membrane stability (increased membrane fluidity) may be fundamental to expression of the neoplastic phenotype. In addition to morphologic (*13*) and biochemical modifications (*14*), the results suggest, and subsequent experiments have confirmed (*15*), that destabilization of macrophage membranes occurs in parallel with activation. Therefore, normal macrophages may not be cytotoxic because their stable membranes do not participate in the fusion reactions required for target cell heterolysis. Likewise, activated macrophages may have little or no cytotoxic effect for normal target cells because the stability of the latter's membranes does not favor the temporary cell fusion reaction required for heterolysis.

The interaction of activated macrophages with tumorigenic cells can result in target cell death. Four hours after challenge, there were 11 3T12 cells per field (at a magnification of $\times 400$) among BCG-activated macrophages, but after 72 hours there was an average of 0.1 3T12 cell per field. The cellular events leading to target cell death were followed by phase contrast microscopy in Sykes-Moore chambers maintained at 37°C: (i) lysosomes of activated macrophage origin accumulated in the cytoplasm of target cells; (ii) cytoplasmic vacuoles in the target cell cytoplasm dilated; (iii) the target cell periphery often began to manifest blebbing (*16*); (iv) the target cell rounded up and often developed violent blebbing, which resulted in target cell fragmentation by pinching off of blebs to form spherical structures identical to those described as apoptotic bodies by Kerr *et al.* (*17*); and (v) all cytoplasmic movement ceased and the target cell eventually disappeared from the cover slip surface. Cell death can occur without blebbing or cell fragmentation. When there was evidence of target cell injury, the activated macrophage or macrophages that were the source of the heterolysosomes have often moved out of the microscopic field and may have made contact with another tumor cell. Target cell destruction in this system could be misinterpreted as an autolytic rather than a largely heterolytic process unless this sequence was followed in situ during an 18- to 48-hour period.

This cytotoxicity system may be an in vitro model with broad biological significance. Certain types of cell death which occur during normal embryonic development may involve macrophage participation (*18*). Trypan blue (*19*) and hydrocortisone (*20*) have been found to be teratogenic in some model systems, and in our studies they inhibit the activated macrophage cytotoxic reaction. This is circumstantial evidence that macrophages could be effector cells during some types of tissue remodeling which occur in normal embryogenesis. In invertebrates, macrophages function in the absence of lymphocytes, plasma cells, and immunoglobulins (*21*). Phylogenetically as well as ontogenetically, macrophages may be a fundamental surveillance system on which specific immunologic mechanisms are superimposed (*22*).

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22. Our studies show that host resistance to tumor development and certain intracellular pathogens is related and mediated at the effector level by activated macrophages (*1, 2*). This suggests that membrane destabilization may also promote membrane fusion reactions within the vacuolar system of activated macrophages resulting in enhanced delivery of lysosomal contents to pathogen containing cytoplasmic vacoules.
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Lectin Binding Saccharides on a Parasitic Protozoan

Abstract. Leishmania donovani *promastigotes were specifically agglutinated by concanavalin A and phytohemagglutinin P. Somatic-somatic, flagellar-somatic, and flagellar-flagellar type agglutination was observed with the lectins. Enzyme-treated promastigotes gave reduced lectin agglutination reactions. The results suggest that complex saccharide moieties are randomly distributed on the surface of this organism.*

Leishmania donovani, a protozoan pathogen of humans, has a life cycle which includes a flagellated extracellular promastigote stage in the gut lumen of sandfly vectors, and a proliferative intracellular amastigote stage in macrophages of vertebrate hosts. Recently, several cytochemical techniques (*1*) were used jointly with electron microscopy to demonstrate polysaccharide-like materials on the surface membranes of *L. donovani* promastigotes (*2*). Further, isolated and solubilized promastigote pellicular membranes separated by disc-gel electrophoresis contain at least three major glycoproteins (*3*).

No report exists on specific terminal saccharide residues on the surfaces of any parasitic protozoan. Inasmuch as plant agglutinins (lectins) have provided highly specific means for studying terminal residues of polysaccharides and glycoproteins on the surfaces of diverse cell types (*4*), several lectins were used to ascertain what, if any, were their effects on promastigotes of *L. donovani*.

Chromatographically pure concanavalin A (Con A; Miles-Yeda), specific for terminal residues similar to α-D-glucose and α-D-mannose (*5*), was used in the agglutination experiments. Unpurified phytohemagglutinin M (PHA-M) and phytohemagglutinin P (PHA-P, Difco), both having an affinity for *N*-acetyl-D-galactosamine–like residues (*6*), also were used.

L. donovani strain 1-S promastigotes were obtained in primary culture from amastigotes isolated from infected hamster spleens. Promastigotes from the

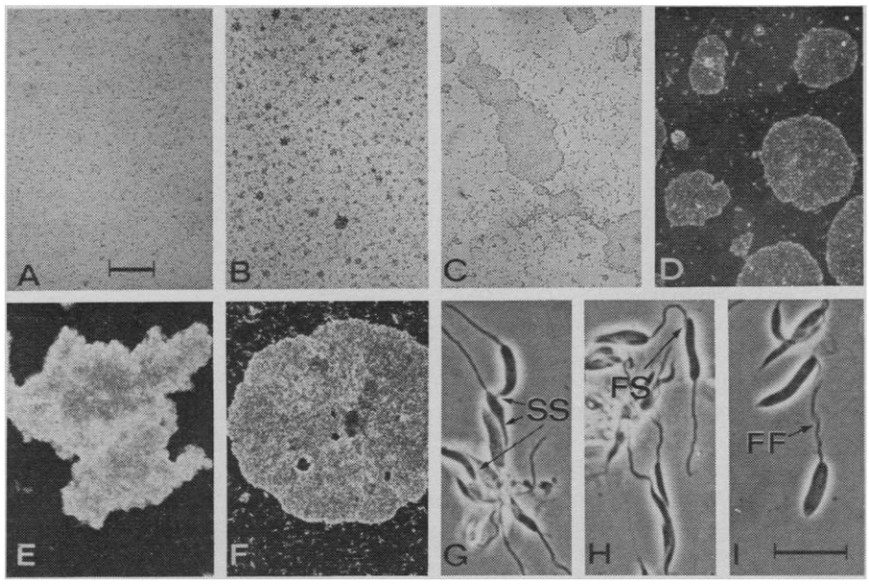

Fig. 1. (A to F) Types of agglutination reactions obtained with *L. donovani* promastigotes and the various lectins as described in Table 1 [the bar is equivalent to 100 μm and applies to (A to F)]. (A) Promastigote control. (B) ± Agglutination. (C) Promastigote 1+ reaction. (D) Type 2+ agglutination. (E) Type 3+ reaction. (F) Strong 4+ promastigote agglutination. (G to I) Types of promastigote agglutination encountered with the several lectins [the bar represents 10 μm and applies to (G) to (I)]. (G) Somatic-somatic agglutination (arrow, *SS*). (H) Flagellar-somatic agglutination (arrow, *FS*). (I) Flagellar-flagellar agglutination (arrow, *FF*).

second transfer of these cultures were grown in bulk in a complex liquid medium (*7*) at 26° ± 0.5°C. At late log phase (~ 132 hours in culture) the organisms were harvested by centrifugation at 4300*g* for 10 minutes at 4°C and washed four times by centrifugation in cold 0.01*M* phosphate-buffered 0.15*M* saline (PBS), *p*H 7.5. Promastigotes were resuspended in PBS, and the suspensions were adjusted with PBS to contain 2×10^8 organisms per milliliter. This cell concentration was used in all experiments.

For agglutination experiments, 0.05 ml of the cell suspension and then an equal volume of the lectin solution were placed on glass slides and mixed by tilting the slides gently for 3 minutes at 24°C. After this, the slides were examined immediately with a light microscope. The agglutinations were scored from 0 (no agglutination) to 4+ (virtually complete agglutination) (Table 1). Controls were set in parallel on the same slide with an appropriate inhibitor saccharide solution substituted for the PBS before the addition of the lectin.

Promastigote agglutination with the various lectins was specific and occurred only when the lectins were present in the medium (Fig. 1, A to F). No spontaneous agglutination of the parasites occurred in the absence of the lectins during the course of the experiments. Agglutination did not occur with Con A when inhibitory concentrations of a competitive sugar were present. Borderline agglutination (that is, ± reactions) did occur, however, at high concentrations of PHA-P in the presence of a competitive saccharide. Several types of promastigote agglutination were observed. These were categorized as somatic-somatic (SS), flagellar-somatic (FS), and flagellar-flagellar (FF) agglutinations, respectively (Fig. 1, G to I).

Trypsinization of the cells neither inhibited nor prompted the agglutination reactions. Dextranase, which catalyzes the hydrolysis of α-1,6-glucan bonds (*8*), considerably reduced the Con A reactivity and almost abolished the PHA-P agglutinability of promastigotes. Similarly α-amylase, which catalyzes the hydrolysis of α-1,4-glucan bonds of polysaccharides containing three or more internally linked D-glucose units (*9*), reduced the lectin agglutination of cells. No loss of cell viability was apparent after any of the enzyme treatments. Results similar to those presented above for the living parasites were obtained also with promastigotes formalinized by the Nicolson method (*10*).

The above results suggest that *L. donovani* promastigotes have polysaccharide residues randomly distributed on their external membranes and that some of these residues are composed of α-1,4- and α-1,6-glucan–linked D-glucose–like units. Further, they suggest that at least some of the residues present in the promastigote surface polysaccharide also contain *N*-acetyl-D-galactosamine–like sugars.

Table 1. Summary of agglutination results obtained with *L. donovani* promastigotes and the several lectins. All enzyme treated cells were incubated for 15 or 30 minutes at either 24° or 37°C. Then after being washed four times in cold PBS, the organisms were used in the agglutination assays. All results were identical regardless of incubation times and temperatures.

Inhibitor	Inhibitor concentration	Lectin concentration (μg/ml)						
		0	10	30	60	120	240	500
		Concanavalin A						
None	0	0	±	1+	2+	3+	4+	4+
Sucrose	0.2*M*	0	0	0	0	0	0	0
α-Amylase*	250 unit/ml	0	0	±	1+	1+	2+	2+
Dextranase†	200 unit/ml	0	0	±	1+	1+	2+	2+
Trypsin‡	200 unit/ml	0	±	1+	2+	3+	4+	4+
		Phytohemagglutinin P						
None	0	0	0	0	±	1+	2+	3+
N-Acetyl-D-galactosamine	0.2*M*	0	0	0	0	0	±	±
α-Amylase*	250 unit/ml	0	0	0	0	0	±	±
Dextranase†	200 unit/ml	0	0	0	0	0	±	±
Trypsin‡	200 unit/ml	0	0	0	±	1+	2+	3+
		Phytohemagglutinin M						
None	0	0	0	0	0	0	0	±
N-Acetyl-D-galactosamine	0.2*M*	0	0	0	0	0	0	0

* Promastigotes were incubated in α-amylase (porcine pancreas, Worthington) at *p*H 6.9. † Cells were treated with dextranase (*Penicillium* sp., Worthington) at *p*H 7.0. ‡ Promastigotes were incubated in trypsin (Worthington, twice crystallized) at *p*H 7.9.

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Fate of α-Bungarotoxin Bound to Acetylcholine Receptors of Normal and Denervated Muscle

Abstract. *In organ culture, α-[^{125}I]bungarotoxin bound to extrajunctional receptors of denervated muscle is lost from the tissue at a more rapid rate than the toxin bound to the junctional receptors of normal muscle. The rapid loss of toxin from denervated muscle can be blocked by inhibitors of energy production and protein synthesis, and may reflect turnover of the toxin-receptor complex in the membrane.*

The acetylcholine receptor in vertebrate skeletal muscle is a membrane protein whose interaction with the transmitter acetylcholine causes a change in the permeability of the muscle membrane to sodium and potassium ions. Normally the receptors are almost exclusively confined to the region of the muscle surface underlying the nerve terminal (*1*). After denervation, however, the receptors occupy the entire muscle surface although the peak receptor concentration in the extrajunctional membrane never reaches that of the end plate membrane (*2*). Since the receptor is an identifiable component of the muscle membrane, the change in acetylcholine sensitivity that occurs after denervation offers an opportunity to study the mechanism by which a cell regulates the distribution of a particular protein on its surface. We have attempted to follow the fate of acetylcholine receptors in skeletal muscle by binding to them a radioactively labeled, small protein toxin—α-[^{125}I]-bungarotoxin—that binds tightly and specifically to the receptor (*3, 4*). Loss of radioactivity from normal and denervated muscles was then followed in organ culture.

Normal and denervated diaphragms of rats (50 to 70 g) were labeled by injection of α-[^{125}I]bungarotoxin (12 μg per 100 g of body weight) into the thoracic cavity. The extent of labeling was comparable to the maximal labeling obtained by incubation of diaphragms with toxin in vitro and permitted survival of at least 75 percent of the animals (*5*). Three hours after the injection, the diaphragms were removed with a small border of ribs attached and transferred to organ culture (*6*). After three changes of medium at 30-minute intervals to reduce levels of free toxin in the tissue, one hemidiaphragm was taken to determine the amount of radioactivity initially bound (0-hour sample). The other hemidiaphragm was cultured an additional 24 hours before analysis. Because the diaphragm can be conveniently divided into regions containing end plates and regions devoid of end plates, the radioactivity specifically bound to the end plate region could be calculated. To eliminate variation arising from differences in the extent of initial binding among animals, radioactivity bound to the end plate at 24 hours was expressed as a percentage of that present at 0 hour for each animal. The loss of radioactivity from denervated muscles was examined in similar experiments in animals whose left hemidiaphragms had been denervated for 5 days prior to the injection so that extrajunctional acetylcholine sensitivity would be high. Portions of the same denervated hemidiaphragm were analyzed before and after culturing. Only tissue lacking end plates was examined in order to restrict the analysis to those receptors which appear after denervation. Further details of the experiment are given in (*7*).

When hemidiaphragms from normal rats were labeled and cultured, very little loss of radioactivity from the muscles occurred. After 24 hours in culture, 79 ± 7 percent (mean ± S.E.M.) of the radioactivity specifically associated with the end plate region remained in the tissue. To determine whether the retention of radioactivity by the muscles occurs through readsorption of dissociated toxin, muscles were cultured with and without a large excess (20 μg/ml) of unlabeled toxin in the medium. No difference in the amount of radioactivity lost from the tissue was observed in the two cases. Thus in normal muscles, most of the toxin-receptor complex which is formed appears to be stable over a 24-hour period. In contrast, radioactivity associated with the extrajunctional toxin-receptor complex of denervated muscle is rapidly lost from the tissue. Only 19 ± 2 percent of the radioactivity originally associated with regions devoid of end plates from denervated hemidiaphragms remained after 24 hours in culture. To investigate the mechanism of this rapid loss of radioactivity, we labeled denervated muscles and cultured them in the presence of an inhibitor of protein synthesis. The addition of cycloheximide (10 μg/ml) to the culture medium caused 61 ± 5 percent of the radioactivity to be retained. This concentration of cycloheximide blocks 95 percent of the protein synthesis in rat diaphragm (*8*).

The release of radioactivity from denervated muscle was further examined by measuring its rate of accumulation in the culture medium over the 24-hour period. Denervated left hemidiaphragms were cultured as described above, except that the medium was changed five times at 30-minute intervals, and the final incubation medium contained 2 μg of unlabeled toxin per milliliter. Samples of medium were then taken at 4- to 5-hour intervals, and at the end of the experiment the hemidiaphragms were analyzed for remaining radioactivity. Parallel cultures of the innervated right hemidiaphragms were used as a measure of release from sources other than the extrajunctional receptors. The percentage of radioactivity remaining associated with extrajunctional receptors in the muscle was then calculated for the various times and plotted on semilogarithmic coordinates (Fig. 1). After an initial lag of 1 to 2 hours, the release of radioactivity occurred as a single first-order process. In two such experiments, half-times of 7.8 and 8.4 hours were obtained for the rate of release, and 86 and 85 percent, re-