

TRYPANOSOMA CRUZI
Factors Modifying Ingestion and Fate
of Blood Form Trypomastigotes*

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Recent observations have shown that metacyclic trypomastigotes from culture are readily ingested by resident mouse macrophages, subsequently escape from the endocytic vacuole, and replicate in the cytoplasm (1). In contrast, macrophages activated in vivo (2) or exposed to lymphokines in vitro (3) are capable of destroying ingested metacyclic forms. This response is associated with the production of H₂O₂ and perhaps other O₂ intermediates (4).

Our studies have now focused on the blood form trypomastigotes (BFT) isolated from infected mice. These organisms differ from metacyclic trypomastigotes in being more virulent for experimental animals, having a thick surface coat and exhibiting a characteristic S-shaped appearance. In this communication we demonstrate that BFT of both the CL and Y strains of *Trypanosoma cruzi* resist ingestion by mouse macrophages. An antiphagocytic substance can be removed from their surface by brief trypsin digestion, and this treatment, as well as opsonization with antibody, promotes ingestion without modifying their intracellular fate in resident or inflammatory mouse macrophages.

Materials and Methods

Parasites. The Y and CL strains of *T. cruzi* were obtained from Dr. R. Nussenzweig (New York University School of Medicine, New York). Parasites were maintained by weekly transfers in 18- to 20-g male A/J mice (The Jackson Laboratories, Bar Harbor, Maine). BFT were obtained from 7-d-infected mice and isolated by density centrifugation on a Ficoll-Paque column (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.). Blood from infected mice was diluted 1:1 with phosphate-buffered saline (PBS) (Grand Island Biological Co., Grand Island, N. Y.) and trypomastigotes recovered at the interface between plasma and the Ficoll-Paque with other slowly sedimenting particles (platelets and a few lymphocytes and monocytes). Cells were then washed once at 400 *g* for 10 min and subsequently at 150 *g* for 10 min to remove platelets. With this procedure erythrocyte-free trypomastigotes were obtained in high yield. Metacyclic trypomastigotes were purified as previously described (3).

Macrophages. Normal mouse peritoneal macrophages were obtained from female Swiss mice maintained at The Rockefeller University (New York). Cells were harvested according to the methods of Cohn and Benson (5) and cultivated on 13-mm, round, glass coverslips in 16-mm Linbro plates (Linbro Chemical Co., New Haven, Conn.) in Dulbecco's medium (Grand Island Biological Co.) that contained 10% heat-inactivated fetal bovine serum (FBS) (Grand Island Biological Co.), 100 U/ml penicillin, and 100 µg/ml streptomycin (D10 FBS). Inflammatory macrophages were obtained from female Swiss mice injected intraperitoneally 4 d earlier with

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1 ml of a 1% solution of proteose-peptone (Difco Laboratories, Detroit, Mich.) Macrophages were harvested and cultivated as described above.

Lymphokine-activated Macrophages. Normal resident peritoneal macrophages were exposed 2 h after explanting to D10 FBS that contained 25% *T. cruzi*-induced lymphokine or 25% control lymphokine, as previously described (3)

Trypsinization of Parasites. Blood form or metacyclic trypomastigotes were washed once in Hanks' balanced salt solution (HBSS) (Grand Island Biological Co.) and incubated in 500 µg/ml of trypsin (type III from bovine pancreas that was twice crystallized, dialyzed, and lyophilized; Sigma Chemical Co., St. Louis, Mo.) in Dulbecco's medium for 15 min at 37°C. Trypsinization was stopped by the addition of 30% heat-inactivated FBS. The parasites were washed once in HBSS and resuspended to the desired concentration in Dulbecco's medium

Hyperimmune Serum Hyperimmune serum was obtained by immunizing mice with 5×10^6 live culture forms of CL or Y strains of *T. cruzi* in PBS. Animals were then injected four times at 15-d intervals with 10^5 live BFT. Blood was collected 1 wk after the last injection, serum prepared and then stored at -70°C . Such sera have a preponderance of IgG antibody (6) A 1:4 dilution of heat-inactivated opsonizing serum in Dulbecco's medium was added to the purified parasites and maintained in the macrophage cultures throughout the 3-h infection period.

Infection of Cells and Evaluation of Macrophage Trypanocidal Activity. Infection of cells and evaluation of macrophage trypanocidal activity was performed according to methods previously described (1).

Blocking of Macrophage Fc Receptor. 24-h explanted macrophages were washed three times in HBSS and incubated for 30 min at 4°C with 30 µg/ml in PBS of 2.4 G2. This is an IgG monoclonal antibody against the mouse Fc receptor (7) and was kindly provided by Dr. Jay Unkeless (The Rockefeller University). Cells were then washed once in HBSS and exposed for 3 h to a suspension of parasites that contained the same concentration of anti-Fc antibody

Results

The Interaction of Macrophages with BFT. The exposure of resident macrophages to large numbers of BFT of either the CL or Y strains lead to the ingestion and parasitization of <3% of the cells. The multiplicities employed (1:2 and 1:3) were sufficient to infect >35% of the similar cells with metacyclic forms. This striking difference in uptake suggested the presence of a potent antiphagocytic material on the surface of BFT. This possibility was first explored by modifying the parasite surface with proteases. Brief trypsin treatment of BFT (15 min, 500 µg/ml) and enzyme inactivation with heat-inactivated fetal calf serum was accomplished without modification of parasite motility or viability. The addition of trypsinized organisms to macrophages (Table I) lead to a 10-fold enhancement in the entry of organisms. This effect was unrelated to the presence of serum per se. Both the CL and Y strain

TABLE I
Effect of Trypsin Treatment on the Uptake of BFT of T. cruzi by Mouse Peritoneal Macrophages

Strain	Treatment of parasites		Percentage of infected macrophages at 3 h	Percentage of infected macrophages at 72 h	Parasites/100 macrophages at 72 h
	Trypsin	Serum			
CL	-	-	2.5	2.5	36
	+	-	26	25	388
Y	-	-	2.5	3.5	26
	+	-	17	18	155

behaved in a similar fashion, whereas metacyclic trypomastigotes were unaffected by trypsinization.

The Additive Roles of Trypsin and Hyperimmune Serum. The trypsin digestion of the parasite surface still allowed the expression of opsonizing antibody (Table II). In fact, opsonization with antibody has an additive effect on trypsin-treated parasites at the high parasite:cell ratios employed. It also promoted the uptake of nontrypsinized parasites. This effect was seen with both fresh and heat-inactivated serum and occurred only if the concentration of specific antiserum was maintained throughout the entire infection period. The effect of hyperimmune serum in enhancing ingestion was mediated via the Fc receptor on the macrophage surface. Table II illustrates that treatment of macrophages with a monoclonal antibody against the Fc receptor II (7) blocked the uptake of previously opsonized BFT but did not influence cell parasitization with trypsin-digested organisms. Similar information was obtained with the Y strain.

The Fate of BFT Within Resident and Activated Macrophages. Because it was now possible to parasitize macrophages in vitro with BFT, it was next of importance to examine their intracellular fate. Neither trypsin nor hyperimmune serum influenced parasite viability in either resident or inflammatory macrophages, and, by 72 h, extensive replication of the CL and Y strains had occurred. In contrast, when taken up by activated macrophages (lymphokine induced) the vast majority of the organisms were promptly destroyed (Table III). In fact, in vitro activated macrophages

TABLE II
Effect of Hyperimmune Serum and Trypsin on the Uptake and Fate of BFT by Mouse Peritoneal Macrophages*

Macrophage treatment with anti-Fc antibody	Treatment of parasites		Parasites/100 macrophages at 3 h	Parasites/100 macrophages at 72 h	Percentage of infected macrophages at 72 h
	Trypsin	Serum			
None	-	-	Undetectable	15	1
2 4 G2	-	-	Undetectable	4 5	0 5
None	-	+	35	230	20
2 4 G2	-	+	Undetectable	7	1 5
None	+	-	18	130	9
2 4 G2	+	-	14	102	8
None	+	+	58	344	38
2 4 G2	+	+	17	97	9 5

* CL Strain

TABLE III
Fate of Trypsinized and Opsonized BFT of T. cruzi in Macrophage Populations

Treatment of macrophages	Treatment of parasites		Parasites/100 macrophages at 72 h	Percentage of infected macrophages at 72 h
	Trypsin	Serum		
Medium alone	+	-	231	26
Medium plus lymphokine	+	-	40	11
Medium plus control supernate	+	-	269	25
Medium alone	+	+	558	54
Medium plus lymphokine	+	+	0	0
Medium plus control supernate	+	+	634	62

appear to kill serum-opsonized blood forms to a greater extent than nonopsonized organisms.

Discussion

These experiments indicate that BFT obtained from mice acutely infected with either the Y or CL strain, which, under normal conditions, resist in vitro uptake by macrophages, can be rendered susceptible to phagocytosis. This can be accomplished either by mild proteolysis with trypsin and/or opsonization with hyperimmune mouse serum. This suggests the presence of an antiphagocytic factor on the blood forms that is not demonstrable in the metacyclic trypomastigotes propagated in acellular cultures.

The enhanced ingestion of BFT after treatment with trypsin and hyperimmune serum appears to occur by separate mechanisms. Hyperimmune serum and, presumably, its content of IgG antibody opsonizes the organisms in a classical fashion, and binding and interiorization take place via the Fc receptor. Therefore, a monoclonal antibody (2.4.G2) directed against this receptor effectively negates any opsonizing effect. Trypsin, on the other hand, appears to be removing an antiphagocytic factor and promoting ingestion via a non-Fc-mediated mechanism. This may represent the previously described protease-sensitive macrophage receptor that modulates the uptake of culture form trypomastigotes (1). Both trypsin and hyperimmune serum combine to mutually enhance parasite uptake in an additive fashion.

The antiphagocytic factor is found not only in the CL strain of *T. cruzi*, reported to be a myotropic strain and unable to enter macrophages (8), but also in the Y strain, considered to preferentially parasitize macrophages. We predict that some of the in vitro differences observed between these strains (9) may be abolished by trypsin digestion. Once taken up by macrophages in vitro the BFT behave in a fashion identical to the cultured trypomastigotes. That is, the organisms proliferate within the resident or inflammatory macrophage and are destroyed in the lymphokine-activated cell. The consistent effect of hyperimmune serum in enhancing the destruction of BFT in activated macrophages is of interest. Possibly, antibody coating the parasites modifies its escape from the vacuolar system in a fashion similar to that described for vaccinia virus (10). In any event, it represents another example of combined humoral and cell-mediated immunity and deserves more careful scrutiny. As opposed to prior descriptions (8), we found no difference in the fate of opsonized parasites of either strain in normal macrophages. In contrast, opsonization enhanced their killing by activated macrophages, despite the increase in their uptake. This suggests that the role of antibody in vivo may be dependent upon the presence of active cell-mediated immunity. This could explain some of the controversies surrounding the protective effects of passively transferred immune serum (6).

The relevance of these findings to other in vivo events in both animals and man remains to be explored. It seems possible, however, that through immunophagocytosis one could remove organisms from the blood stream and focus them into mononuclear phagocytes that at least have the potential for destroying them. This might prevent the persistent parasitization of other organs, such as the heart, and the possible sequelae of chronic trypanosonemia.

We have considered that the antiphagocytic substance may either be a component of host origin that coats the parasite preventing uptake, or a parasite product that is induced in the in vivo environment. Current analysis of the surface topography of

blood and culture forms by means of enzymatic iodination should answer these questions and will form the subject of a future communication.

Summary

Blood form trypomastigotes of the Y and CL strains of *Trypanosoma cruzi* were tested for their ability to enter and infect mouse peritoneal macrophages. Both strains failed to enter macrophages in appreciable numbers, whereas metacyclic trypomastigotes purified from acellular cultures were ingested with ease. Macrophage parasitization was enhanced manyfold after the removal of an antiphagocytic substance by trypsinization. This occurred without modification of parasite viability. Opsonization with hyperimmune mouse serum also enhanced the uptake of blood form trypomastigotes by macrophages. This effect was mediated by the macrophage Fc receptor. The effects of serum and trypsinization were additive at high parasite:cell ratios. Neither trypsin-mediated nor antibody-dependent opsonization of the organisms modified the fate of either strain within resident macrophages. However, lymphokine-activated macrophages were capable of destroying both strains, and antibody opsonization further enhanced this process.

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