RECEPTOR FOR IMMUNOGLOBULIN Fc ON PATHOGENIC BUT NOT ON NONPATHOGENIC PROTOZOA OF THE TRYPANOSOMATIDAE*

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The mechanisms parasites have developed to escape immune surveillance are currently under investigation in many laboratories, because their elucidation may provide new approaches to the prevention of the diseases the parasites cause. For a detailed description and experimental evidence of these various mechanisms, see the recent review by Bloom (1).

One possible means of evasion is the uptake of host antigen by the parasite (2). This phenomenon has been amply studied and documented in schistosomiasis. The acquisition of mouse antigens (3–5), human A, B, and H blood group antigens (6, 7), glycolipids from erythrocytes (8), serum factors (9–11) and, more recently, mouse histocompatibility antigens (12) have been demonstrated in this parasite and offer an effective mechanism for antigenic mimicry of the host and subsequent escape from the immune response.

This same parasite has been used to study the possibility of immunological blockade (13). Host immunoglobulin has been shown to occur on the surface of *Schistosoma mansoni* (14, 15), and it has also been determined that this immunoglobulin is heterospecific, excluding the possibility of a specific parasite-antigen host-antibody interaction (15). These studies also suggest that the host immunoglobulin adsorbed by the parasite is oriented so as to have a Fab region of the molecule available, possibly representing a specific binding of the immunoglobulin through a receptor for Fc present on the membrane of *S. mansoni*. Indeed, receptors for IgG Fc, human β_2-microglobulin (16), and C1q (17) on this parasite have recently been reported.

Host immunoglobulins have also been demonstrated on the surface of blood stream forms of many trypanosomes: *Trypanosoma congolense* (18, 19), *T. brucei gambiense* (20, 21), *T. brucei rhodesiense* (21), *T. cruzi* (22, 23), *T. musculi* (24), and *T. lewisi* (25–28). Other host proteins, such as albumin, have also been detected on the surface of protozoa (28), although in one study with *T. cruzi* (29), attempts in this direction proved negative. It has been suggested (30) that the surface immunoglobulins are responsible for the persistence of adult forms of *T. lewisi* in the circulation of rats.

The precise nature of this adsorption in protozoa has not been determined, despite

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its potential role in parasite escape mechanisms. In this report we use a rosette test to demonstrate the presence of a receptor for IgG Fc on the surface of pathogenic but not of nonpathogenic protozoa of the Trypanosomatidae. Besides its importance in the role already suggested, the presence of an Fc receptor in protozoa poses an interesting problem because of the phylogenic distance between protozoa and trematoda and protozoa and mammalia, thus justifying attempts to elucidate the nature of the adsorption of host immunoglobulin to these parasites.

Materials and Methods

Parasites. Promastigote forms (flagellated culture forms) of *Leishmania mexicana amazonensis* JOS (kindly supplied by Dr. C. A. Cuba Cuba, Universidade Federal de Brasília, Brazil) and H21 (provided by Wellcome Parasitology Unit, Instituto Evandro Chagas, Belém, Pa, Brazil), strains, *Leishmania donovani* 1S strain (kindly donated by Dr. Dennis Dwyer, Laboratory of Parasitology, National Institutes of Health, U. S. A.), *L. mexicana mexicana* 5 strain and *L. enrietti* (kindly donated by Dr. Gabriel Grimaldi, Jr., Instituto Oswaldo Cruz, Rio de Janeiro, Brazil) were maintained in Nicolle, Novy, and McNeal's modified medium (NNN)[1] by weekly subinoculations. For the rosette test, promastigotes were filtered through gauze, washed three times in cold RPMI 1640 tissue culture medium (Grand Island Biological Co., Grand Island, N. Y.) and counted in a hemocytometer chamber and adjusted to 20×10^6 parasites/ml.

Amastigotes (tissue forms of *L. mexicana amazonensis* H21 and *L. donovani* 1S) were obtained from hamsters infected 2–3 mo previously with either parasite. The animals were anesthetized with ether and the tissue (spleens for *L. donovani* amastigotes and skin for *L. mexicana* amastigotes) were removed and teased in Hanks' balanced salt solution (HBSS), and the parasites were then obtained by differential centrifugation. The number of amastigotes used was 20×10^6/ml of medium. Trypomastigotes (blood stream forms) of *T. cruzi* Y strain were obtained in the following manner. On the seventh day of infection, mice were bled through the retroorbital plexus by a capillary tube. The blood was centrifuged at 1,000 rpm for 5 min to pellet erythrocytes. The blood stream forms contained in the supernate were washed three times in RPMI medium supplemented with 5% fetal calf serum (FCS) at 10,000 rpm/20 min. The number of parasites was adjusted to 20×10^6/ml of medium. Nonpathogenic protozoa used were promastigotes of *Herpetomonas samuelpessoai*, *Crithidia harmosa*, *Crithidia acanthocefali*, *Leptomonas samueli*, *Crithidia deanei*, and *Crithidia fasciculata* (kindly supplied by Dr. Jayme Angluster, Departamento de Microbiologia Geral, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil) and epimastigotes of *Trypanosoma mega* (kindly supplied by Dr. Eliana Barreto Bergter, Departmento de Microbiologia Geral, Institutode Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil). All were maintained in NNN with subinoculations at appropriate intervals. They were prepared for the rosette test as described for promastigotes of *Leishmania.*

When performed, enzymatic treatment of all forms consisted in using equal volumes of a suspension of 10^8 parasites/ml of medium and a solution of 1 mg/ml of trypsin (type III, two times crystallized, Sigma Chemical Co., St. Louis, Mo.). The protozoa were incubated at 37°C for 30 min, washed five times in cold RPMI, and resuspended to 20×10^6 parasites/ml.

Antisera. Anti-sheep erythrocyte (SRBC) serum was prepared by immunizing two white rabbits with a 2% suspension of SRBC in complete Freund's adjuvant followed by intravenous boosts of a 1% suspension of SRBC (0.5 ml). IgG and IgM antibodies were prepared from anti-SRBC pooled sera by ammonium sulfate precipitation and column chromatography on Sephadex G-200. The 19S fraction (IgM) was further purified on a Sepharose 4B staphylococcal protein A column (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.). $F(ab')_2$ anti-SRBC was prepared by enzyme digestion of IgG with pepsin (Sigma Chemical Co.), 1 mg/100 mg protein, followed by column chromatography on Sephadex G-150 (31). The reagent was further purified by affinity chromatography on staphylococcal protein A-Sepharose.

[1] *Abbreviations used in this paper:* FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; NNN, Nicolle, Novy, and McNeal's modified medium; PBS, phosphate-buffered saline; SRBC, sheep erythrocytes.

Other anti-SRBC sera used were obtained by immunizing guinea pigs and mice with a 2% suspension of SRBC in complete Freund's adjuvant.

Fc. Fc was prepared by enzyme digestion of a rabbit IgG fraction (obtained by DEAE-cellulose chromatography of normal serum) with papain (type II, Sigma Chemical Co.) at an enzyme:protein ratio of 25 mg/100 mg. Purification was carried out by ion-exchange chromatography on a CM-cellulose column followed by extensive dialysis in the cold (4°C) against water until crystals were formed (32).

Sensitization of Erythrocytes. SRBC were incubated with a subagglutinating dose of IgG, IgM, or $F(ab')_2$ anti-SRBC at room temperature for 20-30 min. The sensitized erythrocytes were then washed five times at 1,500 rpm with HBSS, resuspended to a 0.5% suspension in RPMI, and stored at 4°C.

Rosette Test. The rosette test was carried out in round-bottomed microplates (Linbro Div., Flow Laboratories, Inc., Hamden, Conn.) in the following manner. To 25 μl of protozoa (20×10^6/ml of medium) was added 25 μl of a 0.5% suspension of freshly sensitized SRBC. This represents the ideal proportion of protozoa to SRBC to obtain a high percentage of rosettes. The protozoa-SRBC mixture was then pelleted by centrifugation at 1,500 rpm for 5 min at room temperature. The cell pellets were then gently resuspended with a Pasteur pipette, a sample was transferred to a hemocytometer chamber, and the number of clumps of protozoa-SRBC (rosettes) were counted.

Scanning Electron Microscopy. Samples were kindly processed by Dr. Wanderley de Souza as follows. The rosetted parasites were allowed to adhere to cover slips that had been covered with 0.5% poly-DL-lysine (Sigma Chemical Co.) for 30 min. The cover slips were then washed with phosphate-buffered saline (PBS), and the adhered cells were fixed with 2.5% glutaraldehyde for 1 h at room temperature, postfixed with 1% O_sO_4 for 1 h, dehydrated with ethanol, critical-point dried in CO_2, covered with a layer of gold, and observed in a Jeol JSM-25 SII scanning electron microscope.

Results

Rosette Formation between L. donovani 1S Promastigotes and SRBC Sensitized with Various Immunoglobulins. The presence of a receptor for immunoglobulin Fc on the surface of pathogenic protozoa of the Trypanosomatidae was established by a rosette assay in which SRBC sensitized with subagglutinating doses of IgG, IgM, or $F(ab')_2$ were mixed with promastigotes of *L. donovani* 1S. This mixture was pelleted by centrifugation, resuspended, and examined for the formation of protozoa-SRBC clumps (rosettes). The results presented in Table I show that a high percentage of *L. donovani* 1S promastigotes form rosettes with SRBC sensitized with IgG but practically none with SRBC sensitized with IgM or $F(ab')_2$. None form rosettes with control SRBC. Fig. 1A and Fig. 2 show typical rosettes formed between promastigotes of *L. donovani* 1S and sensitized SRBC. No rosettes formed (Fig. 1B) between these sensitized erythrocytes and *H. samuelpessoai* are seen, thus excluding trapping of the parasites by the sensitized SRBC. These results suggest that the parasite specifically binds the immunoglobulin molecule through the Fc region.

TABLE I

Rosette Formation between Leishmania donovani 1S Promastigotes and SRBC Sensitized with Different Classes of Antibodies

RPMI	Sensitizing antibody		
	IgG	IgM	$F(ab')_2$
–	++++	±	±

–, Absence of rosettes; ±, <100 rosettes/10^4 protozoa; ++++, >400 rosettes/10^4 protozoa.

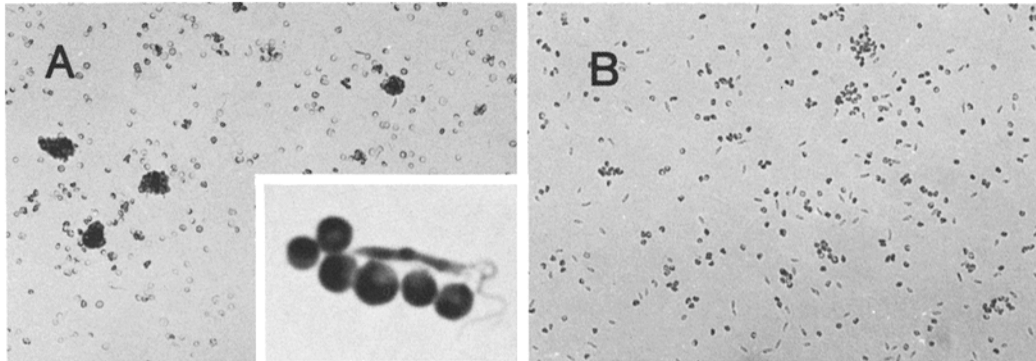

FIG. 1. Rosettes between IgG-sensitized SRBC and (A) promastigotes of *L. donovani* 1S. × 160; (inset in A) Giemsa stain of a typical rosette. × 1,000. (B) absence of rosettes between the sensitized SRBC and promastigotes of *H. samuelpessoai*. × 160.

Immunoglobulin Fc Specifically Inhibits Rosette Formation. In some experiments protozoa were incubated with rabbit Fc or gammaglobulin before the rosette test. The preincubations were as follows. To 40×10^6 protozoa/ml of medium was added an equal volume of RPMI, different concentrations of Fc (0.7–0.01 mg/ml of PBS), or heat-aggregated (60°C/10 min) normal rabbit gammaglobulin. The mixtures were incubated at room temperature for 30 min and then rosetted formed with IgG-sensitized SRBC. Dose-response inhibition was observed when parasites were incubated with purified rabbit Fc or aggregated gammaglobulin, whereas incubation with medium or Fab had no inhibitory effect (Fig. 3).

Enzyme Sensitivity and Regeneration of Fc Receptors in L. donovani 1S. In some experiments, protozoa were treated with trypsin as described in Materials and Methods to determine the sensitivity of the immunoglobulin Fc receptor to this enzyme and its subsequent regeneration. Promastigotes of *L. donovani* treated with trypsin were incubated with sensitized and control SRBC and pelleted by centrifugation at 1,500 rpm for 5 min at room temperature. Pellets were resuspended and observed immediately after centrifugation and at intervals of 15 and 60 min (Table II), as described above. Practically no rosettes were formed immediately after treatment with trypsin. However, further incubation of the cell pellets resulted in regeneration of the parasites' Fc receptors. After 60 min of incubation they showed the same ability to form rosettes as nontreated parasites. Not shown are the results of experiments performed with other pathogenic protozoa that also demonstrate enzyme sensitivity and subsequent regeneration of the IgG Fc receptor. In all of the experiments performed, the parasites did not bind control SRBC after enzyme treatment.

Only Pathogenic Trypanosomatidae Present a Binding Site for Immunoglobulin Fc. In this series of experiments, different species and forms of Trypanosomatidae were used to determine the distribution of the Fc binding site among the members of this family and their growth forms. It is evident from the results shown in Table III that only pathogenic Trypanosomatidae present a receptor for immunoglobulin Fc; nonpathogenic protozoa do not form rosettes with sensitized SRBC. Promastigotes of the genus *Leishmania* and epimastigotes (insect gut form and culture form) of *T. cruzi* formed significant quantities of rosettes ($>400/10^4$ protozoa). However, when *L. donovani* amastigotes were used, almost all of the parasites formed rosettes (Table III).

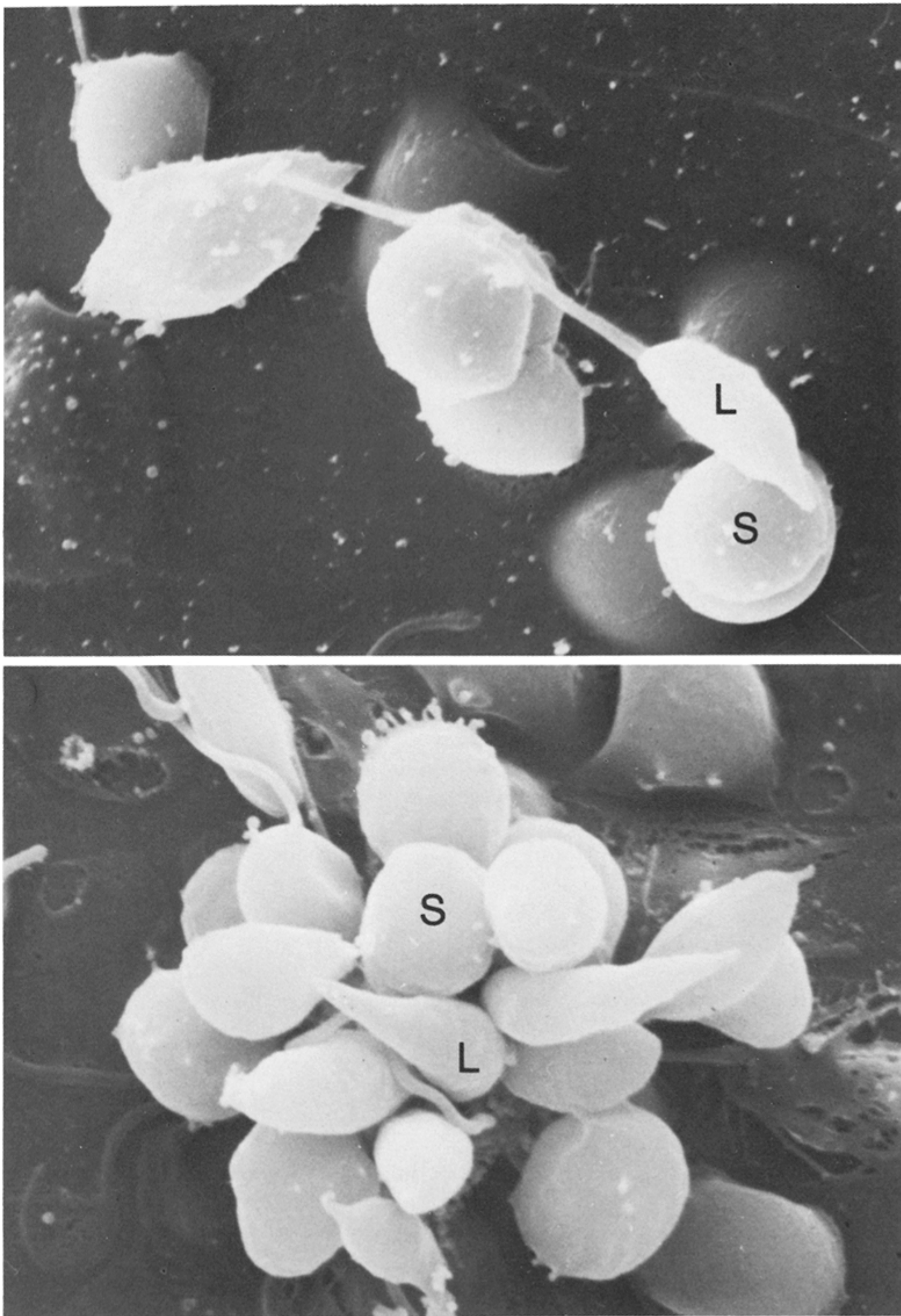

FIG. 2. Scanning electron micrographs of rosettes formed between *L.* donovani 1S (L) and IgG-sensitized SRBC (S). × 7,300.

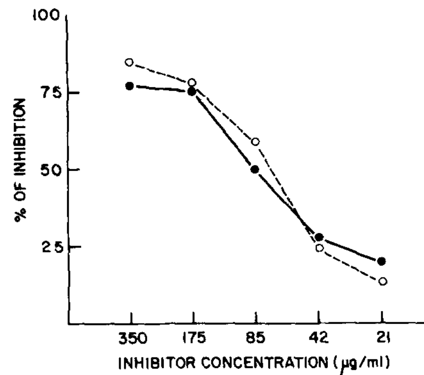

FIG. 3. Specific inhibition by normal rabbit Fc of rosette formation between leishmanias and IgG-sensitized SRBC. The percent inhibition was calculated by dividing the number of rosettes formed in the presence of the inhibitor by the number of rosettes formed without inhibitor. The points represent the average of quadruplicate determinations. (○) Parasites incubated with aggregated normal rabbit gammaglobulin. (●) Parasites incubated with normal rabbit Fc. Parasites were also incubated with 350 to 21 μg/ml of Fab, which did not inhibit rosette formation (data not shown).

TABLE II

Trypsin Sensitivity and Regeneration of IgG Fc Receptor in Promastigotes of Leishmania donovani 1S

Time after enzyme treatment	Rosette formation	
	Trypsin-treated leishmanias	RPMI-treated leishmanias
min		
0-5	±	++++
15	++	++++
60	++++	++++

±, <100 Rosettes/10^4 protozoa; ++, up to 200 rosettes/10^4 protozoa; ++++, >400 rosettes/10^4 protozoa.

In all experiments, none of the species or growth forms formed rosettes with control SRBC. Surprisingly, purified blood stream forms of *T. cruzi* did not form rosettes in significant quantities. To test whether this could be due to the binding of host proteins (immunoglobulin?) to the parasite's surface, possibly through an immunoglobulin Fc receptor, these forms were treated with trypsin in an attempt to remove the protein. Interestingly, the treated forms now formed great quantities of rosettes (>400/10^4 protozoa) with SRBC sensitized with IgG (Table IV). As described above, control SRBC did not form rosettes with the enzyme-treated parasites.

Discussion

The experiments described in this report were performed in an attempt to elucidate the nature of the adsorption of host immunoglobulin to the surface of some members of the Trypanosomatidae. By using a simple rosette test, we gained evidence that this adsorption represents a binding of the host immunoglobulin specifically through an Fc receptor. Several members of this family formed clumps (rosettes) with SRBC sensitized with IgG. This could represent a nonspecific adsorption of the immuno-

TABLE III
Rosette Formation between Sensitized SRBC and Various Genera and Forms of Trypanosomatidae

Pathogenicity	Protozoan	Rosettes	
		SRBC sensitized with IgG	SRBC
Pathogenic for mammals	*L. donovani* 1S (promastigote)	++++	–
	L. donovani 1S (amastigote)	100%*	–
	L. mexicana amazonensis H21 (promastigote)	++++	–
	L. mexicana amazonensis H21 (amastigote)	100%	–
	L. mexicana mexicana 5 (promastigote)	++++	–
	L. enriettii (promastigote)	++++	–
	T. cruzi Y (epimastigote)	++++	–
	T. cruzi Y (trypomastigote)	±	–
Nonpathogenic for mammals	*T. mega* (epimastigote)	–	–
	H. samuelpessoai (promastigote)	–	–
	C. harmosa	–	–
	C. deanei	–	–
	C. fasciculata	–	–
	C. acanthocefali	–	–
	Leptomonas samueli	–	–

–, Absence of rosettes; ±, <100 rosettes/10^4 protozoa; ++++, >400 rosettes/10^4 protozoa.
* No free amastigotes were seen in any of the 100 fields examined.

TABLE IV
Rosette Formation between Trypsinized and Nontrypsinized Blood Stream Forms of Trypanosoma cruzi and SRBC Sensitized with Specific IgG Antibodies

Treatment	Rosettes	
	SRBC	Sensitized SRBC
RPMI	–	±
Trypsin (1 mg/ml, 30 min, 37°C)	–	++++

–, Absence of rosettes; ±, <100 rosettes/10^4 protozoa; ++++, >400 rosettes/10^4 protozoa.

globulin on the parasite's surface. To exclude such a possibility, SRBC sensitized with IgM or $F(ab')_2$ were used in the rosette assay. In this series of experiments practically no rosettes were formed, offering more stringent evidence for the involvement of the Fc portion of the immunoglobulin in the reaction with the parasite's surface. These experiments also exclude the possibility of a carryover phenomenon (trapping) being responsible for the clumps formed between the parasites and the sensitized SRBC, because, although microagglutinations were seen with SRBC sensitized with subagglutinating doses of IgM or $F(ab')_2$, no parasites were seen trapped in them. Also important in rejecting this possibility is the fact that SRBC sensitized with IgG anti-SRBC do not form mixed clumps with nonpathogenic parasites. Furthermore, purified rabbit Fc by itself inhibits rosette formation. The Fc receptor is not specific for rabbit Fc, because rosettes formed between SRBC sensitized with mouse or guinea pig sera and the parasites are also seen (data not shown). This receptor is not nonspecifically adsorbed to the parasite when in the host or in culture media containing protein,

because it is sensitive to trypsin digestion and is regenerated after a short period of time (1 h).

Many authors have demonstrated the presence of host protein on the surface of various blood stream trypanosomes. However, Andrade (29), who used *T. cruzi* isolated from guinea pigs, was unable to detect host immunoglobulin by an immunofluorescence technique. This finding could be due to the fact that the rabbit antiserum used in the test was raised against a wide range of guinea pig proteins. This would certainly decrease the sensitivity of the test if only a limited range of guinea pig proteins was adsorbed to the parasite's surface. The findings of Giannini and D'Alessandro (28) also seem to conflict with our results. These authors found that blood stream forms of *T. lewisi* having little or no detectable surface IgG can adsorb host IgG from immune (ablastic) serum but not from normal rat serum, indicating that this adsorption is due to specific antibody. These findings could be due to the specificity of the fluoresceinated anti-rat IgG used, which is directed to IgG heavy chains and thus to Fc. Therefore, this fluorescein-conjugated anti-rat antibody would not efficiently detect polyspecific host immunoglobulin bound to the parasite's surface by the Fc region, especially if this portion of the immunoglobulin is hidden in the parasite's cell membrane. The fact that blood stream forms absorb IgG from immune serum but not from normal serum might signify that the Fc receptors present on the parasite's surface are already occupied with heterospecific IgG, whereas the specific (ablastic) antibody from immune serum is always readily adsorbed. This does not exclude the importance of the acquisition of host molecules such as IgG as a means of evading the immune response, because this form of protection could be crucial in one of the phases of infection, other escape mechanisms being equally important for the establishment of the host-parasite balance. Recent studies (33, 34) describe structural changes in the tegument and spontaneous loss of surface antigens in *S. mansoni*, which also represent possible escape mechanisms in a metazoan organism and must be considered in protozoa. Such is the case in *Entamoeba*, in which surface redistribution and release of antibody-induced caps has been described (35).

Our results obtained with bloodstream forms of *T. cruzi* further emphasize this point. These forms do not form rosettes after purification from host blood. But after enzyme treatment with trypsin, great quantities of rosettes can be formed with sensitized SRBC. This could be due to host proteins (IgG) already bound to the parasite's surface, thus occupying available Fc receptors. Treatment with trypsin would remove host protein from the parasite's surface, making IgG readily adsorbable. That trypsinization removes substances that protect trypomastigotes from host defenses has recently been shown by Nogueira et al. (36), who found that brief trypsinization removes an antiphagocytic substance from the surface of *T. cruzi*, promoting ingestion by mouse macrophages. In addition, Kipnis et al. (37) found that *T. cruzi* trypomastigotes, which do not activate complement by the alternative pathway, acquire the ability to do so after trypsinization.

Many authors (16, 17, 28, 30) have suggested that host protein antigen uptake by parasites offers a means of escape from host immune responses by disguising parasitical antigenic determinants. We believe that this is the case for the Fc receptor present on the parasites examined in this paper. This receptor offers a means of active adsorption of host protein by the parasite. We observed that only the pathogenic Trypanosomatidae examined presented this phenomenon to a greater or lesser degree, depending

on the form studied. None of the nonpathogenic Trypanosomatidae examined formed rosettes. For nonpathogenic forms, escape from host immune response is not essential for survival, and they would not be selected by evolutionary pressure for the presence of an Fc receptor.

One cannot necessarily correlate virulence with the presence of nonspecific host immunoglobulin on the surface of different pathogenic Trypanosomatidae, and, as a consequence, with the presence of an Fc receptor. The balance between host and parasite defense systems is delicate, and the parasite Fc receptor is not the only means by which a parasite can establish itself in a host. Evolutionary pressures would select parasites that can escape host immune response and at the same time maintain an equilibrium with the host.

Summary

Members of the Trypanosomatidae were studied for their ability to acquire host IgG through a possible Fc receptor. A simple rosette test was devised in which the different species and forms of protozoa were mixed with SRBC sensitized with subagglutinating doses of IgG, IgM, and $F(ab')_2$ anti-SRBC, and the pelleted mixture was observed for the number of clumps (rosettes) formed between the parasites and SRBC. Rosettes were formed between parasites and SRBC sensitized with IgG but not with IgM or $F(ab')_2$, indicating the presence of a receptor for IgG Fc.

The specificity of this receptor for Fc was confirmed by inhibition experiments with normal rabbit aggregated gammaglobulins or with purified normal rabbit Fc. The receptor is sensitive to treatment with trypsin but regenerates after a short period of incubation (1 h), which indicates that it is synthesized by the parasite itself. Interesting was the observation that only pathogenic members of the Trypanosomatidae formed rosettes with sensitized SRBC. In none of the nonpathogenic forms studied could we demonstrate the Fc receptor. Also important was the finding that freshly isolated blood stream forms of *Trypanosoma cruzi* from infected mice did not form rosettes. However, after trypsinization, these forms clearly displayed the ability to do so, possibly indicating a previous acquisition of the host IgG by the parasites in the mouse blood stream.

These findings point to a possible and important means of parasite evasion of the host immune response by masking their surface with host IgG.

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