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PRESENCE OF IMMUNOGLOBULINS ON THE SURFACE OF BLOODSTREAM TRYPANOSOMA CRUZI. CAPPING DURING DIFFERENTIATION IN CULTURE (*)

Judith KLOETZEL (1) and Maria P. DEANE (2)

SUMMARY

Immunological differences between bloodstream trypomastigotes of F and Y strains were demonstrated. F strain trypomastigotes obtained at the peak of parasitemia, between the 30th and 45th day of infection, had host immunoglobulins of the IgM and IgG classes attached to their membrane, demonstrated by direct immunofluorescence test. These globulins were not demonstrable on Y strain bloodstream trypanosomes collected on the 7th day of infection, when parasitemia peak is reached. F strain bloodstream trypomastigotes and Y strain trypomastigotes obtained from tissue cultures and previously incubated **in vitro** with specific antibodies, showed capping of these immunoglobulins, when differentiating in LIT medium.

INTRODUCTION

The course of infection by **Trypanosoma cruzi** in mice varies according to the trypanosome strain. It is either 1) of a fulminating type, with parasitemia peak around the 7th day infection or, 2) parasites may accumulate steadily in the host bloodstream, killing the animal after a longer period of time, or 3) disappearing gradually or rather quickly after this peak, which may be as late as the 45th day (1, personal observations). The mechanisms through which the infection may be controlled by the host have not been satisfactorily clarified, although both humoral and cellular factors seem to be involved [9,10].

Immunoglobulins may play a role in removing parasites from circulation, although they might act otherwise. For instance, in the case of schistosomes, host components have been demonstrated on the parasites [13,14], and were believed to protect them against recognition by the host immune mechanisms.

Capping of immune complexes on cell membranes has been demonstrated in several systems, including **Leishmania enrietti** [5]. It is an expression of the membrane's mobility. After capping, complexes may be either shed or interiorized, and the phenomenon may play a role in the evasion of parasites from the host immune mechanisms.

The present paper deals with F [3] and Y [12] strain trypanosomes. In young mice F strain has a tendency to provoke a gradually ascending parasitemia, reaching a peak between the 30th and 45th day of infection. Broad forms predominate, and parasitemia gradually decreases, with a tendency to became chronic (personal observations). The Y strain on the other hand has a predominance of slender and intermediate forms, with parasitemia peak on the 7th day of infection, and a mortality of practically 100% on the 12th day [1].

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(1) Instituto de Medicina Tropical de São Paulo and Departamento de Medicina Preventiva — Faculdade de Medicina da USP.

(2) Present address: Facultad de Medicina, Univ. de Carabobo, Valencia, Venezuela

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This paper demonstrates an immunological difference between these strains. We show that F strain bloodstream trypomastigotes have host immunoglobulins attached to their surface, while Y strain trypomastigotes have not. These immunoglobulins are capped when trypanosomes are seeded in culture medium.

MATERIAL AND METHODS

Bloodstream trypanosomes — Bloodstream trypanosomes were collected at the peak of infection of each strain. The handling of blood varied according to the strain.

F strain trypanosomes were collected between the 30th and 45th day of infection of 20g swiss albino outbred mice, by bleeding anesthetized mice through heart puncture. No anti-clotting agent was used, in order to minimize action of platelets, which seem to aid destruction of these extremely fragile forms [4]. Clotting was almost immediate, the clot was broken and centrifuged (2,000 x g, for 1 min), serum containing trypanosomes collected, spun down, and parasites washed 3 times in 0.85% NaCl solution, each wash with about 50 times packed trypanosome volume.

In one experiment trypanosomes were harvested on the 11th day of infection.

In one experiment 3 mg (0.15 ml) of a water solution of Enduxan (N.N bi-1 -cloroethil-diamide of the N'-O-propilene ortophosphoric esther) (Pravaz-Recordati), were administered i.v. to, mice one day before infection. After this immunosuppression, trypanosomes were collected on the 7th day, and processed as above.

Y strain parasites were harvested on the 7th day of infection, mouse blood being collected in 3.8% Sodium Citrate solution. Blood was centrifuged (2,000 x g, 1 min) and incubated 15 minutes at 37°C, thus facilitating the movement of trypanosomes trapped between cells to the upper layers. Buffy layer and plasma contained over 80% of the original number of trypanosomes, which were treated as above.

In one experiment Y strain trypanosomes were collected on the 7th day of infection without anti-clotting agent.

All centrifugations and handling of parasites were carried out at 4.°C unless otherwise specified.

Tissue culture trypanosomes — These were obtained by infecting LLC-MKR 2 line cell monolayers, grown in Medium 199 (with Hanks' salts — Flow Laboratories, U.K.), plus 10% heat inactivated bovine serum. Infection was made with Y strain bloodstream trypomastigotes, obtained as above and suspended in Medium 199, plus 5% heat inactivated bovine serum. After infection, medium was always maintained with 5% serum.

The supernatant was changed every third day, and from the 6th day onward contained an abundant number of parasites which had evolved in these cells.

Anti T. cruzi strain specific sera — Y strain: This was obtained by i.p. inoculation of adult mice with 200 bloodstream Y strain trypomastigotes per mouse. The survivors (about 50%) were challenged after 2 months with 200.000 organisms per animal, blood collected after 7 days, and sera stored at -20°C.

F strain: Mice which had survived an initial infection of about 1 x 10^5 bloodstream trypomastigotes, were challenged after 3 to 4 months with 2 x 10^5 homologous organisms per animal, and blood collected and stored as above.

Sensitization of Y strain bloodstream and tissue culture trypomastigotes — Y strain bloodstream trypomastigotes collected and washed as above, were incubated in homologous immune mouse serum at 37°C, 30 minutes, washed 3 times in PBS (0.15 M phosphate buffered saline, pH 7.2). The same was done with tissue culture trypanosomes.

Conjugates — Fluorescein Isothyocianate (FITC) labeled-rabbit anti-mouse gammaglobulins with a F/P ponderal ratio 5, was kindly given to us by Dr. Mario Camargo, of this Institute, and had been prepared and titrated as previously described [7].

FITC Goat anti-mouse IgM and IgG were purchased from Meloy Laboratories, Inc. (Biological Products Division, 6715, Electron Drive, Springfield, Virginia 22151, USA). The anti IgM had a F/P ponderal ratio of 5.6 and the anti IgG of 3.8.

Immunofluorescence test — Trypanosomes obtained as described above, were fixed

overnight at 4°C, in PBS containing 2% formalin, washed once in PBS and suspended in PBS. This suspension was placed with a pipette on glass slides in small areas drawn with nail polish, 1 drop/area, dried at 37°C for a few minutes and stored at -20°C until use.

The direct immunofluorescence test was carried out by incubating antigen slides with FITC conjugated rabbit antimouse gammaglobulin diluted at 1:50, anti IgM at 1:30, 1:60 and 1:75, and anti IgG at 1:10 and 1:30, for 30 minutes at 37°C in a moist chamber. Dilutions were made in 0.1% Evans blue in PBS. These dilutions had previously been assessed as giving specific results in another system. Slides were washed in 3 changes of PBS, dried, mounted with buffered glycerine, pH 8.0 and observed in a Zeiss binocular microscope, provided with dark-field illumination, using a 40 x oil immersion objective, a HBO-200 lamp as light source, a KP 500 filter as exciter and no. 50 filter as a barrier.

As a control slides were also reacted with goat anti-rabbit gammaglobulin FITC conjugated serum.

Non conjugated rabbit anti-mouse gammaglobulins at 1:10 dilution in PBS was used for specific inhibition controls.

Indirect immunofluorescence staining was carried out by incubating slides for 30 minutes at 37°C with homologous anti **T. cruzi** antibody, washing them in 3 changes of PBS, and proceeding as for the direct test. In all tests a control, incubated with normal mouse serum was included.

Differentiation studies — Bloodstream forms of the F strain, collected and treated as above, or without washing, were suspended in LIT medium [2], incubated at 28°C. After varying periods of time, a sample was washed 3 times in PBS and treated as for the other immunofluorescence studies.

Y strain tissue culture trypomastigotes were incubated 30 minutes at 37°C in homologous anti **T. cruzi** mouse serum diluted at 1:50 in LIT medium, washed 3 times and resuspended in the same medium. After 4 and 20 hours they were again washed 3 times in PBS, and prepared for immunofluorescence as above. A control incubated in normal mouse serum was included.

RESULTS

F strain — The direct immunofluorescent test of F strain bloodstream trypomastigotes collected between the 30th and 45th day of infection was positive with FITC antimouse immunoglobulin serum and the anti mouse IgM serum, and slightly positive with anti mouse IgG serum at all dilutions tested. Trypanosomes showed either a bright outline or a patchy pattern (Fig. 1).

The anti-gammaglobulin and anti-IgG reaction was inhibited by previous incubation with non conjugated rabbit anti-mouse gamma-globulins. However, this serum did not inhibit the anti IgM reaction.

F strain bloodstream trypomastigotes collected on the 11th day of infection and on the 7th day of infection of immunosuppressed mice, were negative in the direct test.

When the 30-45 day infection bloodstream trypomastigotes were seeded in LIT medium, incubated at 28.°C fixed after 24 hours, and submitted to anti-mouse gamma-globulin FITC, serum, a number of forms in the process of differentiation showed a bright fluorescent cap (Figs. 2-3) while others had bright overall staining. When these fixed slides were submitted to indirect fluorescence with specific anti **T. cruzi** antibodies all forms had overall staining.

In all reactions a control with goat anti-rabbit gammaglobulin FITC conjugated serum was included, and all controls were negative.

These results refer to at least four experiments each.

Y strain — No immunofluorescence on Y strain bloodstream trypomastigotes collected on the 7th day of infection, either with Sodium Citrate (four experiments) or without anticlotting agent (one experiment) was detected with FITC anti-mouse gamma-globulins and the anti mouse IgG sera; a very weak reaction was observed with the anti IgM serum at a 1:30 dilution, but not at 1:60 and 1:75. This reaction was not inhibited by rabbit anti-mouse gamma-globulins.

Result of the indirect immunofluorescence test with anti **T. cruzi** serum was positive,

with homogeneous staining of parasites. However, when live Y strain bloodstream trypomastigotes were incubated with mouse anti **T. cruzi** serum, washed and fixed, and then submitted to anti-mouse conjugate the same kind of patchy staining as observed with the F strain direct immunofluorescence could be observed (Fig. 4).

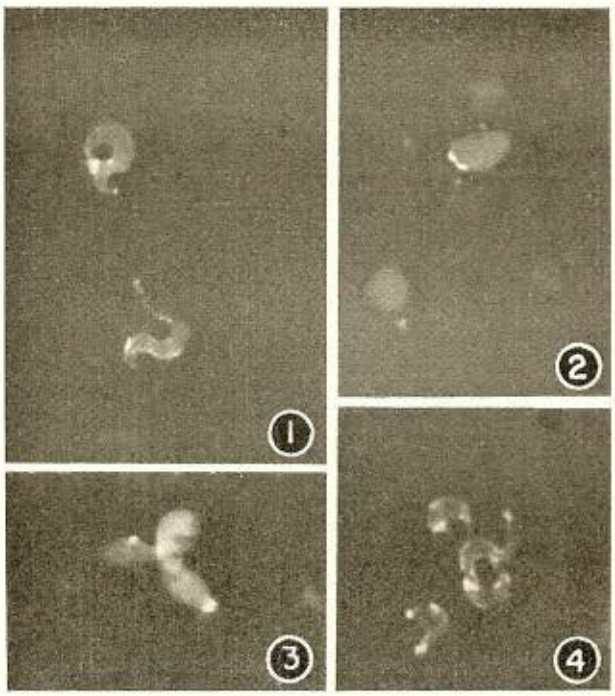

Fig. 1 — Direct immunofluorescence of F strain bloodstream trypomastigotes, with rabbit anti-mouse FITC.
Figs. 2 and 3 — Direct immunofluorescence of F, strain bloodstream trypomastigotes after differentiation in LIT medium. Note capping.
Fig. 4 — Immunofluorescence of Y strain bloodstream trypomastigotes incubated alive in specific anti **T. cruzi** mouse antibody, fixed, and reacted with rabbit anti-mouse FITC.

The same happened with Y strain tissue culture trypomastigotes when sensitized with anti **T. cruzi** serum.

Sensitized tissue culture forms were seeded in LIT medium and observed after 4 and 24 hours. These had forms with the same kind of capping of the F strain trypomastigotes, besides those which were uniformly stained.

In all experiments controls incubated with normal mouse serum, were negative.

DISCUSSION

The F and Y strains have different characteristics. When F strain trypanosomes are inoculated in mice parasitemia is initially very low and the peak of infection is reached between the 30th and 45th day. Only about 20% of animals die at this peak, after which parasitemia falls off. Morphologically the great majority of trypanosomes are of the broad type (personal observations). Also most F strain bloodstream trypanosomes are killed by macrophages **in vitro** [11].

Y strain, on the other hand, has a peak of parasitemia on the 7th day, with an absolute predominance of slender and intermediate forms, and almost 100% mortality by the 12th day [1]. **In vitro,** macrophages are infected with these forms, sustaining their intracellular development [11].

We now describe another difference in bloodstream forms of these strains obtained at the peak of infection, namely, that F strain forms have immunoglobulins attached to their membrane, while these could not be demonstrated for Y strain trypanosomes. These differences could be due to several causes, such as: 1) The time at which parasitemia peak is reached. With the protracted type of parasitemia of F strain there is ample opportunity for the synthesis of antibodies, which could then attach to circulating parasites. With Y strain, the short time elapsed between parasite inoculation and parasitemia peak would not allow for this antibody synthesis. In fact, HANSON [6] has shown that IgM and IgG antibodies started appearing in mouse circulation after two weeks of **T. cruzi** infection, the antibody peak being around 6 weeks. He did not specify the strain of parasites used, however, in his case the maximum antibody titer was observed just after parasitemia peak. The characteristics of these strains are altered with difficulty, namely, a precocious infection is hardly obtained with the F strain even using weanling mice and massive inoculum and thus in only one instance we were able to collect a sufficient number of parasites of the F strain on the 11th day of infection, which had no demonstrable immunoglobulins on their membrane; 2) It may well be possible that broad forms have the capacity to absorb host immunoglobulins on their surface, while slender forms do not. The immunoglobulins observed on F strain trypanosomes surface may play a role in the subsequent disappearance of parasitemia, either through immune lysis, with collaboration of platelets, or through opsonization, in analogy of what happens in other protozoa, where opsonized parasites are killed, while those without antibody thrive within cells [7].

These points are being investigated.

As mentioned under Materials and Methods, in most experiments F strain trypomastigotes were harvested from clotted blood, since they have a tendency to be destroyed by platelets, while Y strain forms, which do not present this problem, were collected from citrated blood because this gives a higher trypanosome yield. However, in one experiment, Y strain forms were also collected from clotted blood, with results identical to the other Y strain experiments.

However, at the dilutions used, forms originating from initial infections and from immunosuppressed animals, which presumably would not be synthetizing antibodies, did not stain. This attests to the specificity of the reaction.

When trypomastigotes are seeded in LIT culture medium and incubated at 26-28°C, they start differentiating, events corresponding roughly to the cycle in the invertebrate host. When these optimal conditions are not observed, degeneration ensues rapidly in acellular media. Both F strain bloodstream trypomastigotes and Y strain tissue culture forms, the latter with **in vitro** passively attached antibodies, were kept under these conditions. After fixation they were submitted to anti-mouse immunoglobulin FITC. Under these conditions a number of forms presented immunofluorescence at one pole. We interpret this phenomenon as capping of immune complexes on the parasite's membrane, which has been described for other cells, including **Leishmania enrietti** [5]. However in those systems the second ligand was involved in the capping process, while in our case capping occurred with the first ligand, the second ligand only revealing the phenomenon. However, when the fixed slides were submitted to indirect immunofluorescence test, using a mouse anti **T. cruzi** serum as the first ligand, all forms showed bright overall staining. This would indicate that the polarized fluorescence is not due to the exclusive localization of antigenic determinants at these points, but patching and capping had indeed occurred with the first ligand, and that antigenic determinants were being resynthetized on the cell membrane.

Capping may have other biological implications. It has been postulated that the fact that **L. enrietti** is able to evade intracellular killing by macrophages may be due to capping of immunoglobulins, rendering parasites resistant against destruction processes [10]. Since only a certain number of immunoglobulin-coated parasites cap, these may correspond to the small percentage of F forms which are not killed within macrophages [11].

This point is also being investigated.

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RESUMO

Presença de imuneglobulinas na superfície de formas sanguícolas do T. cruzi. "Capping" no decorrer de sua diferenciação em meio de cultura

Foram demonstradas diferenças imunológicas entre tripomastigotas sanguícolas das cepas F e Y do **Trypanosoma cruzi.**

Os tripomastigotas de cepa F, obtidos no pico da parasitemia, entre o 30º e o 45º dia de infecção, apresentara imuneglobulinas do hospedeiro, das classes IgM e IgG, ligadas à sua membrana, demonstradas por meio de reação de imunofluorescência direta. Estas imuneglobulinas não foram demonstráveis nos tripanosomas sanguícolas de cepa Y, colhidos no 7.º dia de infecção, pico da parasitemia desta cepa.

Os tripomastigotas sanguícolas de cepa F com imuneglobulinas em sua membrana, e tripomastigotas de cepa Y obtidos de cultivo de tecido, previamente incubados **in vitro** com anticorpo específico, quando em diferenciação em meio de LIT, apresentaram "capping" destas imuneglobulinas.

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