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INTERIORIZATION OF *TRYPANOSOMA CRUZI* INTO MAMMALIAN HOST CELLS IN THE LIGHT OF THE PARASITE MEMBRANE CHEMICAL COMPOSITION

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Apparent parasitemia at the acute phase of Chagas' disease can be viewed as a reflection of the liberation to the blood parasites that have previously multiplied inside host-cells soon after infection. The appearance of circulating anti-*T. cruzi* antibodies, together with the induction of cell-mediated immunity (for reviews see Colli, 1979; Brener & Camargo, 1982; Scott & Snary, 1982), seems to control the infection, probably by killing the majority of the parasites that are liberated in the subsequent bursts. Specific anti-*T. cruzi* antibodies are detected from the beginning of the infection process. It was possible to demonstrate that passive transfer of sera from chronically infected to non-infected animals partially protects against inoculation of virulent homologous or heterologous *T. cruzi* strains (Brener, 1980).

Nevertheless, the mechanisms involved in the control of parasites in the chronic phase of the disease are unknown. This control is "loose", since no spontaneous cure of the disease has been reported. Recently, an interesting correlation between an ongoing infection and the presence of complement-mediated lytic antibodies against the trypomastigote forms was observed (Krettli & Brener, 1982). This class of antibodies has not always been found to be associated with that responsible for conventional serological diagnosis (Krettli, Cançado & Brener, 1982). These results were reinforced by the work of Andrews et al. (1985) and Yoshida (1985), who have found high titers of anti-trypomastigote complement-mediated lytic antibodies in animals immunized with trypomastigotes inactivated, respectively, with 8-methoxy psoralen or merthiolate or by heat-killing.

To date, however, it has not been possible to obtain total protection against *T. cruzi* infections in experimental animals. Several attempts to obtain sterile immunity using attenuated or killed parasites and subcellular fractions of parasites as antigens were unsuccessful (Brener & Camargo, 1982). Such attempts were made either with epimastigotes (which are non-infective and thus might not carry the relevant antigens) or with trypomastigotes under conditions which may have destroyed their ability to infect host-cells. A fairly good protection using heat-killed or merthiolate-inactivated trypomastigotes has recently been reported (Yoshida, 1985); nonetheless, transient parasitemias could not be avoided upon subsequent challenge of the immunized animals with virulent *T. cruzi*.

A high protection against challenge infections has been obtained by Andrews et al. (1985) upon immunization of mice with high doses of trypomastigotes previously inactivated by treatment with 8-methoxy psoralen followed by 365 nm UV irradiation. After inactivation, the parasites remained motile and were able to interiorize into non-phagocytic host cells. Interestingly, the interiorized parasites were unable to divide and, eventually, were destroyed. Accordingly, no significant pathological lesions have been found in the organs of the immunized animals. The authors could not, however, claim that a sterily immunity had been achieved since they encountered two positive hemocultures from blood of the immunized mice, indicating that a total parasite inactivation had not been attained. The strong humoral immune response elicited in the immunized mice, together with the findings of undetectable parasitemia and 100% survival, favor the suggestion that the efficiency of immunization may be related to the maintenance of surface structures, important to parasite interiorization into host cells. The relevant epitopes should either belong to antigens released by metabolically active trypomastigotes, a conclusion also advanced by Krettli & Brener (1982), or be particularly sensitive to chemical and physical perturbation, thus explaining why excessive manipulation of the immunizing agent results only in partial protection against infection (Andrews et al., 1985). Whether these putative interiorization blocking antibodies are related to the complement-mediated lytic antibodies still remains to be investigated.

The trypomastigote form, unlike the epimastigote form, is able to invade non-phagocytic host cells and resists the extremely agressive lysosomal milieu. Both characteristics are related to the capacity of the infective form to recognize the surrounding environment. These characteristics are most probably mediated by the parasite plasma membrane. Thus, it is important to decipher in a sistematic fashion, the chemical composition of the parasite plasma membrane, including the definition of the antigenic composition of this organelle.

Conditions affecting the association of *T. cruzi* trypomastigotes with non-phagocytic vertebrate cells

Partial inhibition of the infection of cultured epithelial cells was found to occur in the presence of inactivated immune sera from infected animals and humans (Zingales et al., 1982). This result has been further confirmed by comparison of the effect of increasing concentrations of monovalent Fab' fragments

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from normal and chagasic immunoglobulins (Colli et al., 1984). Inhibition, however, has never been found to be complete, great variability being observed when several sera were compared. This variability might reflect the variation in titers of several (possibly unrelated) antibody activities present in immune sera (cf. Krettli & Brener, 1982), not to mention the apparent tendency of trypomastigotes to shed bound antibodies from their surfaces (Schmuñis et al., 1978; Katzin et al., 1984).

Adhesion and internalization of trypomastigotes into non-professional phagocytes are steps which have been operationally separated from each other (Henriquez, Piras & Piras, 1981a; Colli, Andrews & Zingales, 1981; Andrews & Colli, 1982).

Piras, Piras & Henriquez (1982) observed that trypomastigotes freshly released from infected cell monolayers have a lower infective capacity than the same parasites incubated in cell-free medium containing fetal calf serum. This observation has been confirmed by us (Kuwajima & Colli, 1983). Puromycin blocked the development of adhesion and interiorization, while actinomycin and tunicamycin interfered with internalization but not with adhesion. We also have found an inhibition of trypomastigote interiorization by tunicamycin, an effect totally reversible 4 h after removal of the drug (Zingales et al., 1985). Pactamycin, a drug which promotes an irreversible inhibition of protein synthesis, also inhibits adhesion and interiorization (Lima & Kierszenbaum, 1982).

Henriquez, Piras & Piras (1981a) have described a 4-to 6-fold stimulation of adhesion upon trypsin treatment of freshly released parasites, but this enhancement became less evident (and eventually disappeared) when parasites that were already partially or totally activated were employed. Incubation of parasites in cell-free media containing protease inhibitors blocked the enhancement of adhesion (Piras, Piras & Henriquez, 1983).

Our experience with trypsin treatment of the tripomastigotes is somewhat different. Treatment of trypomastigotes with 5 to 150μg/ml trypsin (SA = 43μmol p-toluene-sulfonyl-L-arginine methyl ester min^{-1} mg^{-1} at 25°C, pH 8.2 in the presence of 0.01 MCa^{2+}) inhibits internalization (Andrews, Katzin & Colli, 1984). Adhesion was not quantitated, but from microscopic observations it would appear that trypsinized parasites are more difficult to remove from the surface of epithelial host cells than non-trypsinized parasites. Since trypsin treatment of trypomastigotes removes almost all proteins from the surface (Andrews, Katzin & Colli, 1984), it is possible that the increase in adhesion reflects a non specific effect resulting from the exposure of lipid residues on the surface. At present, however, the source of the conflicting results reported by these two laboratories is unknown.

Several laboratories have investigated the effects of lectins on interiorization. Results vary, depending upon the experimental protocol, e.g., whether parasites or host cells are preincubated with the lectin or whether the lectin is maintained in the medium throughout the infection period. The results tend to favor the interpretation that lectins can enhance parasite internalization by acting as bridges between specific ligands present on both the host cell and parasite surfaces, thus maintaining proximity between the interacting cells (Piras, Piras & Henriquez, 1983; Colli et al., 1984). This also appears to be the mechanism by which fibronectin stimulates *T. cruzi* interiorization in fibroblasts and macrophages (Ouassi et al., 1984; Wirth & Kierszenbaum, 1984).

In an attempt to identify the carbohydrate residues involved in the infection process, several laboratories tried to use free carbohydrates to inhibit *in vitro* infection of cell monolayers. Of all carbohydrates tested, N-acetyl-D-glucosamine appeared to be the best inhibitor (Andrews & Colli, 1981; Henriquez, Piras & Piras, 1981b; Crane & Dvorak, 1982; Piras, Piras & Henriquez, 1983; Colli et al., 1984). Nonetheless, recent reports claim the isolation of *T. cruzi* clones which infect cell monolayers even in the presence of high concentrations of this aminosugar (cf. Dvorak, 1984).

Despite some residual controversies, which might in reality be a consequence of the great genetic variability observed among *T. cruzi* isolates, stocks and clones, the available data favor the hypothesis that a specific ligand-receptor mechanism is involved in the interaction of *T. cruzi* with host cells. The surface of *T. cruzi* trypomastigotes apparently contains one or more proteins, at least one of which is a glycoprotein with N-glycosidic linked oligosaccharides, that are involved in adhesion and/or penetration of the parasite into host cells.

Parasite surface proteins: possible role in infection

The majority of the trypomastigote surface proteins (Zingales et al., 1982) are glycoproteins with affinity for Con A (Katzin & Colli, 1983). Another glycoprotein, having a molecular weight of 85,000 (Tc-85) (Katzin & Colli, 1983), a pI ranging from 6.3-7.5 and presenting charge heterogeneity (Andrews, Katzin & Colli, 1984), was found to have high affinity for WGA. An antiserum prepared in rabbits against Tc-85 inhibited the interiorization of *T. cruzi* into LLC-MK_2 cells by 20-40%. IgG isolated from this serum inhibited internalization by 70% (Colli et al., 1984). A monoclonal antibody prepared against Tc-85 seems to be inhibitory to the internalization step (Alves, Abuin & Colli, in preparation).

Treatment of trypomastigotes with trypsin removes almost all of the surface proteins and promotes an inhibition of trypomastigote interiorization (Andrews, Katzin & Colli, 1984). Removal of trypsin was accompanied by the synthesis of the full surface pattern, with concomitant recovery of the interiorization capacity.

Tunicamycin treatment of trypomastigotes inhibited their interiorization into LLC-MK_2 cells by up to 80%. Labeling of tunicamycin-treated parasites with (^{35}S) methionine indicated a modification of the molecular weights of several *T. cruzi* antigens. After 6 h treatment with the drug, the antigens of Mr 170-175,000, 120-125,000, 90-95,000 and 85,000 disappeared, with concomitant appearance of the 155,000, 105,000 and 75,000 antigens. The disappearance of Tc-85 upon treatment with tunicamycin was confirmed by WGA-Sepharose affinity chromatography. Likewise, the other modified antigens no longer bound to Con A-Sepharose (Zingales et al., 1985). These observations reinforce the suggestion that Tc-85 may play a role in parasite interiorization, but do not exclude the possibility that other glycoproteins with N-glycosidic linked oligosaccharides might also be important in the adhesion and/or internalization steps.

Several laboratories have analysed the protein composition of the parasite surface. Despite some conflicting data, it is generally agreed that the surface of the trypomastigote form is more complex than that of the epimastigote form (Zingales et al., 1982; Katzin & Colli, 1983; Andrews, Katzin & Colli, 1984). It is also widely accepted that trypomastigotes have a prominent (if not dominant) cluster of proteins with M.W. ranging from 70,000 to 100,000. Several laboratories have reported that a glycoprotein of Mr 90,000, originally described by Snary & Hudson (1979), is common to the three differentiation stages of *T. cruzi*, whereas two other laboratories failed to find the 90,000 protein in, at least, epimastigote forms (Nogueira, 1983; Lanar & Manning, 1984). Andrews, Katzin & Colli (1984) found that the 90,000 glycoprotein appears in both differentiation stages, but is less pronounced in the epimastigote form.

Recently, analyses of the surface proteins present in several clones from *T. cruzi* strains led to the conclusion that epimastigotes (Zingales et al., 1984) and trypomastigotes (Plata, Garcia Pons & Eisen, 1984) from different origins have highly conserved common cross-reactive antigens. The latter authors found, however, many strain-specific antigens in trypomastigotes, indicating the highly heterogeneous nature of *T. cruzi* strains at the genetic level. Genetic diversity of *T. cruzi* is also apparent from the work of Dvorak and collaborators (Dvorak, 1984; Kirchhoff et al., 1984), who have found different surface reactivities of strains and clones to a monoclonal antibody. Thus, genetic variability, previously shown with zymodemes (Miles et al., 1978) and schizodemes (Morel et al., 1980) also seems to be expressed at the surface level, perhaps explaining charge heterogeneity (cf. Andrews, Katzin & Colli, 1984; Lanar & Manning, 1984) and the variable recognition of individual antigens by different immunesera.

Do sialyl residues of *Trypanosoma cruzi* glycoproteins and glycolipids play a role in host cell infection?

The presence of sialic acids on the surface of *T. cruzi* epimastigotes and trypomastigotes is quite well established (for references see Souto-Padrón et al., 1984). Pereira et al. (1980) found that the binding of WGA to epimastigotes was due mainly to sialic acids. A number $n = 3 \times 10^6$ residues per cell with an affinity constant $Ka = 2\text{-}3 \times 10^6\ M^{-1}$ was found for WGA binding to epimastigotes (Pereira et al., 1980; Katzin & Colli, 1983). Trypomastigotes exhibited at least two binding sites for the lectin, one of higher affinity but lower capacity ($n_1 = 1.2 \times 10^6$, $Ka_1 = 33 \times 10^6\ M^{-1}$) and the other of lower affinity and higher capacity ($n_2 = 5.9 \times 10^6$, $Ka_2 = 1.5 \times 10^6\ M^{-1}$) (Katzin & Colli, 1983). Direct determination of sialic acids following neuraminidase treatment of epimastigotes gave values ranging from 10^6 to 10^7 molecules per parasite (Pereira et al., 1980; Confalonieri et al., 1983; Schauer et al., 1983). Interestingly, no labeled proteins were retained when ^{131}I surface labeled epimastigote lysates were chromatographed on a WGA-Sepharose column (Katzin & Colli, 1983), suggesting that the WGA receptors of epimastigotes are preferentially associated to macromolecules incapable of binding to lectin columns and/or sialoglycolipids. More recently, Pessolani et al. (1984) obtained a sialogalactomannan containing 10% protein by hot phenol-water extraction of epimastigote forms.

The presence of sialic acids in the epimastigote forms of *T. cruzi* has been demonstrated chemically. While Schauer et al. (1983) reported that the main species present were N-acetyl and N-glycolyl neuraminic acids, Confalonieri et al. (1983) provided evidence for the existence of O-alkyl substituted sialic acids in the glycolipid fraction. This apparent discrepancy may reflect changes in the composition of the medium. In fact, at least in the case of epimastigotes, the condensing enzyme for synthesis of sialic acid from N-acetyl-D-mannosamine is apparently lacking (Schauer et al., 1983).

The sialoglycolipids in epimastigotes encountered by Confalonieri et al. (1983), have been shown to be endogenously synthesized by the incorporation of labeled palmitic acid and galactose into the neuraminidase-sensitive glycolipid fraction extracted with solvents recommended for the isolation of gangliosides (Lederkremer et al., 1985). Likewise, endogenous synthesis of sialoglycolipids has also been described in trypomastigote forms of *T. cruzi* (Couto et al., 1985). Preliminary chemical analyses of isolated Tc-85 have shown the presence of sialic acid in this glycoprotein (Couto, Katzin & Colli, 1984). Thus, to date, sialic acids have been found to be associated with sialoglycolipids (probably gangliosides) and a galactomannan in epimastigotes and with sialoglycolipids and the glycoprotein Tc-85 in trypomastigotes.

If the suggestion that sialic acid cannot be endogenously synthesized (Schauer et al., 1983) is confirmed, *T. cruzi* would have to obtain this sugar from the surrounding environment, mainly through the hydrolysis of sialic acid-containing compounds (cf. Schottelius, 1984). This hypothesis is reinforced by the observation of Pessolani et al. (1984) that the sialogalactomannan did not contain sialic acid when the epimastigotes were grown in a serum-free medium. This would theoretically be possible since *T. cruzi* contains a neuraminidase which is expressed on the membrane and secreted to the medium (Pereira, 1983; Pereira, MEA apud Nogueira, 1983). The specific activity of the enzyme is 6- to 15-fold greater in trypo-

mastigotes than in epimastigotes (Pereira, 1983). Several substrates were hydrolysed by the *T. cruzi* neuraminidase, the best being α_1-acid glycoprotein (orosomucoid), fetuin and sialyl-lactose. This enzyme is active against human cells since incubation of freshly obtained trypomastigotes with human erythrocytes released sialic acid in a time-dependent fashion. Red blood cell desialylation also appeared to occur in experimental animals during the course of infection with *T. cruzi* (Pereira, 1983), a finding highly relevant for the pathogenesis of Chagas' disease.

Thus, it is apparent that *T. cruzi* trypomastigotes are "sialic acid eaters", a function that must be related to a specific, and very important, need of the parasite. This function might well be the continuous synthesis of sialic acid-containing molecules crucial to their ability to adhere and penetrate into host cells.

Recently, a stimulation of trypomastigote interiorization into *in vitro* cultured vertebrate host cells by fetuin has been reported (Piras, Piras & Henriquez, 1984; Piras, R., personal commun.). Maximal stimulation was observed when the parasites had been previously treated with trypsin prior to fetuin addition. Other sialoglycoproteins such as transferrin, fibrinogen, α_1-antitrypsin and mucin were also stimulatory, though not as effective as fetuin. Although these results were obtained with trypsinized parasites, a treatment which presumably destroys the neuraminidase (Pereira, MEA apud Nogueira, 1983), one must take into account the fact that interiorization assays are performed by incubating parasites with cells for at least one hour, a period which might be sufficient for the synthesis of new enzyme molecules.

Donation of sialic acid residues could be an alternative explanation for the fibronectin enhancement of *T. cruzi* interiorization into fibroblasts and macrophages (Ouaissi et al., 1984; Wirth & Kierszenbaum, 1984). Fibronectin is also a sialic acid-containing glycoprotein (Mosesson & Amrani, 1980). Although the fibronectin-induced stimulation of *T. cruzi* association to macrophages occurs even with glutaraldehyde-fixed parasites, the need for completion of one or more reactions induced by fibronectin is suggested by the requirement of a 1h preincubation in order to observe this enhanced capacity of *T. cruzi* to associate with macrophages (Wirth & Kierszenbaum, 1984).

Thus, it is possible that sialyl-containing molecules stimulate interiorization of *T. cruzi* into host cells by donating sialyl residues to the parasite surface glycoproteins and glycolipids. If this is the case, sialic acid molecules, recently hydrolysed from donor molecules by the *T. cruzi* neuraminidase, would have to be transported into the cell and processed, presumably via a CMP-sialic acid intermediate, in order to permit their incorporation into recently synthesized glycolipids or glycoproteins prior to their insertion into the surface membrane.

A novel and provocative alternative hypothesis has been advanced by Previato and collaborators (Pessolani et al., 1984; Andrade et al., 1984; Previato, J.O., personal commun.). These authors have found that neuraminidase-treated epimastigotes lose their WGA receptors and acquire the ability to agglutinate with peanut agglutinin. This lectin is specific for galactose, a common subterminal residue in compounds having sialic acid as the terminal residue (cf. also Pereira et al., 1980; Pereira, 1983). The ability to agglutinate with WGA was readily restored in a time-dependent fashion by incubating the neuraminidase-treated epimastigote with fetuin or sialyllactose, but not by incubation with free sialic acid, suggesting that the cell is unable to use the free sugar to sialylate the WGA receptors. This result led the authors to propose that the epimastigote membrane might possess a transglycosylase activity for sialic acid.

This interesting hypothesis will have to be tested with further experimental work, including the demonstration that sialic acid residues can be transferred from a donor to an acceptor (endogenous or exogenous) by acellular *T. cruzi* preparations. Also, since no external source of energy would be available for the resynthesis of the O-bond, the equilibrium reaction would require that the energy of the sialyl-O-R linkage in the donor substrate be preserved in a sialyl-enzyme bond in order to permit the subsequent reaction with the acceptor molecule.

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