LECTIN RECEPTORS AS MARKERS FOR *TRYPANOSOMA CRUZI*

Developmental Stages and a Study of the Interaction of Wheat Germ Agglutinin with Sialic Acid Residues on Epimastigote Cells*

By MIERCIO E. A. PEREIRA,‡ MARIA A. LOURES, FERNANDO VILLALTA, and ARNALDO F. B. ANDRADE

From the Department of Immunology, Institute of Microbiology, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

Chagas's disease is a chronic, debilitating, incurable disease affecting at least 12 million people in Central and South America (1, 2). *Trypanosoma cruzi*, the etiological agent, exists in three morphologically distinct forms related to different environments where it lives (1, 3): the amastigotes, which represent the multiplying stage found within the cells of mammalian hosts; the trypomastigotes, which are present in the bloodstream and in infected tissues of mammalian hosts, in the lumen of the rectum of the transmitting triatomid insect, and in culture; and the third form, epimastigotes, which are found intracellularly in mammalian hosts as a multiplying form in the vector's digestive tract, and in culture. Although bloodstream, insect, and culture trypomastigotes are highly infective, culture epimastigotes have very little, if any, capacity to initiate infection in the mammalian host (3). Although the morphologically defined forms of *T. cruzi* perform different functions in the life cycle of the parasite, the molecular mechanisms underlying the distinct behaviors remain unknown.

The majority of exposed plasma membrane proteins are glycoproteins (4, 5), and there is increasing evidence that glycoproteins are of major importance in the maturation and regulation of functions displayed by cells such as those of the immune system (6, 7). On the other hand, much of the work concerning structure and function of cell surface carbohydrates is derived from studies with lectins, a class of sugar-binding proteins of nonimmune origin (8–10).

In our study a systematic analysis of cell surface glycoproteins and/or of glycolipids of *T. cruzi* developmental stages was undertaken, using ~30 highly purified, glycosidase-free lectins of various carbohydrate specificities as probes. The results showed that some lectins display unique selectivity in interacting with trypanosome forms as determined by agglutination and binding assays. It also showed that trypomastigotes obtained from blood of infected mice and from culture differed in their surface carbohydrates. Moreover, sialic acid residues detected and quantitated in epimasti-

* Supported by contract 527/CT from the Brazilian National Research Council (CNPq), Brazilian Fund for Science and Technology (FINEP), the Council for Graduate Studies, (CEPG) of the Federal University of Rio De Janeiro, and the Rockefeller Foundation.

‡ Present address: Division of Geographic Medicine, Tufts University, Boston, Mass.

gotes were found to be the specific receptor sites for wheat germ agglutinin (WGA)[1], which was one of the lectins that reacted exclusively with epimastigote cells; some biophysical and biological parameters of the sialyl receptor-WGA interaction are also presented.

Materials and Methods

Parasites. The Y strain of *T. cruzi* (11) was used throughout. Epimastigotes were from 3-d-old cultures in ox liver-infusion tryptose supplemented with 10% fetal calf serum at pH 7.2 (LIT) (12). The cells were washed five times with chilled 0.01 M Na-phosphate-buffered saline (PBS) that contained 0.5% bovine serum albumin (BSA), centrifuged at 1,971 *g* for 15 min, and resuspended to the desired concentration in the same buffer: such cultures usually contained >99% epimastigotes as determined by microscopic examination of Giemsa-stained smears.

Trypomastigotes were both from culture and from blood of infected mice. To obtain trypomastigotes differentiated in vitro, the parasites were grown at pH 6.7 in a modified LIT medium in which tryptose had been substituted for lactalbumin hydrolysate to favor the differentiation of epimastigotes into the metacyclic forms of *T. cruzi* (13). Parasites were harvested from 15-d-old cultures (usually >15% trypomastigotes), washed five times with 0.01 M Hepes-buffered TC-199 (Grand Island Biological Co., Grand Island, N. Y.), pH 7.2, and 25% fresh guinea pig sera was added to the pellet to lyse epimastigotes (14). Live trypomastigotes were separated from the dead cells by means of a discontinuous Metrizamide gradient (15), washed two times with PBS-0.5% BSA and resuspended to the desired concentration in the same buffer. The Metrizamide was from Nyegaard and Co., A/S, Oslo, Norway, obtained as a gift from Dr. Hannah Lustig Shear, Division of Parasitology, New York University, New York. The entire purification procedure was repeated (usually once) until preparations contained >99% viable (motile) culture trypomastigotes and intermediate forms. Bloodstream trypomastigotes were isolated as follows: SW/55 outbred mice were inoculated with 10^6 parasites intraperitoneally 7 d before harvesting; immediately after bleeding, heparin (5–10 U/ml) was added to the parasitized blood, which was then repeatedly washed (60 *g* for 10 min) with TC-199 (Difco Laboratories, Detroit, Mich.) until it produced a trypanosome-free pellet. The combined trypanosome-containing supernates were centrifuged at 1,086 *g* for 15 min, the cells washed two times with TC-199, resuspended in 1–2 ml of the Ca^{++}-containing buffer, and then incubated in a 37°C water bath for 10 min to agglutinate the platelets. In some experiments bovine collagen (type II; Sigma Chemical Co., St. Louis, Mo.) was slowly added (final concentration: 0.5 mg/ml) to the mixture before incubating at 37°C to facilitate the clumping of platelets. At the end of this period, the cell suspension was overlayered on a discontinuous 15/20% Metrizamide gradient, and live trypomastigotes were recovered at the 15/20% interface after centrifugation at 1,086 *g* for 60 min. The Metrizamide gradient step was repeated at least twice to get a reasonably pure preparation of blood trypomastigotes. The collected cells were washed in PBS-0.5% BSA and resuspended to the desired concentration in the same buffer and immediately used for agglutination or binding assays. Only those trypomastigote preparations contaminated with <1% platelets and erythrocytes were used.

Amastigotes were obtained as a slight modification of the procedure of Leon et al. (16). Briefly, spleen and liver tissues from mice that had been used for trypomastigote purification were removed and homogenized in 0.05 M PBS that contained 0.2% glucose and 1 mM EDTA, pH 7.4. The homogenate was filtered through gauze and incubated at 29°C with constant agitation in the presence of DNAase (10 μg/ml for 5 min) and trypsin (2 mg/ml for 15 min). The suspension was then filtered and centrifuged at low speed (60 *g* at 4°C for 10 min) to

[1] *Abbreviations used in this paper:* BP, *Bauhinia purpurea* lectin; $BS\text{-}IA_4$, blood-group-A-specific lectin from *Bandeiraea simplicifolia*; $BS\text{-}IB_4$, blood-group-B-specific lectin from *Bandeiraea simplicifolia;* BS-II, *Bandeiraea simplicifolia* lectin II; BSA, bovine serum albumin; Con A, concanavalin A; DGal, D-galactose; DGalNAc, *N*-acetylgalactosamine; DGlcNAc, *N*-acetylglucosamine; DMan, D-mannose; HP, *Helix pomatia* lectin; K_0, association constant(s); LCL, *Lens culinaris* lectin; LIT, ox liver-infusion tryptose supplemented with 10% fetal calf serum at pH 7.2; PBS, phosphate-buffered saline; PL-hog A + H, polyleucyl hog gastric blood group A + H substance; PNA, peanut agglutinin; RCAI, *Ricinus communis* I; WGA, wheat germ agglutinin; WFH, *Wisteria floribunda* hemagglutinin.

separate the blood cells from the parasites, the supernate layered on a linear sucrose gradient (0.25–0.7 M), and centrifuged at 255 *g* at 4°C for 15 min. The parasites were found in the upper layer; they were washed two times (3,015 *g* for 15 min) with Hanks' balanced salt solution and found to be a mixture of amastigotes (usually >90%), long slender forms, and some erythrocytes. However, because trypomastigotes and epimastigotes, rather than amastigotes, are highly motile, the cells were resuspended in Hanks' balanced salt solution (Grand Island Biological Co.), pelleted at 3,015 *g* for 15 min, and left in a 37°C water bath for 60 min to allow the motile forms to move up into the supernate. This step was repeated until trypomastigotes and epimastigotes were <1% in relation to the amastigotes. If erythrocytes were present, they were separated from the amastigotes in a discontinuous 15/20% Metrizamide gradient and the parasites collected as for blood trypomastigotes. When the cell preparation yielded >99% amastigotes, they were resuspended in PBS-0.5% BSA and used for lectin-binding activity.

Isolation of Lectins. All seeds were purchased from F. W. Schumacher Co., Sandwich, Mass. and from local supermarkets in Rio de Janeiro, Brazil. Pokeweed roots were collected in Riverside Park, New York, and *Ricinus communis* seeds were harvested periodically from a single group of plants in Volta da Jurema Beach, Ceará, Brazil. Adult horseshoe crabs (*Limulus polyphemus*) were from Marine Biological Labs., Woods Hole, Mass. Lyophilyzed albumen glands of the snail *Helix pomatia*, as well as the dried sponge *Axinella polypoides*, were a gift from Dr. Hagen Bretting, University of Hamburg, Federal Republic of Germany.

Lectins were purified by affinity chromatography with a variety of immunoadsorbents as previously described (10). The references for specific details on the purification of individual lectins are described elsewhere (8–10). Jacaline, the α-D-galactose (DGal)-binding lectin from *Artocarpus integrifolia*, was isolated using polyleucyl hog gastric blood group A + H substance (PL-hog A + H) and elution with 0.05 M melibiose (M. F. C. Lima and M. E. A. Pereira. Manuscript in preparation.). The blood-group-A-specific lectin from *Bandeiraea simplicifolia* (BS-IB_4) (17) was tested as a crude extract after adsorption of the *N*-acetylglucosamine (DGlcNAc)-(for *Bandeiraea simplicifolia* lectin II [(BS-II]) and *N*-acetylgalactosamine (DGalNAc)-the blood-grous-B-specific lectin from *Bandeiraea simplicifolia* (BS-IA_4) binding proteins onto PL-hog A + H, the agglutination of *T. cruzi* was performed in the presence of 0.05 M DGlcNAc and DGalNAc to block any residual BS-II and BS-IA_4 activity, respectively; such adsorbed extract agglutinated human B erythrocytes specifically. The purity of the isolated lectins was assessed by polyacrylamide gel electrophoresis at pH 8.8 (18) and 4.5 (19). All lectins were tested for the presence of glycosidases using α- and β-*p*-nitrophenyl glycosidases of DGal, DGalNAc, and DGlcNAc, all obtained from Sigma Chemical Co. This was done because glycosidases may be present in the affinity-purified materials (10) and may affect or even disguise the putative biological activity of lectins (20). None of our preparations had detectable glycosidases.

The lectin from *Vicia villosa* (7) and *Geodia cydodonium* (21) were gifts from Dr. Hans Wigzell, University of Uppsalla, Uppsalla, Sweden, and Dr. Hagen Bretting, respectively. Some of the ricin and *Wistaria floribunda* mitogen used in our studies were also gifts from Dr. S. Olsnes, University of Oslo, Oslo, Norway through Dr. Gerald T. Keusch, Tufts University, Boston, Mass. and from Dr. Shunji Sugii, Columbia University, New York, respectively. Concanavalin A (Con A) and pokeweed mitogen purchased from Sigma Chemical Co. were also used in some of our studies, as was WGA obtained from Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, Calif. through the generosity of Dr. Justus Schottelius, Institute of Tropical Medicine, Hamburg, Federal Republic of Germany.

Radioiodination of Lectins. WGA was iodinated with ^{125}I by the method of Hunter (22) using a 40-s exposure to the chloramine T in the presence of 0.1 M DGlcNAc to protect the sugar binding sites, and the lectin was freed from excess of reagents by extensive dialysis against 0.01 M PBS that contained the anion-exchange resin AG 21K mesh 20–50 (Bio-Rad Laboratories, Richmond, Calif.) to facilitate removal of unreacted ^{125}I. The minimum agglutinating dose of ^{125}I-WGA was found to be the same as that of the unlabeled lectin, as determined with rabbit erythrocytes and *T. cruzi* epimastigotes. The sp act ranged from 3.6×10^4 to 8.9×10^4 cpm/μg of protein; 88–94% of the radioactive material was ether insoluble and precipitable by 7% trichloroacetic acid.

Lectin Binding to T. cruzi. Binding studies were performed in 7- × 75-mm glass-stoppered

tubes that had been presoaked overnight with 20 mg/ml BSA before use. *T. cruzi* developmental stages, obtained as described above, were resuspended in Hanks' balanced salt solution-0.5% BSA at appropriate concentrations. For determination of binding parameters, the reaction mixture contained a fixed number of washed cells that ranged from 2.0×10^6 to 5.4×10^6, depending on the experiment, plus varying amounts of ^{125}I-WGA in 150 μl of PBS that contained 750 μg of BSA. After incubation at 23°C for 90 min with occasional mixing, the cells were washed three times with 3 ml of chilled PBS-0.5% BSA at 1,086 *g* for 10 min in the cold, and the amount of bound ^{125}I determined in a gamma counter (Beckman Instruments, Inc., Fullerton, Calif.). Appropriate corrections were made for nonspecific binding to the tubes, which usually accounted for <3% of the total counts bound. The amount of free ^{125}I after the second washing was found to be negligible.

To study the inhibition of binding by competing lectins or saccharide haptens (23), assay conditions were selected such that the lectin and the *T. cruzi* epimastigote receptor site concentrations were near their association constant (K_0) values. Thus, the reaction mixtures contained 40 pmol of ^{125}I-WGA and 2×10^6 *T. cruzi* epimastigotes/150 μl of reaction volume in PBS that contained 750 μg of BSA plus varying amounts of the competing lectin being studied for inhibitory activity (up to 800 pmol). The cells were added last. After 60 min at 23°C, cell-bound radioactivity was determined as described above.

Agglutination. Agglutination assays (24) were performed with a Terasaki microtitrator (Cooke Engineering Co., Alexandria, Va.) with 0.025-ml loops and suspensions that contained 1×10^8–2×10^8 parasites/ml or 2% erythrocytes. Equal volumes of the cell suspension and the lectin dilution were mixed, placed at room temperature (23°C) for 1 h, and read. The agglutination of the parasites were always scored visually with a hand lens after gently resuspending the settled cells and by microscopic observation. Agglutination-inhibition assays were done at room temperature with 4 agglutinating U of lectin.

Enzyme Treatment. For trypsinization, a suspension of washed parasites (8×10^8/ml) in PBS (0.01 M, pH 7.4) was treated with trypsin (Bacto-trypsin; 1.0 mg/ml; Difco Laboratories) at 37°C. For sialidase treatment, cells were incubated under the same conditions in PBS, pH 6.0, that contained 0.1 U of *Clostridium perfringens* sialidase/ml (type IX; chromatographically pure; Sigma Chemical Co.). In some experiments 2-deoxy-2,3-dehydro-*N*-acetyl neuraminic acid was added to the sialidase solution and the mixture incubated with the cell suspension; this compound was a generous gift from Dr. Roland Schauer, Christian-Albrechts-Universitat, Kiel, Federal Republic of Germany. After 60 min, samples were withdrawn, immediately cooled, and the enzyme-treated cells (as well as untreated ones under the same conditions) were washed five times with PBS-0.5% BSA and resuspended at suitable concentrations for agglutination or binding assays. The supernates of neuraminidase-treated cells were taken for the determination of free sialic acid (25). Parasites were also treated with lyzozyme (Sigma Chemical Co.) 400 μg/ml for 10 min at 23°C, the cells washed, and resuspended as above.

The efficacy of enzyme treatment on the parasites was verified by treating human O erythrocytes with trypsin and neuraminidase under the same conditions as that of the parasite, and then determining the activity of appropriate lectins toward the treated erythrocytes. Thus, the titer of soybean agglutinin for trypsinized erythrocytes increased ~100-fold compared to untreated cells (26), whereas peanut agglutinin, which did not agglutinate (1.2 mg/ml) the erythrocytes, had a hemagglutinating activity of 0.5 μg/ml for the neuraminidase-treated cells (27). The activity of lyzozyme on bacteria was checked using group C *Streptococcus* that was obtained from Dr. L. J. Kunz, Massachusetts General Hospital, Boston, Mass.

Miscellaneous. Proteins or glycoproteins were coupled to cyanogen bromide-activated Sepharose as described previously (28). Double diffusion in agar was done using 1.5% agar (Special Noble Agar; Difco Laboratories) in 0.05 M sodium barbital buffer (pH 7.4) according to the procedure described by Crowle (29).

Results

Analysis of Lectin Agglutinating Characteristics of T. cruzi Developmental Stages. The agglutination of epimastigotes, bloodstream and culture trypomastigotes, and amastigotes with lectins of various specificities is shown in Table I. To facilitate comparative

TABLE I

Activity of Lectins of Various Specificities for T. cruzi Developmental Stages *

	Blood group specificity	Minimum concentration required to agglutinate			
		Epimastigote	Trypomastigote		Amastigote
			Blood-stream	Culture	
			μg/ml		
I. DGlcNAc-binding lectins					
WGA	None	25.0	>1,000.0	>1,000.0	>800.0
Aaptos papillata lectin II	None	71.0	381.0	47.0	>762.0
BS-II	None	115.0	>1,275.0	>1,275.0	>1,275.0
II. DGalNAc-binding lectins					
WFH	None	37.0×10^{-3}	2,263.0	92.0×10^{-6}	>6,035.0
Sophora japonica	A + B	125.0	>1,000.0	>1,000.0	>1,000.0
Phaseolus vulgaris	None	>1,520.0	300.0	>1,520.0	300.0
Glycine max	None	250.0	250.0	62.0	250.0
BP	None	93.5	375.0	>1,500.0	>1,500.0
HP	A	32.0	>718.0	>718.0	>718.0
III. DGal-binding lectins					
PNA	None	>2,400.0	>2,400.0	>2,400.0	300.0
RCAI	None	162.0	325.0	82.0	325.0
Ricinus communis II (ricin)	None	7.8	15.0	3.9	3.9
Geodia cydodonium	None	50.0	50.0	25.0	50.0
Artocarpus integrifolia (jacaline)	None	82.0	41.0	41.0	82.0
Axinella polypoides	None	64.0	64.0	32.0	ND‡
IV. DMan-binding lectins					
Con A	None	62.0	125.0	62.0	62.0
LCL	None	375.0	128.0	128.0	375.0
Pisum sativum	None	237.0	950.0	712.0	950.0
V. Sialic acid-binding lectin					
Limulus polyphemus	None	187.0	1,500.0	750.0	>1,500.0

* Average of three experiments.
‡ ND, not done.

analysis, the lectins have been classified according to their sugar and blood-group specificities (10), with the results expressed as the minimum concentration of lectin required to agglutinate the parasites. The lectin-combining site is considered to be most specific to the cell that is agglutinated at the lowest concentration (10, 30). In general, visual assays of agglutination gave the same titer as those determined by observing the agglutination under the microscope, although in some instances, the latter method yielded higher titers, which were then used for the calculation of the specific lectin activity. When epimastigotes or trypomastigotes were agglutinated, mixed types of clumps were observed, i.e., body-body, body-flagellum, or flagellum-flagellum. Whenever a developmental stage was analyzed for agglutination, the whole battery of lectins was used to minimize experimental error. When agglutination assays of live, highly mobile flagellated parasites were checked with the hand lens, the

settling pattern of cells was the converse that of erythrocytes; i.e., the unagglutinated parasites formed a white cloud around the bottom of the microtiter plate, whereas the agglutinated ones sedimented as a pellet. Agglutinations were always checked independently by two of us (M. E. A. Pereira and A. F. B. Andrade).

D-*GlcNAc-binding Lectins.* The most notable feature of the DGlcNAc-binding lectins is the striking specificity of WGA (*Triticum vulgaris*) for *T. cruzi* epimastigotes in that these cells were strongly agglutinated by the lectin (minimum concentration: 25 μg/ml), whereas the other *T. cruzi* stages were not. Indeed, when agglutination was performed in culture forms that contained a mixture of epimastigotes and trypomastigotes, only the former were agglutinated, whereas most trypomastigotes remained free and unattached to the clumps. Similar results were obtained with the commercial preparation of WGA, although the end point was reached at higher concentration. It is interesting that although the binding sites of WGA and *Aaptos papillata* lectin II are complementary to a sequence of three β1-4-linked D-GlcNAc units (*N,N',N''*-triacetylchitotriose) and that the interaction of both lectins with glycoproteins or with cells are affected by sialic acid residues (31–33), *Aaptos papillata* lectin II, although it did not agglutinate amastigotes, did not qualitatively discriminate epimastigotes from trypomastigotes (Table I). Lectin BS-II was also selective in agglutinating epimastigotes, although it was somewhat less active then WGA. Because the sugar specificity and combining-site size of BS-II are quite different from those of WGA and *Aaptos papillata* lectin II (10), these lectins were probably recognizing different receptor sites on epimastigotes; competitive lectin binding studies (see below) were consistent with this hypothesis. Pokeweed mitogen (*Phytolacca americana*) at 1.2 mg/ml and potato lectin (*Solanum tuberosum*) at 0.6 mg/ml did not agglutinate any of the parasite stages. All agglutinations were inhibited by 0.1 M DGlcNAc.

D-*GalNAc-binding Lectins.* Of the 10 lectins tested, *Wistaria floribunda* hemagglutinin (WFH) was the most unusual in that it displayed a unique and amazing selectivity for culture trypomastigotes and epimastigotes: the lectin was capable of agglutinating these cells at a minimum concentration of 92 pg/ml and 37 ng/ml, respectively; and, although bloodstream trypomastigotes were also agglutinated (2.3 mg/ml), the concentration was 2.5×10^7 and 6.2×10^4 times higher, respectively. Amastigotes were not agglutinated. *Wistaria floribunda* mitogen, the other DGalNAc-binding lectin purified from *Wistaria floribunda* seeds, did not agglutinate (1.0 mg/ml) any of the parasite stages.

As with WGA and BS-II, the blood-group-A-specific lectin from the snail *Helix pomatia* (HP) and the blood-group-*A* + B-specific lectin from *Sophora japonica* seeds agglutinated epimastigotes specifically at a minimum concentration of 32 μg/ml and 125 μg/ml, respectively. However, these lectins reacted with receptor sites distinct from those recognized by WGA, as shown by competitive binding studies (see below).

The *Bauhinia purpurea* lectin (BP) agglutinated only epimastigotes and bloodstream trypomastigotes, but it inhibited the movement of all flagellated parasites up to a concentration equivalent to the minimum agglutinating dose (187 μg/ml). Thus, because agglutination and immobilization were prevented by prior incubation of the lectin with hog gastric mucin (1.0 mg/ml) or DGalNAc (10.0 mg/ml), it is apparent that BP lectin reacts with sugar moieties on both epimastigotes and trypomastigotes, although the culture forms of the latter were not agglutinated. It would be of interest

to determine the type and location of the receptors involved in the lectin-mediated immobilization.

The agglutination of *Phaseolus vulgaris* lectins was of significance in that these lectins were the only ones to agglutinate amastigotes and blood trypomastigotes (300 μg/ml) without agglutinating or affecting the movement of epimastigotes and culture trypomastigotes. However, it is not known whether the activity was a result of the leucoagglutinin or hemagglutinin, or to both lectins because the material used contained a mixture of these proteins.

Soybean agglutinin (*Glycine max*) agglutinated all stages to about the same extent. *Maclura aurantiaca* lectin (0.6 mg/ml) and the blood-group-A-specific lectins from *Dolichos biflorus* (2.0 mg/ml), *Phaseolus lunatus* (2.0 mg/ml), and *Vicia villosa* (1.3 mg/ml) did not agglutinate the *T. cruzi* cells at the highest concentrations indicated.

D-*Gal-binding Lectins (Table I).* The most interesting finding was the specific agglutination of *T. cruzi* amastigotes by peanut agglutinin (PNA; *Arachys hypogaea*). PNA is highly specific for the disaccharide DGalβ1→3DGalNAc, but also binds, to a lesser extent, DGalβ1→4DGlcNAc (34); both structures are commonly found in the complex carbohydrates located on cell surfaces, but are available for lectin binding only in undifferentiated, poorly sialylated cells (35). Mature cells do not bind the lectin because the above-mentioned receptors are masked by sialic acid. Thus, the selective agglutination of amastigotes by PNA strongly suggests that these cells are the least sialylated of the *T. cruzi* developmental stages used.

The other D-Gal-binding lectins, *Ricinus communis* I (RCAI), *Ricinus communis* II (ricin), the sponge lectins (*Geodia cydodonium* and *Axinella polypoides*), and jacaline (*Artocarpus integrifolia*) reacted with all forms of *T. cruzi* without selective agglutination. The blood-group-B + H-specific lectins from *Evonymus europaeus* (1.1 mg/ml) and the B-specific lectin BS-IB_4 (titer against blood group B erythrocytes: 1:512) did not agglutinate the parasites at the concentrations indicated.

D*Man-binding Lectins (Table I).* The lectins from *Canavalia ensiformis* (Con A), *Lens culinaris* (lentil), and *Pisum sativum* (pea) reacted with all *T. cruzi* stages without significant quantitative differences. In accord with this, and apparently identical glycoprotein isolated from the three stages by *Lens culinaris* lectin (LCL)-Sepharose affinity chromatography has been reported (36). The agglutination of *T. cruzi* by Con A has already been observed (37, 38).

Sialic Acid-binding Lectins (Table I). The results with WGA and with *Aaptos papillata* lectin II have already been discussed under the heading of DGlcNAc-binding lectins. The other sialic acid-binding protein, the lectin purified from the hemolymph of the horseshoe crab (*Limulus polyphemus*) reacted most strongly with epimastigotes, although trypomastigotes were also agglutinated, but at higher concentrations. In conformity with the PNA data discussed above, the *Limulus polyphemus* lectin did not react with amastigotes, probably because of a lack of cell surface sialic acid residues. The agglutination of epimastigotes and trypomastigotes by the lectin was inhibited by *N*-acetyl neuraminic acid (5 mg/ml).

L-*Fucose-binding Lectins.* The two blood-group-H-specific lectins used, *Lotus tetragonolobus* and *Ulex europaeus* I, did not agglutinate the cells at the highest concentration tested, 1.0 mg/ml and 1.65 mg/ml, respectively.

Preferential Binding of Radiolabeled WGA to T. cruzi Epimastigotes. The results of the agglutination assays demonstrated clearly that lectins discriminate among morpho-

logically and functionally distinct cell populations of *T. cruzi*. Absence of agglutination by lectins, however, does not necessarily mean that the binding protein is not interacting with a particular cell, and it could be a result of, for instance, improper spatial arrangement of lectin receptor sites in the intact cell membrane, in which case the lectin would still react with the cell surface (39). The binding of ^{125}I-labeled WGA to purified *T. cruzi* developmental forms was then studied to find out whether the unagglutinated cells also possessed WGA sites. WGA was chosen as a prototype because of its high specificity for epimastigotes (Table I), because the WGA carbohydrate recognition site is adequately characterized (10) as is its interaction with cells (31) and glycoproteins (32), and finally because the WGA is relatively easy to isolate in high yields (40).

The results seen in Fig. 1 show clearly that WGA preferentially bound to epimastigote forms of *T. cruzi*. Although the other stages bound 5 to 10 times less radioactivity than epimastigotes, the amount of ^{125}I-WGA bound was nevertheless specific, in as much as it could be averted by 0.1 M DGlcNAc. Because culture trypomastigotes were contaminated with epimastigotes, and bloodstream trypomastigotes were contaminated with host cells and epimastigotes, and although these contaminations amounted to <1% (determined by microscopic observation of Giemsa-stained smears), it could very well explain the small degree of binding of radioiodinated WGA to the trypomastigote and amastigote preparations. However, the WGA binding to these latter forms could indeed indicate the presence of a small number of WGA receptor sites on the unagglutinated cells. Whatever it may be, the binding data in Fig. 1 demonstrate that the differential agglutination of *T. cruzi* cells by WGA is correlated with the number of exposed lectin receptor sites on them. If the binding data for epimastigotes are plotted according to the method of Steck and Wallach (41), the number of lectin-binding sites was determined to be $\sim 3 \times 10^6$/cell with an apparent K_0 of 2×10^6 M^{-1} as shown in Fig. 2.

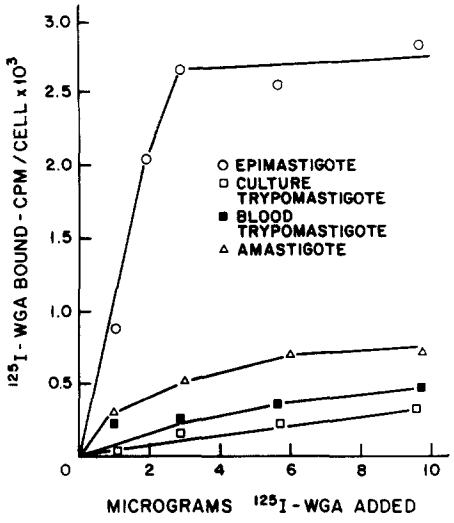

FIG. 1. Binding of iodinated WGA to *T. cruzi* developmental stages. Each point is the mean of three separate binding experiments that differed by ≤10%. Experimental details as described in text.

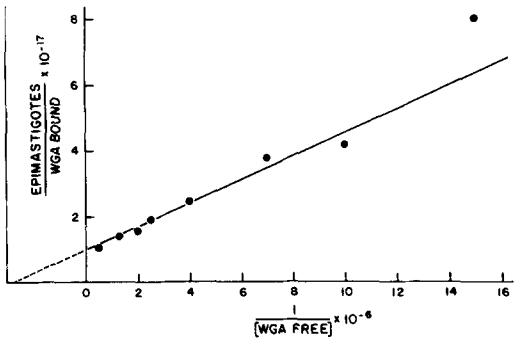

FIG. 2. Binding of ^{125}I-labeled WGA to *T. cruzi* epimastigotes. The data have been plotted by the method of Steck and Wallach (41) according to the equation:

$$\frac{C}{\text{WGA bound}} = \frac{1}{K \cdot n} \cdot \frac{1}{\text{WGA free}} + \frac{1}{n},$$

Where C is the concentration of cells, WGA is the concentration of WGA (molar), n is the number of lectin molecules bound/cell, and K is the association constant of WGA. All points represent average of triplicate experiments.

WGA Interaction with Sialic Acid Residues on T. cruzi Epimastigotes. In view of the specific reaction of WGA with epimastigotes, as determined by agglutination and binding assays, the nature of the WGA receptors was investigated since such receptors might be relevant to the physiology of the parasite.

Complete inhibition of agglutination and of the binding of ^{125}I-WGA to epimastigotes was accomplished by adding 0.1 M DGlcNAc or sialic acid-containing glycoproteins, such as α1-acid glycoprotein (1.5 mg/ml) (kindly provided by Dr. Gilbert Ashwell, National Institutes of Health, Bethesda, Md.), to test tubes or microtiter wells that contained the agglutinated cells. However, these inhibition experiments do not provide any indication of the nature of cell surface receptor sites for WGA and merely reflect an interaction of the lectin with the cells via its carbohydrate-binding regions. Nevertheless, they do imply that the lectin is interacting with cell surface carbohydrate determinants because the radioactivity was completely released by the inhibitors after epimastigotes were incubated with ^{125}I-WGA for 1 h at room temperature (Fig. 5). Similar results were also obtained in inhibition assays performed at 4°C or 37°C, suggesting that very little, if any, pinocytosis of the lectin-receptor complexes was taking place. Direct evidence for the interaction of WGA with sialic acid residues on carbohydrate determinants of the cell surface was then obtained by digesting epimastigotes with *C. perfringens* sialidase (0.1 U/ml at 37°C for 60 min). Such treatment completely abolished the ability of WGA to agglutinate (Table II) and to bind (Fig. 3) epimastigote cells. That sialic acid was, indeed, released by the enzyme treatment was determined by analyzing the supernate of the cell digest for free sialic acid by the periodate-thiobarbituric acid method (25); in this way, 30 μg of sialic acid was liberated from 7.5×10^9 cells (average of two experiments). Preliminary experiments showed that no further sialic acid was released after a 60-min enzyme incubation at 37°C. Similar results were obtained after mild acid hydrolysis (0.1 N HCl at 80°C for 60 min) of the cells. Thus, assuming that the detected sialic acid is *N*-acetyl neuraminic acid (309 mol wt), then one can estimate an average of 7.7×10^6 residues per cell. Additional strong evidence for digestion of epimastigotes by the

TABLE II

Activity of Lectins Tested with T. cruzi Epimastigotes Pretreated with Several Enzymes *

Treatment of cells	Specific lectin activity‡							
	WGA	*Aaptos pallíata* lectin II	BS-II	*Limulus polyphemus*	PNA	WFH	Con A	*Ricinus communis* II (ricin)
				μg/ml				
None	27.5	36.0	159.0	187.5	>1,200.0	2×10^{-2}	16.0	7.8
Sialidase	>1,230.0	190.0	159.0	750.0	75.0	5×10^{-6}	32.0	3.9
Sialidase + inhibitor§	27.5	36.0	ND‖	187.5	>1,200.0	ND	ND	ND
Trypsin	50.0	20.0	159.0	187.5	>1,200.0	5×10^{-3}	63.0	ND
Lyzozyme	25.0	48.0	119.0	ND	>1,200.0	ND	ND	ND

* 3-d-old cultures of epimastigotes were washed four times with PBS, pH 7.2, resuspended to 8×10^8/ml, treated with *C. perfringens* sialidase (0.1 U/ml at 37°C for 60 min), trypsin (1.0 mg/ml at 37°C for 60 min), or lyzozyme (400 μg/ml at 23°C for 10 min). Untreated cells were those incubated in PBS for 60 min at 37°C. After incubation, cells were washed five times in the cold with PBS-0.5% BSA, resuspended to 1×10^8/ml, and assayed for agglutination.

‡ Average of three experiments.

§ The enzyme (0.1 U/ml) was mixed with 2-deoxy-2,3-dehydro-*N*-acetyl neuraminic acid in a final concentration of 0.9×10^{-4} M for 15 min at room temperature and then added to the epimastigote suspension.

‖ ND, not determined.

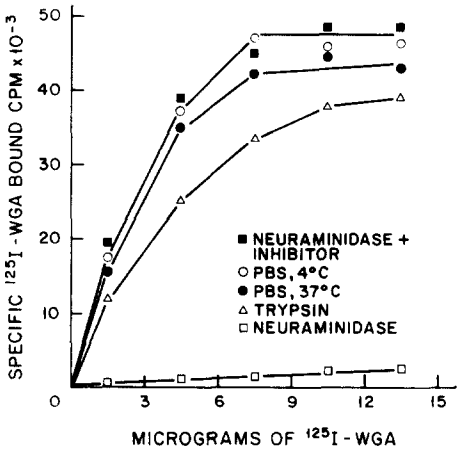

FIG. 3. Effect of trypsin and neuraminidase treatment of *T. cruzi* epimastigotes on ^{125}I-WGA binding. The cells were treated with trypsin and neuraminidase as described in Materials and Methods. The final concentration cells was 2.2×10^6/0.15 ml, and the concentration of the neuraminidase inhibitor 2-deoxy-2,3-dehydro-*N*-acetyl neuraminic acid was 0.9×10^{-4} M (Table II, footnote §).

added sialidase was provided by studies with a competitive inhibitor for bacterial neuraminidase (42), 2-deoxy-2,3-dehydro-*N*-acetyl neuraminic acid. This compound at a concentration of $\sim 1 \times 10^{-4}$ M completely blocked neuraminidase activity on the cells as determined by ^{125}I-WGA binding (Fig. 3) and by agglutination assays (Table II).

The notion that WGA interacts with sialyl compounds on the epimastigote cell surface is further supported by agglutination of sialidase-treated cells using other

lectins (Table II). Thus, the agglutinating activity of the other two sialic acid-binding proteins, *Aaptos papillata* lectin II and *Limulus polyphemus*, was decreased from 36 μg/ml to 190 μg/ml and from 187.5 μg/ml to 750 μg/ml, respectively, after the cells were treated with sialidase. Because the activities were reduced but not abolished, it implies that either the enzyme did not remove all sialic acid or, that these two lectins recognized determinants in addition to sialic acid residues on the cell surface. It is interesting that sialidase treatment did not affect the agglutinating activity of BS-II lectin. This protein, together with WGA and *Aaptos papillata* lectin II, binds D-GlcNAc residues but does not interact with sialic acid ([43]; and M. E. A. Pereira. Unpublished observations.), thus supporting the view that WGA is not interacting with D-GlcNAc residues on epimastigote cells. As already mentioned, PNA does not normally agglutinate mature, fully differentiated cells unless these cells are treated with sialidase to expose its receptor sites (mainly D-Galβ1→3D-GalNAc). That this was also the case with *T. cruzi* epimastigotes is shown in Table II. Another interesting finding is that obtained with WFH since the agglutination titer increased 4,000-fold for the sialidase-treated cells as compared with the untreated ones; thus suggesting that although WFH was the most potent agglutinin for epimastigotes (Table I), some WFH receptor sites are nonetheless inaccessible or masked by the presence of sialic acid in the intact cells. The agglutinating activities of Con A and ricin were not significantly altered after similar sialidase treatment of the cells.

Other enzymes were tested for alterations of lectin-agglutinating activities (Table II). Thus, trypsinization of epimastigotes did not significantly affect agglutination by the lectins tested, except WFH whose activity increased fourfold. Lyzozyme treatment did not alter the titer of any of the lectins used. Those lectins that did not agglutinate epimastigotes remained inactive after the cells were treated with trypsin and lyzozyme (data not shown).

Rapid Regeneration of WGA Receptors. One of the observations about cell surface sialic acid of mammalian cells is its rapid regeneration (6–8 h) after neuraminidase treatment (44). An indication that the cell surface sialic acid of *T. cruzi* epimastigotes is also rapidly recovered after its removal with sialidase was obtained by checking the agglutinating activities of lectins for neuraminidase-treated cells incubated at specified times in growth media. As seen in Fig. 4 A, the ability of WGA to agglutinate neuraminidase-treated epimastigotes was fully restored after incubation of such cells for 4 h at 28°C in LIT medium, whereas the agglutinating activity of PNA was abolished after 2 h. As expected, the activity of Con A was not altered. If the regenerated cells were again treated with neuraminidase, the agglutination pattern with WGA and PNA was that of the original enzyme-digested cells. The possibility exists, however, that the rapid restoration of WGA receptors was a result of simple adsorption of fetuin and of other sialic acid-containing glycoproteins from the LIT medium to the sialidase-treated cells. Thus, LIT medium was passed several times into a 5.0-ml column of WGA-Sepharose 4B (5 mg lectin/ml settled beads) to remove all WGA-binding materials: such adsorbed LIT medium did not inhibit hemagglutination nor the agglutination of *T. cruzi* by WGA. If the neuraminidase-treated epimastigotes were now allowed to grow in this medium, the WGA- and PNA-agglutinating activities were restored and abrogated, respectively, in a similar manner as for the unadsorbed medium (Fig. 4 B). Furthermore, if the sialidase-treated cells were incubated with unadsorbed or adsorbed LIT medium at 4°C for up to 72-h of

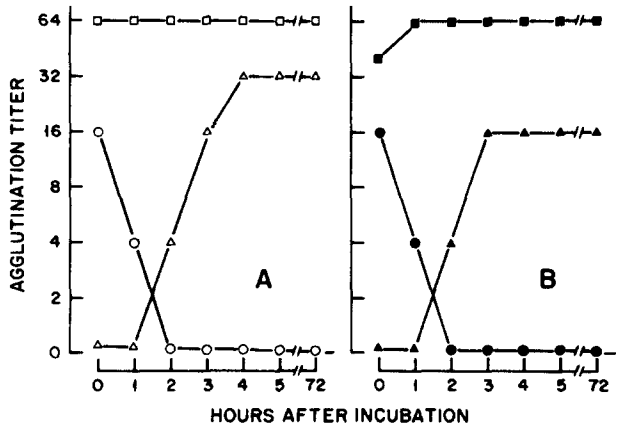

FIG. 4. Reestablishment of lectin-agglutinating characteristics of sialidase-treated epimastigotes in growth media. Cells were treated with *C. perfringens* sialidase as described in Materials and Methods, resuspended to 3.0×10^6 cells/ml in LIT medium (A) and in LIT medium previously adsorbed on a WGA-Sepharose 4B column (B), and then incbuated at 28°C. At specified times, cells were removed, washed five times in PBS-BSA, resuspended to $\sim 10^8$/ml, and tested for agglutination with WGA (△, ▲), PNA (○, ●), and Con A (□, ■). In both LIT and adsorbed LIT, the density of cells increased 30- to 40-fold at the end of 72 h. The lectin agglutination pattern after 4 h of incubation was the same as that of the control cells.

observation, the lectin agglutinating patterns were unaltered (data not shown). Similarly, the original WGA and PNA agglutinating activities were not reformed if the cells were left in non-growth-permitting media such as PBS-0.5% BSA and TC-199 for up to 48 h of observation at 28°C. Finally, WGA agglutinated *T. cruzi* epimastigotes that were growing in a defined, protein-free medium (AR-103) for >50 passages (M. E. A. Pereira and I. Roitman. Unpublished observations.). Thus, taken together, these data provide evidence for the rapid regeneration of WGA sialyl-receptor sites and indirectly suggest that at least most of these receptors were not adsorbed from the growth medium.

Effect of Lectins on ^{125}I-labeled WGA Binding to T. cruzi. To obtain additional information about the membrane binding site for WGA, competitive binding studies were performed using ^{125}I-labeled WGA and unlabeled lectins of various specificities. Because significant errors could result in the following equilibrium studies if direct interaction between lectins occurred in the process of lectin competition for the cell receptor sites, the absence of such interaction was verified by immunodiffusion using WGA and the competing lectins; the results confirmed absence of interaction.

Of the three DGlcNAc-binding proteins tested, only WGA and *Aaptos papilliata* lectin II have in common the ability to react with sialic acid residues. The results shown in Fig. 5 are consistent with sialyl residues on epimastigotes since, although two times less potent than unlabeled WGA, *Aaptos papilliata* II fully inhibited the binding of ^{125}I-WGA, whereas BS-II did not compete significantly for WGA sites even at a concentration 20 times higher on a molar basis. *Limulus polyphemus* lectin did not compete with WGA, thus showing that these two lectins interact with different oligosaccharide moieties, despite the inhibition of LP agglutination by sialic acid. Con A and LCL have sugar specificity completely different from that of WGA (cf. 10), the former interacting mainly with α-linked, six-membered ring of the DMan or D-glucose configuration. Yet on a molar basis, both Con A and LCL were very effective in

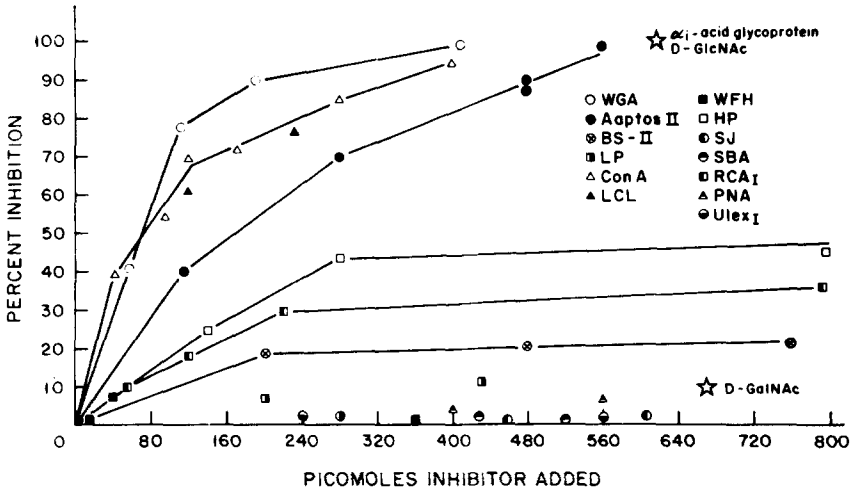

FIG. 5. Inhibition of ^{125}I-WGA binding to *T. cruzi* epimastigotes by competing lectins. Inhibition assays were performed as described in Materials and Methods. The final concentration of DGlcNAc and DGalNAc was 0.1 M, and the final concentration of α1-acid glycoprotein was 1.5 mg/ml. Aaptos II, *Aaptos papilliata* lectin II; LP, *Limulus polyphemus* lectin; SJ, *Sophora japonica* lectin; SBA, soybean agglutinin; Ulex$_I$, *Ulex europaeus* I lectin.

competing for WGA sites, thus providing evidence that either Con A, LCL, or WGA sites were on the same oligosaccharide moiety, or that they were very closed spaced. The HP, which, like WGA, reacts with epimastigotes specifically, did not appear to compete for the actual WGA lectin-binding site, as HP inhibition did not increase with higher HP concentration. Similarly, other epimastigote agglutinins tested (WFH, *Sophora japonica*, soybean agglutinin, and RCAI) also did not inhibit WGA binding. As expected, no inhibition was obtained with those lectins that did not agglutinate *T. cruzi* (*Ulex europaeus* I and PNA).

Discussion

The results showed quite clearly that lectins can serve as specific markers for developmental stages of *T. cruzi*. Thus (Table I), WGA and the lectins from *Bandeiraea simplicifolia*, *Sophora japonica* and *Helix pomatia* reacted selectively with epimastigotes, PNA with amastigotes, *Phaseolus vulgaris* with bloodstream trypomastigotes and amastigotes, and WFH with culture forms of the trypanosome. Competitive lectin-binding studies demonstrated that the receptor sites for WGA were distinct from those for the other three epimastigote-specific lectins (Fig. 5).

These specific interactions suggest that lectins can be used to isolate *T. cruzi* developmental stages by affinity chromatography (45). Similarly, the lectin-cell interactions described in this paper could also prove valuable for comparing cell surface characteristics of a certain trypanosome stage harvested from different environments; the comparative analysis would be particularly useful if one considers that certain stages such as those of the vector's gut are difficult, if not impossible, to get in high yields.

The results shown in Table I also suggest the occurrence of qualitative and quantitative differences in cell surface carbohydrate composition, as detected by lectins, between bloodstream and culture trypomastigotes, the distinction between

these two morphologically similar stages being most striking with DGalNAc-binding proteins. Thus, WFH was ~25 million times more potent in agglutinating culture than bloodstream trypomastigotes; for comparative purposes, the variation seen with blood-group-specific lectins (10) acting on human erythrocytes is in general <1000-fold! Conversely, BP and the lectin from *Phaseolus vulgaris* were strong agglutinins for bloodstream trypomastigotes, but were inactive toward the corresponding culture stages. However, the pattern of activity of most lectins for the two kinds of *T. cruzi* stages was similar, although as a rule the culture trypomastigotes was more sensitive to the agglutinins than the bloodstream counterparts. Whether structural differences and similarities inferred from these findings are related to the virulence of trypomastigotes remains to be determined, but it certainly provides an interesting investigative tool with which to approach the problem.

The question of infectivity vs. cell surface receptor sites poses a very important and provocative puzzle, mainly because of its therapeutic implications (46). Although lectins detected structural differences between trypomastigotes and epimastigotes, one cannot *a priori* relate these differences to the greater or lesser virulence of the respective trypanosomal stage. However, the finding of sialic acid residues on epimastigote cells is very intriguing. Epimastigote-bound sialic acid was detected both by colorimetric analyses and biologically at a minimum density of $\sim 7.7 \times 10^6$ residues/cell. This value falls within the wide range found for other cells, from 3×10^6 residues per human erythrocyte (47) to 9×10^{18} for the L cell (48). The demonstration that sialic acid residues were a component of the WGA receptors is shown in Table II and Figs. 3–5. It is interesting that the number of WGA receptor sites (Fig. 2) was found to be 3×10^6 per cell compared with 7.7×10^6 residues of sialic acid/cell, thus supporting the contention (32) that one molecule of WGA interacts with multiple sialyl residues of a glycoprotein. Though WGA did not interact with trypomastigotes, these cells were agglutinated by *Aaptos papillata* lectin II and *Limulus polyphemus* lectin, two sialic acid-binding proteins, but not by PNA. Furthermore, preliminary experiments indicate that if trypomastigotes are treated with sialidase from *Vibrio cholerae* they become PNA-agglutinable. These findings strongly suggest that sialic acid residues are also present in trypomastigotes, although they must belong to a class different from those found in epimastigotes, as judged by the differential WGA agglutination. The presence of sialic acid in amastigotes is not supported by the pattern of lectin agglutination, that is, these cells, without sialidase treatment, displayed PNA-induced agglutination, whereas the three sialic acid-binding proteins were inactive.

Sialic acids are widely distributed in nature (49); in protozoa they have been reported in the marine diatom *Nitzschia alba* (50) and in the extracellular malaria parasite *Plasmodium berghei* (51). An extensive literature has been accumulating on the biological importance of sialic acid residues at the surface of the eukaryotic cell (52). One of its roles appears to be the activation of the alternative complement pathway (53). Indeed, preliminary experiments in our laboratory showed that neuraminidase-treated epimastigotes are much more susceptible to the Mg^{++}-dependent lytic action of normal guinea pig serum (54) than the untreated cells. We are ccurrently investigating whether the normally resistant trypomastigote acquires the capacity to activate the alternative complement pathway after treatments that affect cell surface sialic acid.

In conclusion, it is demonstrated that cell surface carbohydrates, detectable by

lectins, are characteristic markers of *T. cruzi* developmental stages and of morphologically defined stages harvested from different milieus. Sialic acid residues were demonstrated on epimastigotes and shown to be the specific receptor sites for WGA. Based on these findings, a new approach for the isolation of trypanosome subpopulations and of cell surface receptor sites can be envisaged. Further analysis of the lectin receptors should allow deeper insight as to how the specialized *T. cruzi* stages exert their biologically relevant functions. Finally, although the above studies were performed using a strain of a human parasite as a prototype, it is obvious that the same facile approach of analyzing cell surface carbohydrate can be applied with success to different strains of the same parasite, to other protozoa, and to morphologically and/or functionally related cells in general.

Summary

Trypanosoma cruzi at various stages of maturation and differentiation have been isolated by conventional cellular fractionation procedures and characterized by cell surface markers using 30 highly purified lectins encompassing all known sugar specificities. Cell surface carbohydrates of the various *T. cruzi* stages were analyzed by agglutination and lectin-binding assays.

Specific receptors for wheat germ agglutinin (WGA), *Helix pomatia*, *Sophora japonica*, and *Bandeiraea simplicifolia* lectin II were found only in culture epimastigotes, whereas peanut agglutinin (PNA) sites were present exclusively in amastigotes, those for *Phaseolus vulgaris* in bloodstream trypomastigotes and amastigotes, and for *Wistaria floribunda* hemagglutinin predominantly in culture forms of *T. cruzi*. The *N*-acetylgalactosamine (DGalNAc)-binding lectin from *Bauhinia purpurea* agglutinated and inhibited the movement of epimastigotes and bloodstream trypomastigotes, but it only inhibited—without agglutinating—culture trypomastigotes. Because both the agglutination and inhibition of movement were reversed by specific sugar haptens, *Bauhinia purpurea* sites were present in all the flagellated parasites. On the other hand, PNA sites were detectable on epimastigotes after the cells were treated with sialidase, whereas, at the same time, WGA receptors were completely removed and those for the other sialic acid-binding proteins, *Aaptos papillata* lectin II and *Limulus polyphemus*, were partially eliminated; moreover, the activity of *Wistaria floribunda* hemagglutinin, a DGalNAc-binding lectin, increased 4,000 times. Trypsinization and lyzozyme treatment of epimastigote cells did not significantly affect lectin agglutination or lectin binding.

WGA reacted solely with sialic acid residues on epimastigote cell surface with an apparent association constant of 2×10^6 M^{-1}, each epimastigote having an estimated average of 3×10^6 WGA sites, as determined by binding experiments and a minimum of 7.7×10^6 sialic acid residues, as calculated by colorimetric method after sialidase digestion. Evidences are presented that the sialyl residues are rapidly regenerated (in ~4 h) and that they, at least for the most part, are not adsorbed from the culture medium. The receptor for the D-mannose-binding lectins (concanavalin A [Con A] and *Lens culinaris*) must either be on the same carbohydrate moiety having the WGA site, or, if in a distinct molecule, both carrier molecules of Con A and WGA sites must be located close to each other in the plasma membrane of the parasite.

We thank Dr. Victor Nussenzweig for iodinating the WGA, Doctors Elvin A. Kabat and Philip

D'Alesandro, Columbia University, Victor and Ruth Nussenzweig, New York University, and Wanderley de Souza and Julio Sharfstein, Biophysics Institute, Rio de Janeiro, for reading the manuscript; all those listed in Materials and Methods and Results for their gifts of the various samples used in this study; and Ms. Delminda Clem for typing the manuscript. M. E. A. Pereira thanks the Nussenzweigs for their encouragement.

Received for publication 12 March 1980 and in revised form 9 June 1980.

References

1. Immunology of Chagas' disease. 1974. *Bull. W. H. O.* **50:**459.
2. Bloom, B. R. 1979. Game parasites play: how parasites evade immune surveillance. *Nature (Lond.).* **279:**21.
3. Brener, Z. 1973. Biology of *Trypanosoma cruzi. Ann. Rev. Microbiol.* **27:**347.
4. Bretscher, M. S. 1973. Membrane structure: some general principles. *Science (Wash. D. C.)* **181:**622.
5. Gahmberg, C. G., P. Hayry, and L. C. Andersson. 1976. Characterization of surface glycoproteins of mouse lymphoid cells. *J. Cell Biol.* **68:**642.
6. Cantor, B., and E. A. Boyse. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. *J. Exp. Med.* **141:**1376.
7. Kimura, A., H. Wigzell, G. Holmquist, B. Ersson, and P. Carlsson. 1979. Selective affinity fractionation of murine cytotoxic T lymphocytes (CTL). Unique lectin specific binding of the CTL associated surface glycoprotein, T 145. *J. Exp. Med.* **149:**473.
8. Lis, H., and N. Sharon. 1977. Lectins: their chemistry and application to immunology: *In* The Antigens. M. Sela, editor. Vol. IV. Academic Press, Inc., New York. 459.
9. Goldstein, I. J., and C. E. Hayes. 1978. The lectins: carbohydrate-binding proteins of plants and animals. *Adv. Carbohydr. Chem. Biochem.* **35:**127.
10. Pereira, M. E. A., and E. A. Kabat. 1979. Immunochemical studies on lectins and their application to the fractionation of blood group substances and cells. *Crit. Rev. Immunol.* **1:**33.
11. Silva, L. H., and V. Nussenzweig. 1953. Sobre uma cepa de *Trypanosoma cruzi* altamente virulenta para o camundongo branco. *Folia Clin. Biol.* **20:**191.
12. Camargo, E. P. 1964. Growth and differentiation of *Trypanosoma cruzi.* I. Origin of metacyclic trypanosomes in liquid media. *Rev. Inst. Med. Trop. Sao Paulo* **6**(Suppl. 3):93.
13. Castellani, O., L. V. Ribeiro, and J. F. Fernandes. 1967. Differentiation of *Trypanosoma cruzi* in culture. *J. Protozool.* **14**(Suppl. 3):447.
14. Muniz, J., and A. Borrielo. 1945. Estudo sobre a ação lítica de diferentes soros sobre as formas de cultura e sanguicolas do *S. cruzi. Rev. Bras. Biol.* **5**(Suppl. 4):563.
15. Nogueira, N., and Z. A. Cohn. 1978. *Trypanosoma cruzi*: in vitro induction of macrophage microbicidal activity. *J. Exp. Med.* **148:**288.
16. Leon, W., F. Villalta, T. Queiroz, and A. Szarfman. 1979. Antibody-induced-capping of the intracellular stage of *Trypanosoma cruzi. Infect. Immun.* **26**(Suppl. 3):1218.
17. Murphy, L. A., and I. J. Goldstein. 1977. Five α-D-galactopyranosyl-binding isolectins from *Bandeiraea simplicifolia* seeds. *J. Biol. Chem.* **252:**4739.
18. Laemmli, U. K. 1970. Clevage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* **227:**680.
19. Reisfeld, R. A., V. J. Lewis, and D. E. Williams. 1962. Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature (Lond.).* **152:**281.
20. Molano, J., I. Polacheck, A. Duran, and E. Cabib. (1979) An endochitinase from wheat germ. Activity on nascent and preformed chitin. *J. Biol. Chem.* **254:**4901.
21. Bretting, H. Purification and characterization of sponge lecitins and a study of their immunochemical and biological activities. Proceedings of an International Colloquium on Sponge Biology. Elsevier North-Holland, Inc., New York. In press.

22. Hunter, W. M. 1973. In Handbook of Experimental Immunology D. M. Weir, editor. Blackwell Scientific Publications Ltd., Oxford.
23. Kawaguchi, T., I. Matsumoto, and T. Osawa. 1974. Studies on competitive binding of lectins to human erythrocytes. *Biochemistry.* **13:**3169.
24. Pereira, M. E. A., and E. A. Kabat. 1974. Specificity of purified hemagglutinin (lectin) from *Lotus tetragonolobus. Biochemistry.* **13:**3184.
25. Warren, L. 1959. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* **234:**1971.
26. Lis, H., B. A. Sela, L. Sachs, and N. Sharon. 1970. Specific inhibition by N-acetyl-D-galactosamine of the interaction between soybean agglutinin and animal cell surfaces. *Biochim. Biophys. Acta.* **211:**582.
27. Bird, G. W. G. 1964. Anti-T in peanuts. *Vox Sang.* **9:**748.
28. Pereira, M. E. A., and E. A. Kabat. 1976. Immunochemical studies on blood groups. LXII. Fractionation of hog and human A, H, and AH blood group active substance on insoluble immunoadsorbents of *Dolichos* and *Lotus* lectins. *J. Exp. Med.* **143:**422.
29. Crowle, A. J. 1973. Immunodiffusion. 2nd edition. Academic Press, Inc., New York. 1.
30. Kabat, E. A. 1976. Structural Concepts in Immunology and Immunochemistry. 2nd edition. Holt, Rinehart & Winston, New York. 1.
31. Adair, W. L., and S. Kornfeld. 1974. Isolation of the receptors for wheat germ agglutinin and the *Ricinus communis* lectins from human erythrocytes using affinity chromatography. *J. Biol. Chem.* **249:**4696.
32. Bhavanandan, V. P., and A. W. Katlic. 1979. The interaction of wheat germ agglutinin with sialoglycoproteins. *J. Biol. Chem.* **254:**4000.
33. Bretting, H., E. A. Kabat, J. Liao, and M. E. A. Pereira. 1976. Purification and characterization of the agglutinins from the sponge *Aaptos papillata* and a study of their combining site. *Biochemistry.* **15:**5029.
34. Pereira, M.E. A., E. A. Kabat, R. Lotan, and N. Sharon. 1976. Immunochemical studies on the specificity of the peanut (*Arachis hypogaea*) agglutinin. *Carbohydr. Res.* **51:**107.
35. Sharon, N., and Y. Reisner. 1979. Lectin receptors as markers for lymphocyte subpopulations in mouse and man, *In* Molecular Mechanisms of Biological Recognition. Elsevier North-Holland, Inc., New York. 479.
36. Snary, D., and L. Hudson. 1979. *Trypanosoma cruzi* cell surface proteins: identification of one major glycoprotein. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **100:**166.
37. Alves, M. J. M., and W. Colli. 1974. Agglutination of *Trypanosoma cruzi* by Concanavalin A. *J. Protozool.* **21**(Suppl. 4):575.
38. Chiari, E., W. de Souza, A. J. Romanha, C. A. Chiari, and Z. Brener. 1978. Concanavalin A receptors on the cell membrane of *Trypanosoma cruzi. Acta Trop.* **35:**113.
39. Nicolson, G. L. 1974. The interactions of lectins with animal cell surface. *Int. Rev. Cytol.* **39:** 89.
40. Bloch, R., and M. M. Burger. 1974. Purification of wheat germ agglutinin using affinity chromatography on chitin. *Biochem. Biophys. Res. Commun.* **58:**13.
41. Steck, T. L., and D. F. H. Wallach. 1965. The binding of kidney-bean phytohemagglutinin by Ehrlich ascites carcinoma. *Biochim. Biophys. Acta.* **97:**510.
42. Meindl, P., G. Bodo, P. Palese, J. Schulman, and H. Tuppy. 1974. Inhibition of neuraminidase-activity by derivatives of 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid. *Virology.* **58:** 457.
43. Wood, C., E. A. Kabat, S. Ebisu, and I. J. Goldstein. 1978. An immunochemical study of the combining sites of the second lectin isolated from *Bandeiraea simplicifolia* (BS-II). *Ann. Immunol. (Paris)* **129C:**143.
44. Hughes, R. C., B. H. Sanford, and R. W. Jeanloz. 1972. Regeneration of the surface glycoproteins of a transplantable mouse tumor cell after treatment with neuraminidase. *Proc. Natl. Acad. Sci. U. S. A.* **69:**942.

45. Pereira, M. E. A., and E. A. Kabat. 1979. A versatile immunoadsorbent capable of binding lectins of various specificities and its use for the separation of cell populations. *J. Cell Biol.* **82:**185.
46. Keusch, G. T. 1979. Specific membrane receptors: pathogenetic and therapeutic implications in infectious diseases. *Rev. Infect. Dis.* **1:**517.
47. Eylar, E. H., M. A. Madoff, O. V. Brody, and J. L. Oncley. 1962. The contribution of sialic acid to the surface charge of the erythrocyte. *J. Biol. Chem.* **237:**1992.
48. Glick, M. C. 1974. Chemical components of surface membranes related to biological properties. *In* Biology and Chemistry of Eucaryotic Cell Surface. E. Y. C. Lee and E. E. Smith, editors. Academic Press, Inc., New York. 213.
49. Tuppy, H., and A. Gottschalk. 1972. The structure of sialic acids and their quantitation. *In* Glycoproteins: Their Composition, Structure and Functions. A. Gottschalk, editor. 2nd edition. Elsevier Scientific Publishing Co., Amsterdam. 403.
50. Sullivan, C. W., and B. E. Volcani. 1974. Isolation and characterization of plasma and smooth membrane of the marine diatom *Nitzschia alba. Arch. Biochem. Biophys.* **163:**29.
51. Seed, T. M., M. Aikava, C. Sterling, and J. Rabbege. 1974. Surface properties of extracellular malaria parasites: morphological and cytochemical study. *Infect. Immun.* **9:**750.
52. Rosenberg, A., and C.-L. Schengrund, editors. 1976. Biological Roles of Sialic Acid. Plenum Publishing Corp., New York. 1.
53. Fearon, D. T. 1978. Regulation by membrane sialic acid of β1H-dependent decay-dissociation of amplification C3 convertase of the alternative complement pathway. *Proc. Natl. Acad. Sci. U. S. A.* **75:**1971.
54. Nogueira, N., C. Bianco, and Z. Cohn. 1975. Studies on the selective lysis and purification of *Trypanosoma cruzi. J. Exp. Med.* **142:**224.