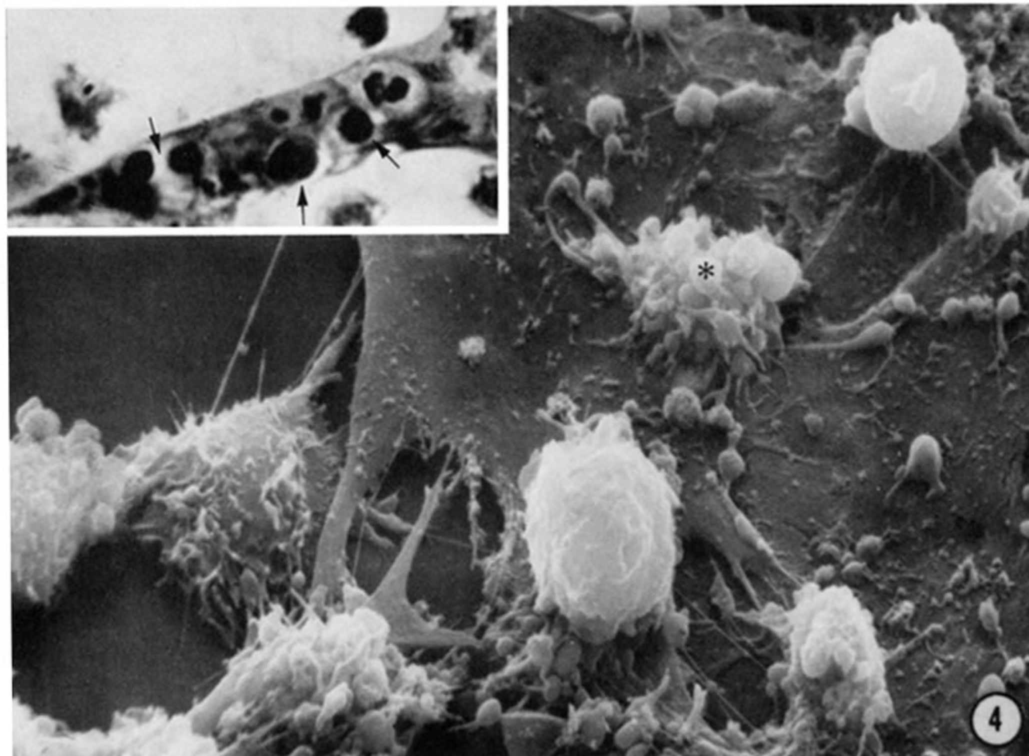
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sensitized lymphocytes with parasitized and nonparasitized allogeneic heart cells. The results of a typical protocol are shown in Table II. In the first experiment, *T. cruzi* parasitized and nonparasitized allogeneic heart cell cultures grown in the presence of fresh rabbit antisera with high hemagglutinating titers were incubated with immune lymphocytes from CIRb-D and AIRb-F5 rabbits. The degree of cytolysis was the same as that observed when normal rabbit serum was used. Moreover, fresh rabbit immune serum as well as fresh normal

TABLE II

Effect of Rabbit Immune Sera on the Cytotoxic Interaction of T. cruzi-Sensitized Lymphocytes with Parasitized and Nonparasitized Allogeneic Heart Cells

Blood lymphocytes	Degree of cytolysis and lymphocyte adherence*							
	Heart cell monolayer cultured in normal Rb serum‡		Heart cell monolayer cultured in Rb immune sera‡		Heart cell monolayer + lymphocytes previously incubated with normal Rb serum§		Heart cell monolayer + lymphocytes previously incubated with Rb immune serum§	
	Parasit.	Nonparasit.	Parasit.	Nonparasit.	Parasit.	Nonparasit.	Parasit.	Nonparasit.
CIRb-D	3+	2+	3+	2+	3+	2+	3+	2+
AIRb-F5	3+	2+	3+	2+	3+	2+	3+	2+
Control Rb-C4‖	±	±	±	±	±	±	1+	1+

* 3+, marked cytolysis and lymphocytic adherence; 2+, moderate degree of cytolysis and lymphocytic adherence; 1+, mild cytolysis and lymphocytic adherence; ±, random cytolysis and lymphocytic adherence.

‡ Heart cells grown in MEM with 10% sera either from normal rabbits or from rabbits immunized with the initial homogenate and cytosol of *T. cruzi*, before incubation with test lymphocytes.

§ Lymphocytes in MEM were incubated with 10% sera either from normal rabbits or from rabbits immunized with the initial homogenate and cytosol of *T. cruzi* for 1 h at 37°C, before transference into heart cell cultures grown in MEM with 10% heat-inactivated horse serum.

‖ Control rabbit C-4, normal rabbit.

rabbit serum proved to be better substrates for growth of allogeneic heart cells than heat-inactivated horse serum at 10% (vol/vol) concentration. The results of this first experiment indicate that rabbit immune sera appear to have no inhibitory effects on the rate of destruction of target cells by *T. cruzi*-sensitized

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FIG. 3. Inset and scanning electronmicrograph are of nonparasitized heart cells incubated with *T. cruzi*-sensitized lymphocytes for 18 h at 37°C. Arrows point to lacunae around dark-staining effector cells penetrating the cytoplasm of the target cell. The counterpart of the light microscopy observation is marked by an asterisk in the scanning electronmicrograph. (LM, Giemsa-May-Grünwald. × 1,200. SEM, gold, 45° angle. × 3,600.)

FIG. 4. Inset and scanning electronmicrograph are of nonparasitized heart cells incubated with *T. cruzi*-sensitized lymphocytes for 18 h at 37°C. Arrows point to lymphocytes crawling beneath the target heart cell. The counterpart of the light microscopy observation is marked by asterisks in the scanning electronmicrograph. (LM, Giemsa-May-Grünwald. × 1,200. SEM, gold, 45° angle. × 3,600.)

lymphocytes. In the second experiment, prior incubation of normal lymphocytes derived from control rabbit C-4 with high titer rabbit antisera against *T. cruzi* antigens resulted in a mild degree of target cell destruction (1+). The incubation of nonsensitized lymphocytes with fresh normal rabbit serum did not result in any significant degree of target cell destruction. Prior incubation of *T. cruzi*-sensitized lymphocytes with rabbit immune sera neither enhanced nor inhibited the rate of destruction of allogeneic heart cells (Table II). The results of this second experiment indicate that fresh *T. cruzi* immune serum appears to give normal, nonsensitized lymphocytes a mild cytotoxicity to target cells.

Localization of Heart Cell Cross-Reacting Antigen.—Results from previous experimentation appear to indicate that *T. cruzi*-sensitized lymphocytes possess properties cytotoxic to nonparasitized allogeneic heart cells but not to allogeneic kidney cells. The hypothesis was tested that normal heart cells contain a cross-reacting antigen with *T. cruzi*, capable of bringing out the cytotoxic effects of immune lymphocytes. Normal heart cell antigens were secured by partition of homogenates of rabbit myocardium into seven subcellular fractions, using the same methods of homogenization and differential centrifugation as those used for partition of homogenates of *T. cruzi*.[1] Transmission electron microscopy of the particulate heart subcellular fractions showed that F1 consisted of nuclei, nuclear membranes, and a few aggregated organelles; F2 showed large vacuoles, mitochondria, and aggregated organelles; F3 was comprised principally of mitochondria; F4 contained long, slender, serpiginous, smooth, tubular structures, probably smooth sarcoplasmic reticulum, and a few lysosomes; F5 consisted of shorter, stubby tubular structures, lysosomes, and vesicles; and F6 was principally formed of small microvesicles.

Tests of inhibition of blood mononuclear cell migration in the presence of equal amounts of protein of the subcellular fractions were done.[2] Sensitized blood mononuclear cells from chronically infected rabbits and actively immunized rabbits were tested. The results of these experiments are summarized in Table III. The degree of inhibition of monocyte migration in the presence of subcellular fractions derived from homogenates of rabbit myocardium was consistently less than the inhibition achieved in the presence of subcellular fractions derived from homogenates of *T. cruzi*. Of the group of actively immunized rabbits, the heart cell antigens present in the F2, F5, and F6 fractions showed marked inhibition of blood mononuclear cell migration, to the level of 25.6 $\pm$ 7.7, 39.5 $\pm$ 7.1, and 25.8 $\pm$ 8.3%, respectively. The degree of inhibition obtained with blood mononuclear cells of a chronically infected rabbit (CIRb-D) was more marked than the degree of inhibition obtained with blood mononuclear cells derived from the group of actively immunized animals (Table III). A very strong inhibition was obtained, to the level of 42.9 $\pm$ 9.1 and 48.9 $\pm$ 9.8%, respectively, when the blood mononuclear cells from CIRb-D were allowed to migrate in the presence of heart cell antigens of subcellular fractions F2 and F5. Tests for the migration of blood mononuclear cells from a normal

TABLE III

*Inhibition of Monocyte Migration in the Presence of the Antigens of Homogenates of T. cruzi Organisms and of Heart Myofibers**

Antigens derived from homogenates of *T. cruzi* and from heart cells	Actively immunized rabbits of groups I and II‡		Chronically infected rabbits§	
	T. cruzi antigen‖	Heart cell antigen¶	*T. cruzi* antigen‖	Heart cell antigen¶
TH (initial homogenate)	47.8 ± 9.8	17.3 ± 6.2	56.7 ± 9.4	29.3 ± 7.2
F1 (350 *g* x 10 min)	52.8 ± 6.9	9.0 ± 3.8	46.3 ± 1.6	30.5 ± 4.1
F2 (700 *g* x 10 min)	48.2 ± 4.7	25.6 ± 7.7	53.7 ± 1.6	42.2 ± 9.1
F3 (5000 *g* x 10 min)	36.5 ± 8.1	16.5 ± 5.6	49.1 ± 4.3	41.1 ± 6.1
F4 (15,000 *g* x 25 min)	41.5 ± 5.3	8.8 ± 5.7	56.4 ± 5.9	32.6 ± 5.7
F5 (30,000 *g* x 35 min)	51.8 ± 7.3**	39.5 ± 7.1‡‡	90.6 ± 12.1§§	48.9 ± 9.8‖‖
F6 (100,000 *g* x 90 min)	42.4 ± 9.1**	25.8 ± 8.3‡‡	76.9 ± 3.9§§	37.6 ± 4.6‖‖
F7 (cytosol)	33.2 ± 2.0**	15.5 ± 0.1‡‡	48.4 ± 2.3§§	24.8 ± 6.5‖‖

* % inhibition = 100 minus average diameter of mononuclear cell migration exposed to antigen divided by the average diameter of mononuclear cell migration in the absence of antigen × 100.

‡ Rabbits immunized with TH and subcellular fractions F1 to F7 derived from homogenates of *T. cruzi* organisms.

§ Rabbits chronically infected with trypomastigotes of *T. cruzi* 1 yr before experiment, now in negative parasitemia.

‖ Blood mononuclear cells were exposed to 150 μg/ml of protein of the *T. cruzi* antigen, TH, F1, F2, F3, F4, and F7. For F5 and F6 100 μg of protein/ml was used.

¶ Blood mononuclear cells were exposed to 150 μg/ml of protein present in the TH and subcellular fractions F1 to F7 derived from homogenate of normal rabbit myocardium.

** F5 vs F7, $t_{11} = 6.069$, $0.005 > P > 0.001$; F6 vs F7, $t_{11} = 2.0172$, $0.1 > P > 0.05$.

‡‡ F5 vs F7, $t_5 = 8.2791$, $0.001 > P$; F6 vs F7, $t_5 = 3.0396$, $0.05 > P > 0.025$.

§§ F5 vs F7, $t_{11} = 5.8708$, $0.005 > P > 0.001$; F6 vs F7, $t_{11} = 10.4$, $0.001 > P$.

‖‖ F5 vs F7, $t_5 = 4.5825$, $0.001 > P > 0.005$; F6 vs F7, $t_5 = 3.5944$, $0.025 > P > 0.02$.

rabbit, control Rb-C4, in the presence of the antigens in the initial homogenate of rabbit myocardium, did not show any significant degree of inhibition when compared with the migration of the blood mononuclear cells in the absence of antigen (6.0 ± 3.6%).

The results of these experiments appear to indicate that rabbit heart cells have a cross-reacting antigen with *T. cruzi*, capable of activating previously sensitized rabbit lymphocytes. Moreover, this cross-reacting antigen is present in its highest concentration in the subcellular F5 fraction (30,000 *g*, 35 min) derived from the initial homogenates of rabbit myocardium, since this fraction gave rise to the highest degree of inhibition of migration of *T. cruzi*-sensitized blood mononuclear cells.

DISCUSSION

T. cruzi-sensitized lymphocytes, collected from chronically infected rabbits and from rabbits actively immunized with the lysosome-rich (30,000 *g*, 35 min) fraction derived from homogenates of *T. cruzi* organisms, destroyed parasitized and nonparasitized heart cells in vitro. The degree of destruction was more marked when *T. cruzi*-sensitized lymphocytes were incubated with parasitized heart monolayers than when *T. cruzi*-sensitized lymphocytes were incubated with the nonparasitized heart monolayers, over a period of 18 h at 37°C. On the other

hand, nonsensitized lymphocytes derived from a normal rabbit, incubated with monolayers of allogeneic heart cells, did not reject target cells. The results of the experiments reported here suggest that sensitized lymphocytes destroyed parasitized target cells at a higher rate than nonparasitized target cells, probably because *T. cruzi* organisms in parasitized cultures may have provided a persistent stimulus for activating the effector cells in the course of the incubation. Examinations made with the scanning electron microscope indicated that the sensitized round lymphocytes with sparse filipodia which were seen at the start of the 18-h incubation were morphologically altered by the presence of both parasitized and nonparasitized heart cells to become larger with many long and broad cytoplasmic ruffles that adhered to the target heart cells before lysis occurred. In support of the observation of the phenomenon of antigen-induced morphologic alterations of the effector cell is the previous documentation that sensitized lymphocytes incubated in the presence of *T. cruzi* trypomastigotes undergo blast transformation and multiplication.[2]

The tissue destruction in the experiments reported here suggests that *T. cruzi*-sensitized lymphocytes have a high degree of cytotoxic specificity. When *T. cruzi*-sensitized lymphocytes from both chronically infected rabbits and actively immunized rabbits were incubated with monolayers of parasitized and nonparasitized allogeneic kidney cells, no destruction could be observed during a period of 18 h at 37°C.

The cytotoxic response of the sensitized lymphocytes used in the experiments reported here might have been stimulated by contaminating heart antigens, normal or altered, from the parent muscle cell culture and not by cross-reacting protozoal antigens. This possible artefact in the system was controlled as follows: (*a*) Trypomastigotes and amastigotes used in the preparations of homogenates and particulate protozoal antigens were judged free of muscle cell contaminants following isolation by the differential centrifugation procedure used as outlined in another study.[1] (*b*) The washed, particulate protozoal antigens of the 30,000 *g*, 35 min fraction derived from these homogenates, which had been previously shown to induce marked cell-mediated immune phenomena in rabbits,[2] did not absorb in end-point dilution tests antibodies to cardiac actomyosin and uterine tropomyosin, two universal major components of the contractile system of muscle cells.[4] (*c*) Inhibition of monocyte migration tests, using blood mononuclear cells from a rabbit immunized repeatedly with homogenates of cultured allogeneic heart cells, were not different from normal controls when allowed to migrate in the presence of *T. cruzi* antigens.[2] These data indicated to us that very little, if any, heart cell antigen was present in the protozoal particles associated with the elicitation of cell-mediated immunity in the rabbits used in the experiments described here.

The results of these experiments suggest that recognition of cross-reactive antigens of the myocardial cell by *T. cruzi*-sensitized lymphocytes may be the

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[4] Teixeira, A. R. L., and C. A. Santos-Buch. The immunology of experimental Chagas' disease. IV. Production of lesions like those of chronic Chagas' disease by multiple injections of subcellular antigens of *T. cruzi*. Comparison with lesions of chronically infected rabbits. Manuscript in preparation.

pathogenetic basis for subsequent target cell destruction. Tests of inhibition of blood mononuclear cell migration designed to localize a cross-reacting heart cell antigen with *T. cruzi*-sensitized lymphocytes showed that heart cell particles in the subcellular (30,000 *g*, 35 min) fraction reacted very strongly with blood mononuclear cells from rabbits chronically infected with *T. cruzi*, and also from blood mononuclear cells of rabbits immunized with the lysosome-rich (30,000 *g*, 35 min) fraction derived from *T. cruzi* homogenates. This observation appears to indicate that the antigens in the cytoplasmic particles present in the 30,000 *g*, 35-min fraction become available on the surface of the myocardial cell to cross-react with the receptors of *T. cruzi*-sensitized lymphocytes.

The cell-mediated immune response is depressed by both inhibitors of RNA synthesis and inhibitors of protein synthesis of effector cells (4–7). Trypsin inhibition of target cell destruction is reversible, and this phenomenon may be attributed to the reappearance of the missing receptor moiety on the lymphocyte surface, either by de novo synthesis and/or by diffusion from a preexisting intracellular pool (8). In the experiments reported here, the intracellular localization of the cross-reacting antigen of the heart cell with *T. cruzi*-sensitized lymphocytes implies that the antigen might be extruded to the surface of the living myocardial cell, suggesting that an energy-dependent mechanism might be involved in target cell/effector cell interaction.

When fresh, high titer rabbit anti-*T. cruzi* antisera did not show toxic effects on cultured heart cells, experiments were further designed to test what role these antisera might play in the cytotoxic interaction of lymphocytes with allogeneic target cells. The incubation of *T. cruzi*-sensitized lymphocytes with allogeneic heart monolayers grown in MEM with 10% rabbit anti-*T. cruzi* antisera did not prevent the destruction of heart cells by sensitized lymphocytes and, furthermore, no depression of the destructive phenomenon was detected in the presence of fresh, complement-rich, rabbit immune sera. Likewise, high titer *T. cruzi* rabbit antisera did not appear to increase the efficiency of sensitized lymphocytes in destroying parasitized and nonparasitized allogeneic heart cells. The observation that *T. cruzi* rabbit antisera did not depress target cell destruction may be explained on the basis that the humoral antibodies were not capable of blocking all the antigenic sites of the target cell. Cytophilic antibodies and complement may play a role in cell-mediated target cell destruction (9–11). In the experiments described here, fresh *T. cruzi* antiserum was capable of inducing a mild degree of target cell destruction in the presence of normal, nonsensitized lymphocytes. However, in view of the very marked degree of target cell destruction carried out by *T. cruzi*-sensitized lymphocytes, it is unlikely that either cytophilic antibody or antibody-complement dependent systems are principally responsible for the degree of cytolysis observed in our studies.

A high percentage of patients with active rheumatic fever appear to have circulating anti-group A streptococcus antibodies cross-reactive to heart myofibers, demonstrable by gel diffusion and immunofluorescence techniques (12). It has been suggested that these cross-reacting antibodies play a role in the pathogenesis of rheumatic heart dis-

ease (13). In this regard, antiheart antibody has been observed in a high percentage of guinea pigs and mice infected with *T. cruzi*, ranging from 56% at 3 mo after infection to 80% after 10 mo (14). Kozma, in 1962, observed that 84% of the patients with chronic Chagas' disease had antiheart antibody demonstrable by gel diffusion tests (14). Whether cross-reacting circulating antiheart antibodies play a principal role in the pathogenesis of the myocarditis observed in patients with chronic Chagas' disease is not supported by the results of the experiments reported here.

The in vitro rejection of rabbit allogeneic heart cells by *T. cruzi*-sensitized lymphocytes was a reproducible phenomenon in this series of experiments, and cellular lysis was accomplished by lymphocytes encrusted on the surface of target cells. Earlier, in 1950, Kidd and Toolan (15, 16) and, in 1952, Ellis and Kidd (17), reported that "sensitized lymphocytes" from resistant immune hosts which had previously overcome the cells of transplanted cancers, specifically destroyed cancer cells in vitro, and the destructive effect occurred "wherever the lymphocytes have penetrated, but not elsewhere." Later, in 1961, Rosenau and Moon (18) observed that close contact of sensitized lymphocytes and target cells appeared to trigger the cytolytic reaction. It is of interest that the in vitro system described here paralleled the observations of Kidd and Toolan, and target cells with adherent *T. cruzi*-sensitized lymphocytes died one after another, whereas many target heart cells remaining in the culture flask after 18 h incubation did not show lymphocytes adherent on their surfaces. The experimental observation that normal, nonsensitized lymphocytes do not destroy target cells has been documented by many investigators in different in vitro systems (15–23). On the other hand, sensitized lymphocytes can differentiate specifically between different allogeneic target cells in different in vitro systems, as shown by Goevarts (21) and later by Solliday and Bach (22) and Brunner, et al. (23).

Acute Chagas' disease is an infection disseminated to various organs and tissues, and many of the lesions are probably due to the action of the intracellular *T. cruzi* organisms (24). In the chronic cardiac form of Chagas' disease, in humans (1, 25, 26) and in experimental animals,[4] death is attributed to the presence of a diffuse myocarditis. Renal lesions are not observed. The myocarditis of chronic Chagas' disease in man (1) and in rabbit[4] is characterized by infiltrates of principally lymphocytes associated with persistent destruction of myofibers in the absence of encysted parasites in situ. Torres, in 1929, was the first to attribute the absence of parasites in situ in the chronic myocarditis of patients with Chagas' disease to an "allergic state" of the host (1). The chronic myocarditis would develop following repeated reinoculations, and the parasite led a transitory existence in cells and rapidly disappeared as a consequence of the immune state (1). This interpretation of the pathogenesis of the chronic myocarditis of Chagas' disease is not supported by the experiments reported here. These results strongly suggest that a parasite-induced, cell-mediated immune reaction directed towards the host cell per se is the basis of myocardial injury in experimental Chagas' disease. Furthermore, these observations suggest that antigens in the myocardial cell cross-react with *T. cruzi*-sensitized lymphocytes that cause target heart cell lesions. Moreover, a single inoculation with *T. cruzi*

1 yr before these studies was sufficient to induce and maintain sensitization of rabbit lymphocytes.

SUMMARY

Experiments that consisted of incubation of *Trypanosoma cruzi*-sensitized lymphocytes derived from chronically infected rabbits and from rabbits repeatedly immunized with a small particle or membrane fraction derived from homogenates of *T. cruzi* forms, showed destruction of allogeneic, parasitized and nonparasitized heart cells in vitro. Mononuclear cells collected from peripheral blood were incubated for 1 h at 37°C to isolate the lymphocytes. Following incubation, over 99% of the cells in the supernate were lymphocytes, which were utilized in these experiments. At the start of these experiments, 70–80% of the sensitized lymphocytes were unattached, small and round, with sparse filipodia. In the ensuing hours, marked heart cell destruction, similar to that seen in an active lesion when lymphocytes invade heart tissue, were observed. After 18 h incubation, about 65–70% of the lymphocytes were attached, larger, and rough surfaced.

Inhibition of monocyte migration tests, each in the presence of the antigens of subcellular fractions of *T. cruzi* organisms and of allogeneic heart myofibers, indicated the presence of a cross-reacting antigen common to both the parasite and the heart in the small particle or membrane fractions. The particulate antigens of the 30,000 *g*, 35-min fraction of heart muscle gave rise to inhibition of monocyte migration as did the counterpart fraction derived from *T. cruzi* organisms.

The destruction of nonparasitized target heart cells by *T. cruzi*-sensitized lymphocytes is an in vitro model of the chronic myocarditis of Chagas' disease, and the recognition of cross-reactive antigens of the host cell by *T. cruzi*-sensitized lymphocytes is believed to be the pathogenic basis for subsequent tissue injury in the chronic phase of this disease.

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