Investigación Clínica 31(1): 17-31, 1990

SPLENOCYTE MEMBRANE CHANGES AND IMMUNOSUPPRESSION DURING INFECTION AND REINFECTION WITH *Trypanosoma cruzi*

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Key words: *Trypanosoma cruzi*, **infection, reinfection, antigen expression, immunosuppression, lymphocyte membrane**

SUMMARY

Antisera to epi- and trypomastigote forms of *Trypanosoma cruzi* were used to detect trypanosome antigens on the surface of lymphocytes from infected mice. Only the antitrypomastigote serum could recognize antigens expressed transiently on the splenocyte membranes from infected animals. The number or structural configuration of Concanavalin A receptors was similarly affected and a clear correlation was seen between these two types of membrane changes and the immunosuppression to mitogens and SRBC presented by the infected mice. Reinfected animals did not show evidences of trypanosome proliferation in blood or tissues nor trypomastigote antigens on splenocytes, but presented a less intense, transient immunosuppression as measured by responsiveness to mitogens and SRBC, suggesting that the primed immune system can eliminate the new parasite inoculum before the host is immunosuppressed and also that the liberation of strong immunosuppressor trypomastigote antigens induce the new state of suppression.

INTRODUCTION

It is well established that the experimental infection with *Trypanosoma cruzi* induces a transient, generalized state of immunosuppression in the host that is overcome during the chronic phase of the disease in the surviving animals (2, 13-15, 24, 26, 32-34). Nevertheless, defining the mechanisms by which this suppression takes place is still a controversial matter since many and sometimes contradictory models of immunosuppression have been proposed to explain this phenomenon, including changes in numbers or proportions of immunocompetent cells (16), polyclonal activation of lymphocytes (9, 28), a direct effect of parasites on the lymphoid cells (17, 24), modulation of the immune responses by suppressor cells that could be either

Recibido: 13-11-89
Aceptado: 07-03-90

T cells (31, 34, 37) or macrophages (18, 26), and the effect of soluble suppressor factors (7, 38).

The absorption of trypanosome antigens on mammalian cells **in vitro** (1, 22, 36) has also been described, so it was the intention of this study to assess if this phenomenon was taking place in the living infected mice to try to correlate it with the observed suppression. Since much attention has recently been paid to the comparison of the antigenic repertoire of the different differentiation stages of *T. cruzi* (4, 23, 41) and to the differences between strains or clones of this parasite (6, 10, 19, 30), we also tried to define the origin of the antigens putatively expressed on lymphocytes from infected mice, keeping in mind that the most successful procedures of vaccination used so far have involved the use of trypomastigote antigens (3, 21, 40, 43), compared to epimastigote antigens.

Furthermore, it was of interest to evaluate the immunological state of convalescent and reinfected-convalescent mice to shed some light on the still controversial issue of concomitant immunity in trypanosomiasis (5, 8, 12, 20, 36) and to find out if the same alterations seen in the immunocompetent cells during the acute phase of the disease were also occurring during reinfection.

MATERIALS AND METHODS

Animals: C3H/He and C57BL/6 male mice (Jackson Laboratory, Bar Harbor, ME), 2 to 3 months old were fed Purina chow and water **ad libitum**. Rabbits and guinea pigs of either sex were provided by IVIC's animal facilities.

Infection and reinfection: Different groups of inbred C3H/He and C57BL/6 mice were infected once with 500 trypomastigotes of the Y strain of *T. cruzi* (39), in order to keep a stock of mice with different periods of infection. The parasites had been maintained in mice by weekly infections and in a synthetic medium supplemented with 5% fetal bovine serum (FBS, GIBCO) (v/v) that permits parasite growth at 26, 30, 34 and 37°C (27). Convalescent mice were reinfected at different times post-infection during the chronic phase of the disease with the same number of parasites (see Table IV).

Anti-culture form trypanosome sera: Epimastigotes grown at 26° or 37°C were concentrated to 2 x 10^8 parasites/ml by centrifugation at 900 x g and washing 3 times with phosphate buffered saline (PBS). Parasite pellets grown at 26°C were resuspended in cold PBS, while the cells grown at 37°C were resuspended in cold PBS supplemented with 0.02 M PMSF (phenyl-methyl-sulphonyl-fluoride, Sigma Chemical Co., St. Louis, MO) in 2% propanol (v/v). These suspensions were sonicated for 5 min at 4°C. Aliquots of 2.5 ml were used to immunize rabbits using the following protocol: 1 dose in Complete Freund's Adjuvant, i.m. in the four limbs; 1 dose in Incomplete Freund's

Adjuvant, s.c. in the back and 1 dose, i.v. in the ear vein, at 15 day intervals. Two weeks after the last injection the animals were bled by cardiac puncture and the sera was prepared. These sera were denominated: anti-Epi-26° and anti-Epi-37°-PMSF.

Anti-blood trypomastigote antiserum: Rabbits were injected every other week with 10^6 trypomastigotes from mouse blood, i.v. during two months. Fourteen days after the last injection the blood was collected by cardiac puncture and the serum obtained was denominated: anti-blood-Tryp-serum.

Anti-sera fractionation and absorption: An aliquot of the anti-blood-Tryp-serum was fractionated by 37% ammonium sulphate precipitation and the precipitate and supernatant fractions were reconstituted to the original volume and extensively dialysed against PBS. Another aliquot was fractionated by affinity chromatography using a Sepharose 4B column coupled to normal mouse serum separating the bound and unbound fractions and reconstituting them to the original volume. A second passage of the pre-absorbed serum did not yield any bound proteins.

Blood from normal and 17-day infected mice was collected in the presence of 3.8% (w/v) sodium citrate. The spleens of these animals were also removed and splenocyte suspensions (3 spleens/group) were prepared to use in the agglutination experiments described below. The citrate blood was separated by centrifugation through Ficoll-Hypaque (29), obtaining the plasma, the leukocyte and platelet and the erythrocyte fractions. Aliquots of rabbit anti-blood-Tryp-serum were absorbed each with these blood preparations from normal and infected mice and tested afterwards in the agglutination assay with normal and 17 day infected splenocytes.

Agglutination: Microtest tissue culture plates (3040, Falcon Labware, Oxnard, CA) were used for the agglutination of spleen or lymph node cells from normal, infected or reinfected mice or normal and infected erythrocytes, using 25 μl/well of lymphocytes at 10^7 cells/ml and erythrocytes at 2% (v/v) and 25 μl/well of absorbed anti-blood-Tryp-serum. The agglutination titers registered after 2 hours of incubation at room temperature were evaluated under the microscope for the lymphocyte and by eye for the erythrocytes. The values are expressed as the dilution at which a 1^+ agglutination pattern was detected. The agglutination titer is defined as the inverse of these maximum dilutions.

Indirect immunofluorescence: Aliquots of 200 μl of spleen cells at 10^6 cells/ml were spun down on glass slides with a Shandon cytocentrifuge at 900 x g for 15 min and fixed in 95% ethanol (v/v) for 30 min. After drying, the slides were incubated with absorbed anti-blood-Tryp-serum for 45 min, washed three times with PBS and stained with fluoresceinated goat anti-rabbit immunoglobulins (FITC-goat-anti-rabbit Ig, Cappel Laboratories), at 1 mg/ml for 45 min. The percentage of fluorescent cells was determined counting the total number of cells under phase contrast and discri-

minating the number of them that showed fluorescence under UV light in 100 fields of each slide (two slides per mouse, three mice per group per experiment).

(^{3}H)-Concanavalin A binding: The mitogenic capacity of (^{3}H)-Con A (New England Nuclear, Boston, MA; 42.4 Ci/mmol) was compared to "cold" Con A, as determined by blastogenic assay using concentrations of 0.5 and 1.0 μg/ml to stimulate normal mouse splenocytes. Once known the mitogenic effectiveness of the radioactive lectin, the binding capacity of (^{3}H)-Con A to normal or infected spleen cells was assessed measuring the amount of radioactivity bound to triplicates of 2×10^5 cells in the presence of 1 μg/ml of (^{3}H)-Con A in RPMI-1640 supplemented with 5% (v/v) FBS, after 4, 8 and 20 hours of incubation with the labelled lectin. The values are the average of two different experiments ± standard deviation. The comparisons of the means was made using the Student-Newman-Keuls Test (42) and differences were considered significative at $P < 0.05$.

Blastogenic assay: A blastogenic assay (26) was used to evaluate the responsiveness to mitogens of normal, convalescent and reinfected mice. Analysis of variance and comparisons of means were made by the Student-Newman-Keuls Test (42) and the differences were considered significative at $P < 0.05$.

Primary response to sheep red blood cells (SRBC): A modification of Jerne's technique (25) was used, in which normal, convalescent and reinfected mice were injected with 0.4 ml of a 10% (v/v) SRBC, i.p., four days before sacrifice and in such a way that they were killed after 17, 24 or 48 days after reinfection. The splenocytes were adjusted to 10^6 cells/ml and the number of PFC was determined. The PFC data were normalized using Gottlieb's method (11) of $\log_2$ transformation previously to the analysis of variance.

Pathological signs: The classical signs of Chagas'disease: parasitemia, splenomegaly, histopathological changes in cardiac and skeletal muscles, mortality, general aspect of mice and recuperation of parasites from spleen cell cultures in vitro (26), were followed-up during the course of infection and reinfection with *Trypanosoma cruzi* and compared to normal controls, when applicable.

RESULTS

Rabbit anti-blood-form trypomastigotes or its fractionated Igs agglutinated 17 day-infected splenocytes at a dilution of 1:64. This antiserum did not agglutinate normal spleen lymphocytes, nor normal or infected lymph node lymphocytes (Fig. 1). Anti-blood-Tryp-serum, absorbed with normal mouse serum maintained the agglutination titer on infected spleen cells. Also, a rabbit anti-normal mouse serum could not agglutinate infected splenocytes. Rabbit antisera against cultured epimastigotes did not agglutinate normal or infected splenocytes (Table I).

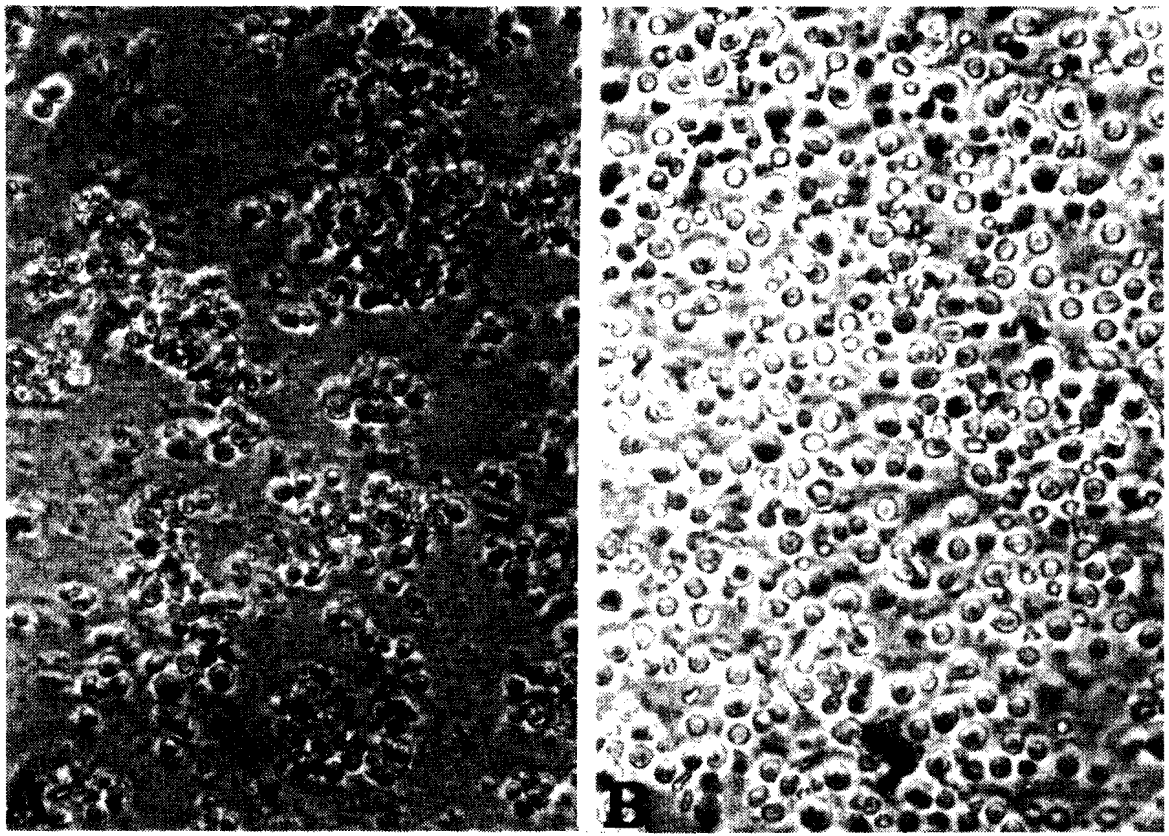

Fig. 1.– Agglutination rabbit anti-blood-form trypomastigotes serum of 17 day-infected splenocytes (A). Absence of agglutination with normal splenocytes (B).

TABLE I

AGGLUTINATION OF NORMAL AND 17 DAY-INFECTED SPLEEN CELLS FROM C3H/HE AND C57BL/6 MICE

Serum preparations	Agglutination titer	
	Normal	17d-infected
NRS	2	2
Anti-NMS	2	2
Anti-Epi-26°	2	2
Anti-Epi-37°	2	2
Anti-blood-Tryp	2	64
Anti-NMS-Igs from anti-Blood-tryp	0	0
Anti-blood-tryp globulin fraction	2	64
Anti-blood-tryp supernatant after ammonium sulphate precipitation	0	0

NRS = Normal rabbit serum
NMS = Normal mouse serum
The results were identical for C3H/He and C57BL/6 strains.
Normal or infected lymph node cells could not be agglutinated by any of the preparations tested.

Absorption of anti-blood-Tryp-serum with normal or 17 day-infected mouse blood leukocytes, erythrocytes or plasma did not change the agglutination pattern on 17 day-infected splenocytes. The absorption with normal or infected erythrocytes cleared out the anti-erythrocytic agglutination activity present in the rabbit serum, without impairing the agglutination titer of the rabbit anti-blood-Tryp-serum on infected spleen lymphocytes (Table II).

In Table III it is shown that the agglutination titer was maximum when using 17 day-infected splenocytes and gradually diminished when spleen cells from mice infected for 30 or 60 days were used, returning to background levels by 90 days of infection. The reinfected animals, convalescent for more than 120 days of infection, and tested at 17, 24 and 48 days of reinfection, did not show any agglutination with anti-blood-Tryp-serum.

Trypomastigote antigens on splenocyte surfaces were detected with a maximum percentage of fluorescent cells at 17 days post-infection: 58 ± 13% in C3H/He and 64 ± 11% in C57BL/6 mouse spleen cells. Lymphocytes from normal, convalescent animals after 120 days post-infection and reinfected-convalescent animals did not show any trypomastigote antigens on their surfaces.

TABLE II

EFFECT OF THE ABSORPTION OF THE RABBIT ANTI-BLOOD-TRYP SERUM WITH DIFFERENT BLOOD COMPONENTS ON THE AGGLUTINATION OF SPLENOCYTES AND ERYTHROCYTES FROM NORMAL AND 17 DAY-INFECTED C3H/HE AND C57BL/6 MICE

	Agglutination titer			
	Splenocytes		Erythrocytes	
Absorbent	Normal	17d-inf.	Normal	17d-inf.
None	2	64	32	32
Normal leukocytes	2	64	–	–
Normal erythrocytes	2	64	0	0
Normal plasma[1]	2	64	–	–
17d-inf. leukocytes	2	64	–	–
17d-inf. erythrocytes	2	64	0	0
17d-inf. plasma	2	64	–	–

– = not done

[1] = Same aliquots of plasma alone without rabbit anti-blood-Tryp serum, could not agglutinate infected spleen cells.

The results were identical for C3H/He and C57BL/6.

TABLE III

AGGLUTINATION OF SPLENOCYTES FROM C3H/HE AND C57BL/6 MICE DURING THE ACUTE, CHRONIC AND REINFECTIONS STAGES OF *T. Cruzy* INFECTION

Time of infection and/or reinfection (days)	Agglutination titer
0	2
5	2
17	64
30	8
60	4
90	2
120	2
120-17	2
120-24	2
120-48	2

The (Ig-anti-normal mouse serum) –depleted rabbit anti-blood-Tryp serum was used for these agglutination experiments.

The binding of Con A (Fig. 2) to spleen cells from different periods of infection was also significantly affected at 17 days post-infection. There were no differences in the total binding after 4, 8 or 24 hours of incubation with the "hot" lectin within each experimental group. Subsequently the binding of Con A returned to normal values.

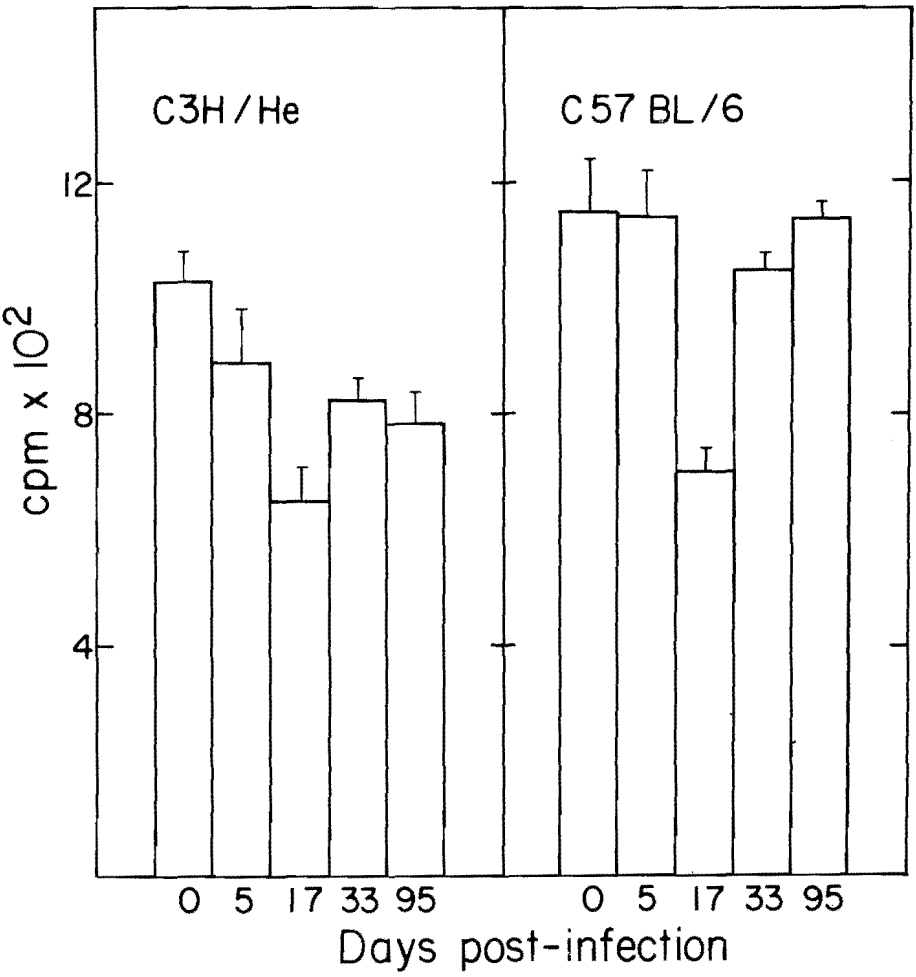

Fig. 2.– Binding of (^{3}H)-Concanavalin A to normal and infected splenocytes from C3H/He and C57BL/6 mice. No differences were found for the total binding at 4, 8 or 20 hours of incubation with the lectin, maintaining through the differences of binding at each period of infection.

The mitogenic response of splenocytes from reinfected-convalescent animals showed a secondary, transient, less intense inhibition and a faster recovery than when infected for the first time (Table IV) between 17 and 48 days after incubation, and also the primary response to SRBC was inhibited in the same way during reinfection recovering the responsiveness by day 48 (Fig. 3).

TABLE IV

BLASTOGENIC RESPONSE TO MITOGENS OF SPLEEN CELLS FROM REINFECTED C3H/HE AND C57BL/6 MICE

Strain	Period of Inf.-reinf. (days)	Percentage of response' Con A	PHA	LPS	DS
C3H/He	17''	25	9	10	10
	105-17	46	54	30	13
C57BL/6	17''	24	23	7	11
	25''	43	27	21	46
	60''	97	111	62	68
	120-17	25	51	28	28
	120-24	35	29	17	51
	135-17	58	89#	55	45
	135-24	65	73	37	61
	150-17	63	89#	52	60
	150-24	68	56	38	84
	165-17	57	90#	32	59
	165-48	168	150	116#	102#

'Percentage of response:

$$\frac{\text{cpm exp with mitogen} - \text{cpm exp without mitogen}}{\text{cpm control with mitogen} - \text{cpm control without mitogen}} = \text{x } 100$$

'' = Data reported by O'Daly et al., 1984 (26)

= Differences are statistically significant at $P < 0.05$.

With regard to the pathological signs of Chagas'disease of infected, convalescent and reinfected animals, infected mice showed evidence of the parasites or its effects through the evaluation of mortality, parasitemia, splenomegaly, presence of parasites in skeletal and cardiac muscles and in splenocyte cultures, agglutination and indirect immunofluorescence of splenocytes, while convalescent or reinfected animals were not positive for any of these parameters.

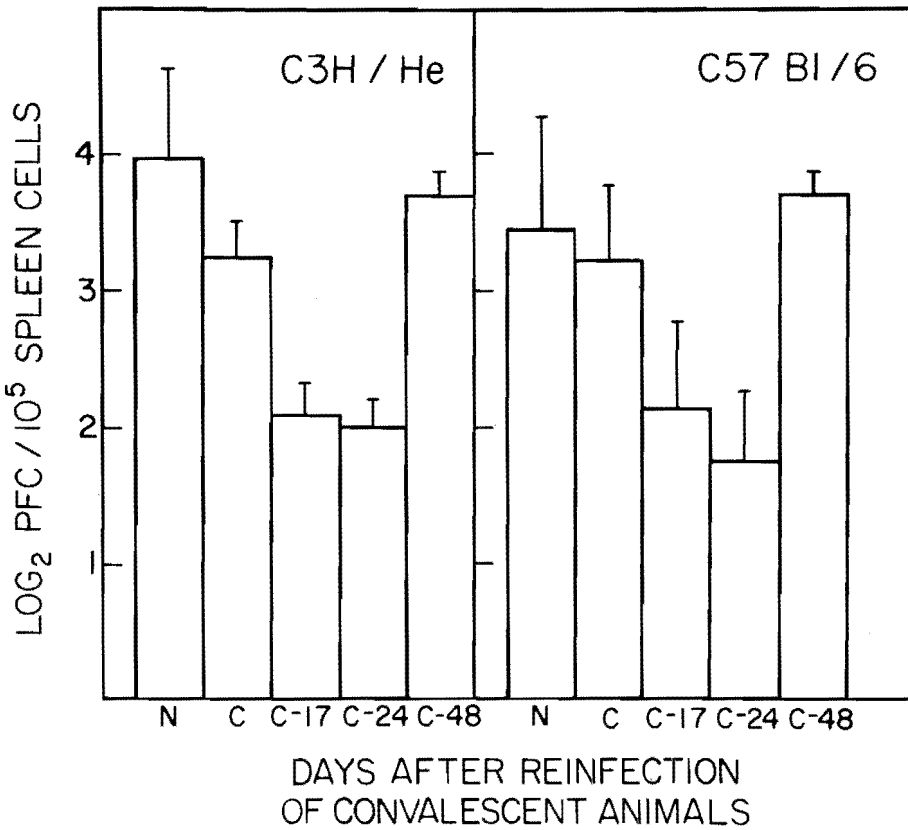

Fig. 3.– Inhibition of the primary response to sheep red blood cells during reinfection with *T. cruzi* of convalescent C3H/He and C57BL/6 mice. Differences are statistically significative at $P < 0.05$ for C-17 and C-24 for both C3H/He and C57BL/6 mice.

DISCUSSION

Two lines of evidences indicate that the splenocytes from mice infected with *T. cruzi* are expressing changes on their membrane surface, that could be correlated with the alteration of the immunocompetence of these lymphoid cells observed during the acute phase of Chagas'disease. First, the presence of new molecules recognized by an antiserum raised against blood-form trypanosomes, which diminished as the disease enters the chronic phase, and second, the alteration in the number or structural conformation of some surface receptors (Con A receptors in our experiments) with similar kinetics, parallel to the decrease in immunogenic responsiveness shown by infected mice during the acute phase of the disease. These surface changes could

very likely lead to an alteration of the immunocompetence of these cells that could be acting concomitantly to other immunosupressor mechanisms during the development of the disease.

On the other hand, the new antigens expressed on the surface of the splenic cells can not apparently be detected neither on the surface of lymphocytes from peripheral blood, which could not absorb the agglutinating activity of the rabbit anti-blood-Tryp-serum, nor on lymph node lymphocytes from 17 day-infected mice, which could not be agglutinated by the rabbit anti-blood-Tryp-serum, suggesting a preferential avidity of binding of trypomastigote antigens on splenocytes in comparison to other lymphocytes, or a compartmentalized processing and expression of parasitic antigens in the spleen.

Interestingly, the splenocytes of reinfected mice did not show any detectable trypomastigote antigens on their surfaces, which suggests that a different mechanisms for the suppression during reinfection, probably mediated by memory-suppressor cells could be taking place since these splenocytes also showed some immunosuppression. The fact that during reinfection parasites are rapidly destroyed within the convalescent host –no evident parasite proliferation was observed with any of the techniques employed– and that nevertheless the cellular and humoral immune responses are suppressed, suggests that the early destruction of the inoculated trypomastigotes liberate strong immunosuppressor antigens, capable of eliciting a secondary response of an immunosuppressor mechanism that takes place right after the immunoprotective response of the immune system has taken effect destroying the parasites.

Trypanosomal antigens present on the surface of splenic cells cannot be recognized by antisera against culture form epimastigotes, suggesting that parasites from culture do not possess the trypomastigote antigens expressed on the surface of the infected spleen cells or that they do not present them in an immunogenic form adequate enough to induce an efficient antibody response in the immunized rabbits. This is an important difference that should be taken into account when analyzing the antigenic repertoire of *T. cruzi* during the isolation of relevant immunoprotective antigens to be used in vaccination protocols. Some attention has been paid to this issue in recent years regarding the conservation or divergence of putatively immunoprotective relevant antigens of *T. cruzi* between strains or even clones of this parasite (4, 6, 10, 19, 23, 30, 41) and it should be pointed out that the best results obtained for the protection of experimental animals has been achieved with antigens present in the trypomastigote form of *T. cruzi* (3, 21, 40, 43). This observation, together with the fact that only a relative effectiveness in protection has been obtained when using epimastigote preparations, indicate that blood form trypomastigotes or its antigens should be tried as immunizing agents for the induction of protection against a challenge of virulent trypanosomes.

Acknowledgments

Mrs. Miriam Pérez is gratefully acknowledged for her excellent secretarial assistance. This work was financed by Petroleos de Venezuela (PDVSA).

RESUMEN

Cambios en la membrana de los esplenocitos e inmunosupresión durante la infección o reinfección con *Trypanosoma cruzi. Serrano L.E. (Centro de Microbiología y Biología Celular, Instituto Venezolano de Investigaciones Científicas (IVIC), Apartado 21827, Caracas 1020A, Venezuela), O'Daly J.A.* **Invest Clín** *31(1): 17-31, 1990.*– Se usaron anticuerpos contra las formas epi y tripomastigotes de *T. cruzi* para detectar antígenos de parásitos en la superficie de linfocitos de ratones infectados. Sólo el suero anti-tripomastigote pudo reconocer antígenos expresados transitoriamente sobre la membrana de los esplenocitos de animales infectados. También se afectó el número o la configuración estructural de los receptores de Concanavalina A y se observó una correlación clara entre estos dos tipos de cambios en la membrana y la inmunosupresión a mitógenos y eritrocitos de carnero. Los animales reinfectados no mostraron evidencia de proliferación de los tripanosomas en la sangre o en los tejidos ni tampoco se vieron antígenos de tripomastigotes sobre los esplenocitos, pero presentaron una inmunosupresión transitoria menos intensa, medida con mitógenos y eritrocitos de carnero, lo cual sugiere que el sistema inmune ya sensibilizado puede eliminar el nuevo inóculo de parásitos virulentos antes de que el hospedador se inmunosuprima y que la liberación de antígenos inmunosupresores de tripomastigotes induce el nuevo estado de supresión.

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