TRYPANOSOMA CRUZI

Surface Antigens of Blood and Culture Forms*

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Recent studies from this laboratory have shown a striking difference in the ability of mouse macrophages to ingest blood-form trypomastigotes (BFT)[1] of the Y and CL strains of *Trypanosoma cruzi*, as compared with metacyclic trypomastigotes grown in culture. This was the result of an anti-phagocytic factor, present on the surface of BFT, that could be either removed by trypsinization or nullified with specific hyperimmune serum. Such treatments were without influence on their intracellular fate when ingested by resident or inflammatory macrophages. However, when taken up by lymphokine-activated macrophages, BFT were readily destroyed, as were the metacyclic trypomastigotes (1).

The presence of this antiphagocytic material lead us to investigate the surface components of both culture-form trypomastigotes and BFT. In this article, we report that BFT possess a major 90,000-relative molecular weight (M_r) surface membrane glycoprotein of isoelectric point (pI) 5.0, which is readily cleaved by trypsin and is precipitated by both human and murine immune sera. This component is absent from metacyclic trypomastigotes and epimastigotes from acellular cultures and may be responsible for the anti-phagocytic effect.

Materials and Methods

Mice.   Male and female A/J, C57BL/10J (The Jackson Laboratory, Bar Harbor, Maine), and Swiss mice (The Rockefeller University, New York) were used at 6–8 wk of age.

Parasites.   The Y and CL strains of *T. cruzi* were obtained from Dr. R. Nussenzweig (New York University School of Medicine, New York) and maintained by weekly passage in A/J mice. BFT were purified from heparinized blood of 7-d infected untreated mice or 5-d infected irradiated mice (irradiated 2 d before infection with 500 rad from a Cesium source). Parasites were collected after cell separation on Ficoll-paque (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.). They were washed once at 4°C in phosphate-buffered saline (PBS), and further purified in a Metrizamide (Nyegaard and Co. A/S, Oslo, Norway) gradient (2) to reduce the number of contaminating mononuclear cells and platelets. Some platelets still remained in samples prepared from nonirradiated mice. Culture-form trypomastigotes were grown from blood stabilates in liver-infusion tryptose (LIT) medium. Metacyclic trypomastigotes were purified from 4-wk old LIT cultures by complement lysis of epimastigotes

[1] *Abbreviations used in this paper:* BFT, blood-form trypomastigotes; BSA, bovine serum albumin; LIT, liver-infusion tryptose; M_r, relative molecular weight; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; pI, isoelectric point; RT, room temperature; SDS, sodium dodecyl sulfate.

followed by separation of live trypomastigotes in a Metrizamide gradient, as previously described (2). Epimastigotes were obtained from 2 wk-old LIT cultures (99% epimastigotes) or as the major form (70–80%) from 4-wk-old samples.

Cell Surface Iodination. Purified parasites were washed twice by centrifugation (400 g for 10 min at 4°C) in PBS and plasma membrane proteins radioiodinated using a slight modification of the lactoperoxidase-glucose oxidase method of Hubbard and Cohn (3). Reagents used were lactoperoxidase (purified grade) from Calbiochem-Berhring Corp., American Hoeclost Corp., La Jolla, Calif. Glucose oxidase (type V), and trypsin (type III from bovine pancreas) from Sigma Chemical Co., St. Louis, Mo. Carrier-free Na[^{125}I] was obtained from New England Nuclear, Boston, Mass. 100% of the parasites were found to be viable and actively motile after radiolabeling. Parasites were washed free of the radiolabel and whole lysates prepared by solubilization in lysis buffer (PBS that contained 0.5% Nonidet P-40 [NP-40; Bethesda Research Labs., Bethesda, Md.], 100 U/ml aprotinin, and 1 mM phenylmethylsulfonylfluoride [Sigma Chemicals Co.]).

[^{35}S]Methionine Labeling of Parasites. Purified parasites were incubated for 3 h at 37°C with 0.5 ml of methionine-free, α-modified Eagle's medium that contained 100 μg/ml bovine serum albumin (BSA) and 50 μCi of [^{35}S]methionine (500 Ci/mmol) (Amersham Corp., Arlington Heights, Ill.).

Immune Precipitation. For isolation of [^{35}S]methionine-labeled cell surface components, parasites were resuspended after radiolabeling in 0.5 ml of PBS that contained 700 μg of human immune IgG or a 1:4 dilution of mouse hyperimmune serum, and incubated for 20 min at 4°C. Cells were then washed by centrifugation in PBS (400 g for 10 min), and resuspended in lysis buffer. After centrifugation (23,000 g for 20–40 min) to remove nuclei and debris, the immune complexes formed in the initial incubation were isolated using Protein A-Sepharose 4B, as described below. ^{125}I-labeled parasite lysates were clarified by centrifugation (23,000 g for 20 min) and 100 μl of clarified supernates were incubated with 35 μg of human immune IgG or 5 μl of mouse hyperimmune serum for 1 h at 4°C, with constant agitation. Antigen-antibody complexes were collected using 30 μl of a 30% suspension of Protein A-Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.), and incubated for 1 h at 4°C with constant agitation. The beads were then centrifuged, washed twice at 4°C in salt-azide buffer (0.2 M NaCl; 12.5 mM potassium phosphate, pH 7.4; and 0.02% NaN₃), twice at room temperature (RT) with a mixed-detergent solution (4) (0.05% NP-40, 0.1% sodium dodecyl sulfate SDS, 0.3 M NaCl, 10 mM Tris-HCl, pH 8.6) and twice again with salt azide buffer. Controls for nonspecific binding were performed by incubation of lysates with 8 μl of normal human serum, 5 μl of normal mouse serum, or with Protein A-Sepharose 4B alone. Antigens were solubilized in 40 μl of electrophoresis sample buffer (2% SDS, 12% sucrose, 0.01% bromophenol blue, 50 mM DTT, and 50 mM Na₂CO₃ buffer, pH 8.6) and boiled for 2 min. All samples were stored at −70°C until used.

Binding to Lectin. 100-μl samples in lysis buffer were incubated for 1 h at 4°C with constant agitation with 50 μl of a 30% suspension of concanavalin A-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, Div. of Pharmacia, Inc.) in PBS. Controls for nonspecific binding were performed by incubating the samples in the presence of 0.25 M α-methyl mannoside (Sigma Chemical Co.). Samples were then centrifuged at 4°C, washed twice in salt-azide buffer at 4°C, twice in detergent solution at RT (0.05% NP-40, 0.3 M NaCl, 10 mM Tris-HCl, pH 8.6) and once more with salt-azide buffer. Samples were eluted in 40 μl of electrophoresis sample buffer and boiled for 2 min.

Polyacrylamide Gel Electrophoresis (PAGE). Samples were electrophoresed in 1-mm thick slab gels with a 4–11% polyacrylamide gradient in the running gel (5). Gels containing ^{35}S-labeled samples were processed for fluorography (6) and exposed on prefogged Kodak X-Omat R-1 film (Eastman Kodak Co., Rochester, N. Y.) at −70°C (7). ^{125}I samples were dried and exposed on Kodak X-Omat R-1 film with image intensification screens (Kronex Lighting Plus; E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.).

Two-dimensional gel electrophoresis was performed as described by O'Farrell (8). For separation in the first dimension, samples were run under equilibrium conditions. The second dimension was run in 4–11% gradient gels as described above.

Mouse Hyperimmune Serum. Hyperimmune sera were prepared by immunizing A/J,

C56BL/10J, or NCS female mice with 5×10^6 culture forms of the CL or Y strain of *T. cruzi* in PBS. Animals were then subsequently injected four times at 15-d intervals with 10^5 live BFT. Blood was collected 1 wk after the last injection, serum prepared, and stored at $-70°$C.

Human Immune IgG. Human immune IgG was isolated from the blood of five patients hospitalized with chronic Chagas' cardiophathy. This was performed with the generous cooperation of Dr. Sonia Andrade and Dr. Zilton Andrade, and Dr. Marcelle Reesink (Department of Pathology, Federal University of Bahia, Brazil). A crude IgG fraction was obtained by sodium sulfate precipitation of a plasma fraction (9). Protein determinations were done by the Lowry method (10).

Results

Externally Disposed Polypeptides of Cultured Epimastigotes and Trypomastigotes, LPO Iodination. Epimastigotes and trypomastigotes of both the Y and CL strains were iodinated and labeled polypeptides displayed on 4–11% gradient SDS-PAGE. Fig. 1, lane A illustrates the pattern obtained when Y strain epimastigotes (2-wk cultures, 99% epimastigotes) were labeled. The same pattern is seen for the CL strain. Fig. 1, lanes B and C show the profile for culture-form trypomastigotes (4-wk cultures, 30% trypomastigotes), when these were labeled before complement lysis and gradient separation, as previously described (11). In each case, only a single 75,000-M_r polypeptide was labeled. If, however, epimastigotes were first lysed with guinea-pig complement and trypomastigotes were then purified and iodinated, the profile was dramatically different (lanes D and E, for Y and CL strains, respectively). Many additional labeled peptides were apparent. The majority of these bands were precipitable with an anti-C3 antibody (not shown), and represent complement components adhering to the surface of trypomastigotes.

Surface Iodination of BFT. The iodination of BFT gave quite a different labeling pattern. It exhibited a major peptide of 90,000 M_r and two minor bands of 50,000 and 55,000 M_r. This pattern was representative of BFT of both the Y (Fig. 2, lane A) and CL (Fig. 2, lane B) strains when obtained from irradiated mice. In contrast,
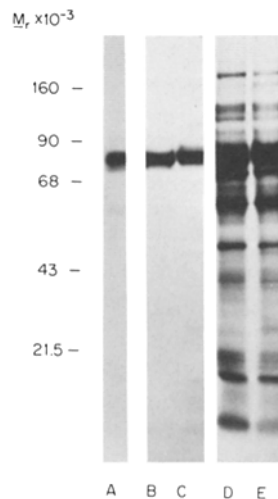

Fig. 1. Iodinated polypeptides on the surface of cultured epimastigotes and trypomastigotes. (A) Epimastigotes. (B, C) Trypomastigotes Y strain (B) and CL strain (C) iodinated before separation from epimastigotes. (D, E) Trypomastigotes, Y strain (D) and CL strain (E) iodinated after separation from epimastigotes.
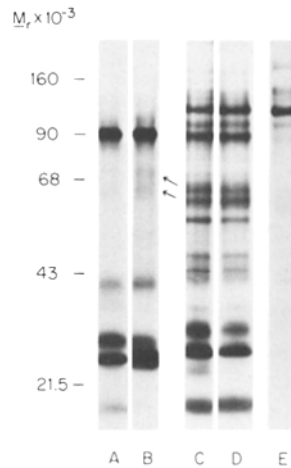
Fig. 2. Iodination profile of BFT. (A, B) Y strain (A) and CL strain (B) isolated from irradiated mice. (C, D) Y strain (C) and CL strain (D) isolated from normal mice. (E) Profile from platelet contaminant obtained by mock isolation of trypomastigotes from noninfected, nonirradiated mice.

labeling of BFT prepared from nonirradiated mice yielded a more complex pattern (Fig. 2C and D), with additional labeled polypeptides. These were thought to arise from platelets that contaminate the preparation. A sham isolation of parasites from uninfected, nonirradiated mice yielded a preparation containing platelets, which upon iodination gave a pattern that accounted for some of the major contaminating peptides (Fig. 2, lane E).

Biosynthetic Labeling of BFT. The possible presence of noniodinated surface polypeptides was analyzed by biosynthetic labeling with [^{35}S]methionine. Sufficient [^{35}S]methionine was incorporated into BFT protein within 3 h to be easily detected (Fig. 3, lane A) in whole-cell lysates. Labeled surface components were identified by incubating viable ^{35}S-labeled parasites with either mouse hyperimmune serum (Fig. 3, lane C) or an IgG fraction from the plasma of a patient with chronic Chagas' disease (Fig. 3, lane B). After incubation at 4°C for 20 min, the organisms were washed, lysed, and immune complexes were isolated by means of Protein A-Sepharose 4B. Label was rapidly incorporated into a 90,000-M_r component, which was the major surface polypeptide recognized by both human and mouse antibodies. Fig. 3, lane D shows that this component binds to concanavalin A-Sepharose 4B and this can be specifically blocked by the addition of 0.25 M α-methyl mannoside (Fig. 3, lane E).

Biosynthetic Labeling of Surface Peptides of Culture-Form Epimastigotes and Trypomastigotes. Both epimastigotes and trypomastigotes purified from acellular cultures also incorporated enough [^{35}S]methionine into their polypeptides within 3 h to yield the whole-cell lysates shown in Fig. 4 (lane A, trypomastigotes; and lane C, epimastigotes). Immunoprecipitation of the surface peptides by human immune IgG is shown in lane B for trypomastigotes and in lane C for epimastigotes. It is clear that the major peptide recognized is again the 75,000-M_r component that was identified previously by iodination.

Effect of Trypsinization on the Pattern of Labeled Peptides. Because trypsinization of BFT resulted in the removal of an anti-phagocytic factor, it was of interest to examine the labeled peptide profile after trypsinization. Comparison of BFT surface polypep-
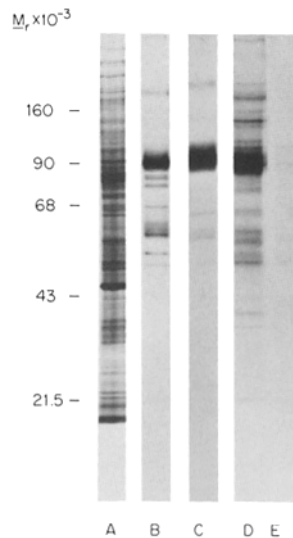
$M_r \times 10^{-3}$

FIG. 3.   [^{35}S]Methionine-labeled molecules isolated from BFT by immunoprecipitation. (A) Whole-cell lysate, 3 h incorporation. (B) Surface components immunoprecipitated with a human immune IgG fraction (patient 1). (C) Surface components immunoprecipitated with a mouse hyperimmune serum. (D) Products labeled of whole-cell lysate (A) bound to concanavalin A-Sepharose 4B. (E) Identical to (D) but in the presence of α-methyl mannoside (0.25 M).
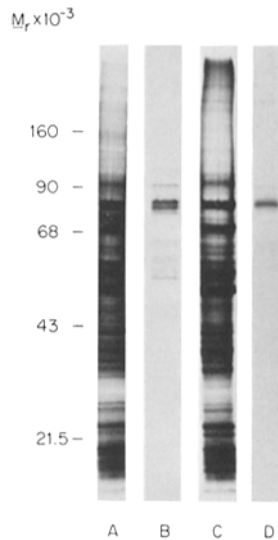
$M_r \times 10^{-3}$

FIG. 4.   [^{35}S]Methionine labeling of culture forms. (A) Y strains trypomastigotes, whole-cell lysate, 3 h incorporation. (B) Y strain, trypomastigotes surface components immunoprecipitated with human immune IgG (patient 2). (C) Y strain, epimastigotes, whole-cell lysate, 3 h incorporation. (D) Y strain, epimastigotes, surface components immunoprecipitated with human immune IgG as in (B).

tides before (Fig. 5, lanes A, B, and E) and after trypsinization (Fig. 5 lanes C, D, and F) reveals the loss of a 90,000-M_r peptide. This is equally true for parasites labeled with ^{125}I (Fig. 5, lanes A–D) and [^{35}S]methionine (lanes E–F). Lanes A and B show immunoprecipitation patterns for Y strain, ^{125}I-labeled BFT with mouse and human

$M_r \times 10^{-3}$
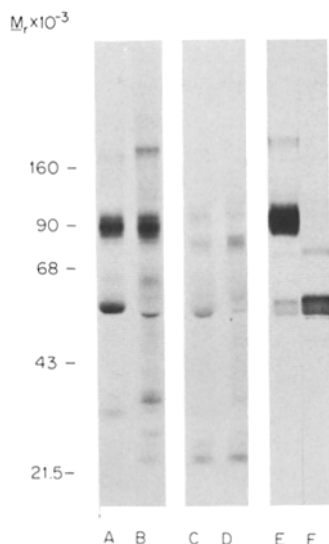
160 –
90 –
68 –
43 –
21.5–

A B   C D   E F

Fig. 5. Effect of trypsin treatment of BFT on the immunoprecipitated proteins. (A) Y strain - immunoprecipitation of ^{125}I-labeled whole-cell lysate with hyperimmune mouse serum. (B) Y strain, profile of ^{125}I-labeled whole-cell lysate with human immune IgG (patient 2). (C) As in (A), parasites trypsinized before iodination. (D) As in (B), parasites trypsinized before iodination. (E) CL strain, [^{35}S]methionine-labeled surface components immunoprecipitated with hyperimmune mouse serum. (F) As in (E) parasites trypsinized before incubation with hyperimmune mouse serum.

TABLE I

Reexpression of BFT Anti-Phagocytic Activity After Trypsinization

Treatment of parasites	Time after trypsinization	Intracellular parasites/100 macrophages	Infected macrophages
	h		%
None		8	1.5
Trypsin	0	63	9
Trypsin	3	25	4
Trypsin + cycloheximide	3	56	10

Average of two experiments. BFT (Y strain) were isolated as described in Materials and Methods. Parasites were exposed to macrophages either immediately, or 3 h after trypsinization.

antibodies, respectively. Lanes C and D represent similar samples except that BFT were trypsinized before iodination. It is clear that the 90,000-M_r component is absent from these preparations. Lane E shows the surface immunoprecipitation pattern for ^{35}S-labeled-CL strain BFT with mouse hyperimmune serum. Lane F is the pattern obtained when BFT are trypsinized after the 3-h labeling period.

Table I shows that BFT begin reexpressing the anti-phagocytic component on their surface within 3 h after trypsinization. This is shown by the inhibition of phagocytosis of trypsinized parasites 3 h after treatment. The presence of 5 μg/ml of cycloheximide inhibits this effect, and it also completely blocks the incorporation of [^{35}S]methionine into BFT polypeptides (not shown).

Trypsinization of epimastigotes and trypomastigotes from acellular cultures had no

apparent effect on either the number or intensity of iodinated or biosynthetically labeled polypeptides.

Surface Antigens Recognized by a Panel of Human Immune IgG. IgG fractions obtained from the plasma of five patients with Chagas' disease revealed remarkably similar immunoprecipitation patterns. Three of such immunoprecipitates are shown in Fig. 6. These were obtained with [^{35}S]methionine labeled BFT of the Y (Fig. 6 A) and CL (Fig. 6 B) strains.

It is noteworthy that the human and mouse antibodies recognize the same polypeptides on the parasite surface (see Fig. 5, lanes A, B, and E). Incubation of the antibodies with ^{35}S-labeled whole-cell lysates of the parasites did not bring down any major peptide in addition to the ones identified at the surface of the parasites (data not shown). Incubation of cell lysates with either normal mouse or human sera does not bring down any labeled bands (data not shown).

Two-Dimensional Gel Electrophoresis Characterization of the Major Surface Peptides of T. cruzi. The charge and heterogeneity of the individual polypeptides was next examined. Two-dimensional analysis of ^{125}I lysates of metacyclic trypomastigotes, derived from acellular cultures, revealed the major 75,000-M_r band which was quite homogeneous and had a pI of 7.2 (Fig. 7 B). The same analysis from ^{35}S-labeled immunoprecipitates of BFT (Fig. 8 B) shows both the 90,000- and 55,000-M_r components, both quite homogeneous. The 90,000-M_r peptide had a pI of 5.0 and the 55,000 a pI of ~4.7. No differences could be detected between the Y and CL strains.

Discussion

In this paper, we employed surface and biosynthetic labels coupled with immunoprecipitation techniques, to study surface antigens of different stages in the life cycle
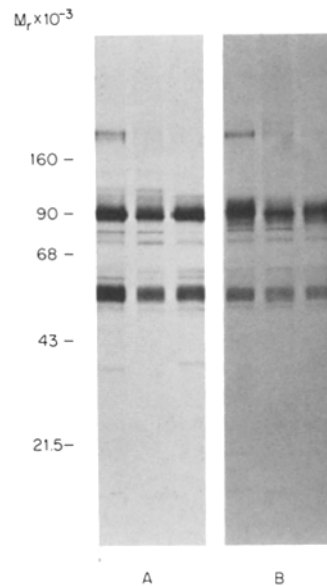

Fig. 6.   Immunoprecipitation profiles of [^{35}S]methionine-labeled blood forms with human immune IgG fractions. (A) Y strain, human immune IgG fractions from patients 4, 5, and 6. (B) CL strain, human immune IgG fractions from Patients 4, 5, and 6.
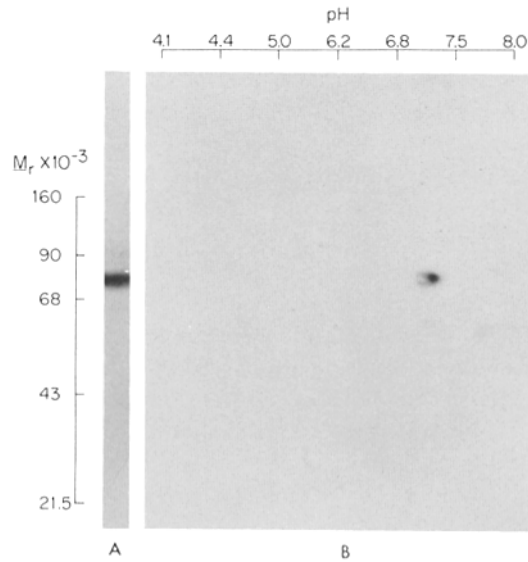
FIG. 7.   Two-dimensional gel electrophoresis of culture-form trypomastigotes' surface peptides. (A) One-dimensional profile of ^{125}I-labeled whole-cell lysate of metacyclic trypomastigotes, CL strain. (B) Two-dimensional profile of the same sample in (A).
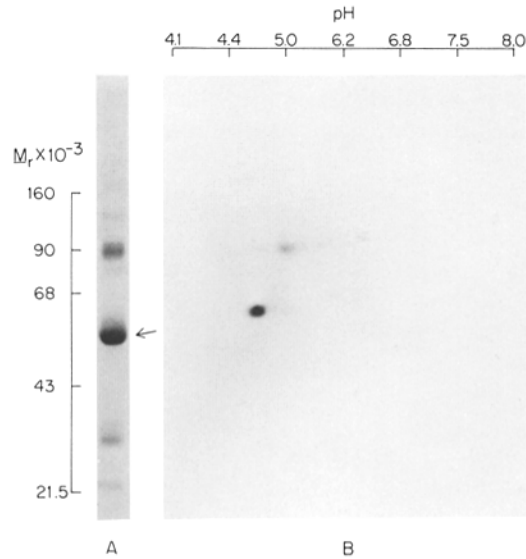

FIG. 8.   Two-dimensional gel electrophoresis of BFT surface peptides. (A) One-dimensional profile of [35S]methionine-labeled blood forms, CL strain; surface components immunoprecipitated with mouse hyperimmune serum. (B) Same sample run in two dimensions.

of T. cruzi. By surface labeling with the LPO technique we demonstrated several peptides on the surface of BFT, one of which is a major glycoprotein of 90,000 M_r. Similar labeling of trypomastigotes and epimastigotes from acellular cultures revealed that the major labeled component was a glycoprotein of 75,000-M_r. Biosynthetic labeling with [35S]methionine also revealed the same polypeptides. In this case,

however, identification required immunoprecipitation with immune sera obtained from both humans and mice.

The 90,000-M_r peptide from BFT was readily removed from the parasite surface by trypsin, and was an acidic glycoprotein with a pI of 5.0. In contrast, the major glycoprotein of both epimastigotes and trypomastigotes isolated from acellular cultures (75,000 M_r) was trypsin insensitive and had a pI of 7.2. The 90,000-M_r glycoprotein found on BFT is likely to be the antiphagocytic surface component (1) in view of its trypsin sensitivity, time of reexpression on the plasma membrane after trypsinization, pI and absence from culture forms.

The presence of an iodinated glycoprotein of 90,000 M_r on the surface of BFT is in keeping with the results of Snary and Hudson (12). These authors reported the presence of this component as the major glycoprotein of all life stages of *T. cruzi*. We find a different component on the surface of epimastigotes and metacyclic trypomastigotes, with a M_r of 75,000. We think it unlikely that the 75,000-M_r component arose by proteolysis of the 90,000-M_r component for the following reasons: (a) We consistently find a single component labeled in iodinated lysates of culture forms, and no other fragments are seen. (b) The samples are lysed in the presence of protease inhibitors, are always handled on ice, and stored frozen at −70°C. (c) All the parasites remained viable throughout the purification procedures. In addition, the surface glycoproteins of the culture forms and those of the BFT have different trypsin sensitivities. Our data also agree with previous observations by de Souza et al. (13) who reported that BFT have a highly negative surface charge and bind large amounts of cationized ferritin, whereas epimastigotes are less negatively charged and bind very little cationized ferritin. It is also possible that the difference between the 75,000- and the 90,000-M_r components is more easily detected in gradient gels.

Two experimental artifacts of labeling are worth discussing in more detail. One is the finding of iodinated complement components on the surface of metacyclic trypomastigotes after purification with guinea pig complement. This suggests that these forms may also be able to trigger the alternative pathway of complement (11) but are resistant to lysis. However, we cannot rule out the initiation of the complement cascade by epimastigotes and its fixation on trypomastigotes as a bystander effect. The other artifact occurs when BFT obtained from nonirradiated mice are surface iodinated. Here, the presence of contaminating platelets complicates the labeling pattern, whereas preparations from irradiated animals reveal a much simpler pattern.

The finding that all mouse and human immune sera tested recognized the same components suggest that these are the relevant immunogens of the parasites. Immunoprecipitation of [^{35}S]methionine-labeled whole-cell lysates did not identify any major additional component not identified by incubation of live parasites with antibodies or by ^{125}I surface labeling. This suggests that surface polypeptides are the dominant immunogenic species, and intracellular parasite components play little, if any, role in the immune response.

We do not as yet understand the immunologic relationship between the 75,000- and 90,000-M_r polypeptides. It is possible that they are closely related peptides, as suggested by Snary and Hundson (12), which differ in terms of glycosylation and/or other posttranslational events. Alternatively, they may be totally unrelated gene products.

Summary

The surface polypeptides of both cultured and blood forms of *Trypanosoma cruzi* were iodinated by the glucose oxidase-lactoperoxidase technique. Blood-form trypomastigotes (BFT) isolated from infected mice displayed a major 90,000-M_r component. In contrast, both epimastigotes and trypomastigotes obtained from acellular cultures expressed a smaller 75,000-M_r peptide. Both major surface components were presumably glycoproteins in terms of their binding to concanavalin A-Sepharose 4B.

Within a 3-h period, both blood and culture forms synthesized their respective surface glycoproteins (90,000 M_r and 75,000 M_r, respectively) in vitro. [^{35}S]methionine-labeled surface peptides were immunoprecipitated with immune sera of both human and murine origin. A panel of sera from patients with chronic Chagas' disease and hyperimmunized mice recognized similar surface peptides. These immunogens were the same components as the major iodinated species.

The major BFT surface peptide was readily removed by trypsin treatment of the parasites, although this procedure did not affect the 75,000-M_r peptide from the culture forms. Two-dimensional polyacrylamide gel electrophoresis revealed that the 90,000-M_r peptide found on BFT was an acidic protein of isoelectric point (pI) 5.0, whereas, the 75,000-M_r peptide from culture-form trypomastigotes has a pI of 7.2. The 90,000-M_r component is thought to be responsible for the anti-phagocytic properties of the BFT (1).

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