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A study of the differential respiratory burst activity elicited by promastigotes and amastigotes of *Leishmania donovani* in murine resident peritoneal macrophages

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Summary. Acridine orange and ethidium bromide and a combination of fluorescent and transmitted light microscopy used in conjunction with the qualitative nitroblue tetrazolium assay for superoxide anion (O_2^-) release demonstrated dramatic differences in the binding of and respiratory burst (RB) activity elicited by promastigotes and amastigotes of *Leishmania donovani* in resident peritoneal macrophages (Mϕ) from C57BL/10ScSn mice. When amastigotes were incubated with Mϕ for 30 min the number of parasites per 100 Mϕ was 2–4-fold higher, a higher proportion of Mϕ became infected and the mean number of parasites per infected Mϕ was higher than in promastigote infections. RB activity was higher for promastigotes than amastigotes both in terms of the percentage of infected Mϕ containing formazan positive parasites and the percentage of individual formazan positive parasites. In an attempt to explain the differential response to promastigotes and amastigotes, RB activity was examined for sodium azide-treated, glutaraldehyde-fixed and heat-killed parasites and for various transformation intermediates between amastigotes and promastigotes. Binding and RB activity were also examined in conjunction with competitive binding assays designed to determine the specific receptors involved in ligand binding of both forms of the parasite to the Mϕ. The results indicate that, while amastigotes may possess an azide-sensitive mechanism which either competes for O_2^- produced or causes localized inactivation of RB activity, this cannot account for the full magnitude of the difference between the two forms of the parasite. The transformation and competitive binding studies suggest that the more likely explanation lies in both qualitative and quantitative differences in the distribution of surface ligands involved in binding the parasite to the Mϕ plasma membrane and that the well characterized mannose/fucose receptor may be important in promastigote, but not amastigote, binding and RB activity.

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INTRODUCTION

The entry of most protozoan parasites into macrophages (Mϕ) occurs in two main stages: attachment of the parasite to the host cell membrane via some form of receptor/ligand binding (Chang, 1981; Mosser & Roberts, 1982; Zenian & Kierszenbaum, 1982, 1983; Zehavi *et al.*, 1983) followed by its enclosure in a vacuole or phagosome with a Mϕ derived lining membrane (Jones, Yeah & Hirsch, 1972; Alexander & Vickerman, 1975; Chang & Dwyer, 1976). One very important event which may occur at the Mϕ surface before or during internalization of the parasite is

triggering of the Mϕ respiratory burst (RB) with the production of potentially harmful reactive oxygen intermediates (Murray & Cohn, 1979; Nathan *et al.*, 1979; Murray, 1981, 1982a). As has been demonstrated for zymosan (Berton & Gordon, 1983), perturbation of the Mϕ membrane by the parasite may activate the membrane bound enzyme NADPH oxidase via the same receptor/ligand interaction involved in attachment. Alternatively, the receptor/ligand interaction may mediate binding only, or binding and phagocytosis, but not RB activity (Wright & Silverstein, 1983). The level of oxidative activity inducible in Mϕ is related both to the species of protozoan parasite (Wilson, Tsai & Remington, 1980; Murray, 1981, 1982a) and to the physiological state of the Mϕ (Murray & Cohn, 1980). In general, activation of the Mϕ enhances the RB. In the case of *Leishmania*, however, the promastigote form of the parasite has been shown to elicit a strong RB in resident peritoneal Mϕ comparable to that elicited by the potent yeast wall extract, zymosan (Murray, 1981). The amastigote form of the parasite elicits a much reduced RB in resident Mϕ (Murray, 1982a).

In an attempt to explain the differential triggering response to promastigotes and amastigotes RB activity is now examined for sodium azide-treated, glutaraldehyde-fixed and heat-killed parasites, and for various transformation intermediates between amastigotes and promastigotes. RB activity is also examined in conjunction with competitive binding assays designed to determine the specific receptor/ligand interactions which occur between mouse resident peritoneal Mϕ and both forms of the parasite. Our results indicate that, while amastigotes may possess an azide-sensitive mechanism which either competes for the superoxide anion (O_2^-) produced or causes localized inactivation of RB activity, this cannot account for the full magnitude of the difference between the two forms of the parasite. The transformation and competitive binding studies suggest that the more likely explanation lies in both qualitative and quantitative differences in the distribution of surface ligands involved in binding the parasite to the Mϕ membrane.

MATERIALS AND METHODS

Mice

Conventionally reared C57BL/10ScSn mice were obtained directly from OLAC (1976) Ltd or were bred in our laboratory from stocks originally purchased from the same source. Male or female mice were used between 6 and 18 weeks of age.

Media

Bicarbonate buffered (pH 7·3) medium 199 (m199) supplemented with 20 mM L-glutamine, 10 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin was used routinely for Mϕ experiments and in preparation of amastigotes. Where stated this was further supplemented with 10% v/v heat-inactivated foetal calf serum (FCS; GIBCO). For promastigote cultures REIX medium (Steiger & Black, 1980), pH 7·3, supplemented with 10% FCS, 75 μg/ml gentamicin, 100 U/ml penicillin and 100 μg/ml streptomycin was used.

Parasites

The L82 Ethiopian strain of *L. donovani*, which has been maintained by continuous hamster passage in our laboratory since 1972, was used for all experiments. Amastigotes were obtained by homogenizing the hamster spleen in m199 10% FCS and centrifuging at 100 *g* for 10 min to remove red blood cells and large cell debris. Residual red blood cells in the supernatant were lysed by addition of 0·05% w/v saponin for 5 min. Parasites were washed by centrifugation at 2000 *g* for 10 min, resuspended in 5 ml and passed three times under pressure through a 26-gauge needle to disperse clumps of amastigotes and to aid in shedding of host phagocytic vacuole from the amastigote surface. Two 2·5 ml aliquots were each layered over 5 ml of a single density (1·037 g/ml) isotonic Percoll solution and centrifuged at 2000 *g* for 45 min to separate the amastigotes from remaining spleen cell debris. The purified pellets of amastigotes were washed, pooled in 5 ml of m199 10% FCS, and again dispersed through a 26-gauge needle. Parasites were then resuspended in a larger volume of m199 10% FCS and placed at 37° in 5% CO_2: 95% air. Amastigotes prepared in this way remain >90% viable for more than 48 hr as assessed by combined dye uptake/dye exclusion fluorescence viability assays following a 10 min incubation with either fluorescein diacetate (5 μg/ml) or acridine orange (5 μg/ml) in conjunction with ethidium bromide (50 μg/ml) (FDA/EB or AO/EB). In this assay viable parasites exclude EB and fluoresce green because of the intercalation of AO with nuclear and kinetoplast DNA or because cytoplasmic esterases

hydrolyse FDA to fluorescein. Non-viable parasites fail to exclude EB and fluoresce red/orange because of the intercalation of EB with DNA. Amastigotes also retain their ability to transform into promastigotes and their infectivity for mice *in vivo* for more than 48 hr after their isolation from hamsters. In the studies reported here amastigotes were, nevertheless, used within 24 hr of preparation. Electron microscopic observations confirmed that the amastigotes were free of host phagocytic vacuolar membranes. Immediately prior to their use in Mϕ experiments the amastigotes were recentrifuged at 2000 ***g*** for 10 min and resuspended in m199 without FCS to give 1×10^8 amastigotes/ml as determined by phase contrast microscopy and a Helber bacteriological counting chamber. An aliquot was serially double diluted to 1:16 and preliminary coverslip infections examined to ascertain the parasite concentration which would result in a mean of 3–5 amastigotes per infected Mϕ after 30 min incubation. This was usually around 2.5×10^6 amastigotes/coverslip by phase count and never exceeded 5×10^6/coverslip.

Promastigotes were obtained by transformation of amastigotes at 26° in REIX 25% FCS. Weekly subcultures were prepared and promastigotes used between 5 and 8 days later. Promastigotes were used between the 7th and 14th subcultures. Viability was assessed using the FDA/EB fluorescence assay and/or flagellar motility. Immediately prior to use in Mϕ experiments promastigotes were spun at 650 ***g*** for 10 min and resuspended in m199 at 5×10^7/ml. A 100 μl aliquot containing 5×10^6 promastigotes was added to each Mϕ culture for *in vitro* infection studies. In one experiment promastigotes were also processed over Percoll in a similar manner to amastigotes except that the washing spins were at 650 ***g*** rather than at 2000 ***g***. Control promastigotes for this experiment were given the same number of washing centrifugations at 650 ***g***.

In other experiments some attempt was made to synchronize the transformation procedure by incubating the amastigotes at 34° for 8 hr in glucose-free Krebs–Ringer phosphate buffer as described by Brun, Berens & Krassner (1976). Amastigotes were then transferred to REIX 10% FCS and incubated at 26° to allow transformation to proceed. Although we did not observe the degree of synchrony described by the previous workers (Brun *et al.*, 1976), cultures did contain high proportions of the different transformation intermediates at various times during a 72-hr transformation period. Transforming cultures were used in Mϕ experiments at 0, 24, 48 and 72 hr. At each time point parasites were examined using the FDA/EB fluorescence assay and scored for amastigotes, enlarged amastigotes (intermediate 1 or I1), elongates without flagellum (I2), elongates with flagellum less than the length of the body (I3), and promastigotes (flagellum longer than the body length). For these experiments 5×10^6 control or transforming parasites were incubated with Mϕ for 60 min.

Additional experiments were performed using heat-killed (56° for 10 min) and glutaraldehyde-fixed (0·125% v/v for 45–60 min) promastigotes and amastigotes. Heat-killed parasites were washed once and glutaraldehyde-fixed parasites three times before being used in Mϕ experiments. Parasites were also pretreated for 1 hr with 20 mM sodium azide (NaN_3) to inhibit superoxide dismutase (SOD) activity. In this case the NaN_3 was kept in the medium during 30 min Mϕ infection experiments.

Macrophages

Resident peritoneal Mϕ were obtained by lavage with 5 ml m199 10% FCS. Cells were plated onto 13-mm washed coverslips in 4-well multidishes (NUNC) to give a total of 8×10^4 large cells/coverslip in 100–150 μl of medium. After 2–3 hr incubation, coverslips were washed twice to remove non-adherent cells and overlaid with 1 ml m199 10% FCS. Cultures were incubated overnight at 37° in 5% CO_2:95% air and used for experiments on the following day.

Single cell/single parasite assay of triggering of the respiratory burst

In earlier studies (Murray, 1981, 1982a) the SOD inhibitable reduction of nitroblue tetrazolium (NBT) by O_2^- to form a blue/black formazan precipitate was used to assess RB activity in response to promastigotes and amastigotes by scoring the percentage of infected Mϕ containing at least one formazan positive parasite (=% Mϕ triggered) in glutaraldehyde fixed preparations. In our experiments we scored both the percentage of Mϕ triggered and the percentage of individual formazan positive Mϕ-associated (attached or internalized) parasites (=% parasites triggering) triggering the Mϕ RB in NBT (1 mg/ml; 0·45 μm millipore filtered) preparations to which AO (5 μg/ml)/EB (50 μg/ml) had been added 10 min prior to scoring. Coverslips were mounted Mϕ side down in a drop of fresh m199 on slides with 11-mm diameter depressions and examined immediately using a com-

bination of fluorescent and transmitted light on a microscope fitted with a high pressure mercury source and an epifluorescence condenser containing a 450–490 nm band pass filter and a 525 nm barrier filter. With this fluorescent method Mϕ were unfixed, by the criteria given above the viability of both Mϕ and extracellular parasites could be determined, and the Mϕ-associated parasites were easily visualized. Only viable Mϕ were scored for parasite binding and RB activity. Appropriate controls were examined to ensure that the presence of the AO/EB did not inhibit RB activity and hence formazan deposition. Although it is possible to quantify RB activity by spectrophotometric and fluorimetric assays of O_2^- and hydrogen peroxide (Murray, 1981, 1982a), the number of Mϕ required to obtain measurable levels of activity would be prohibitive for the number of different treatments we wished to examine. Also, it would not be possible in such experiments to determine the response of individual Mϕ to individual parasites.

Inhibition of parasite binding

Monosaccharides [D-mannose, D-glucose, L-fucose, D-fucose, D-galactose and N-acetyl-D-glucosamine; SIGMA] were tested in competitive binding assays at 50, 100 and 200 mM concentrations. Yeast mannan from *Saccharomyces cerevisiae* (SIGMA M3640, isolated with cetavalon) was tested at 1·25, 2·5, 5 and 10 mg/ml. Mϕ were overlaid with parasites and the monosaccharides or mannan in m199 without FCS and incubated for 30 min. For all treatments duplicate coverslips were prepared in the presence or absence of NBT (=four coverslips/treatment). In some experiments, the effect of the highest monosaccharide concentrations on the binding and RB response of promastigotes opsonized by incubation with *L. donovani* IgG positive human serum was examined. Coverslips were washed three times in sterile phosphate buffered saline (pH 7·3) and processed for microscopy with AO/EB or by fixation in Bouin's solution and staining with Giemsa. Mϕ were scored individually for RB activity and/or for the number of attached or internalized parasites. The sum of attached and internalized parasites was taken as a measure of total parasite binding during the 30 min. Although comparable estimates of total parasite binding were obtained in the presence or absence of NBT there was some indication that heavy deposition of formazan around the parasite could prevent internalisation.

Interpretation of NBT assays in inhibition studies

If parasite attachment is inhibited by any treatment a simultaneous reduction in the amount of O_2^- and H_2O_2 released would be expected. The question we addressed with our single cell/single parasite assay was whether blocking entry by one receptor which causes RB activity might result in entry of the parasite by another 'non-triggering' receptor or vice versa. This would lead to a difference in the proportion of individual bound parasites triggering the RB in treated cultures relative to the control. To ensure that a reduction in the proportion of individual formazan positive parasites was not an artefact of the overall reduction in binding we measured Mϕ RB activity for serial double dilutions of promastigotes which gave infection rates ranging from 5 to 170 parasites per 100 Mϕ. At the lower infection rates sufficient Mϕ were counted to allow a minimum of 50 individual promastigotes to be scored.

RESULTS

Baseline parasite binding and triggering response of resident peritoneal macrophages

Dramatic differences in binding of and RB activity elicited by promastigotes and amastigotes of *L. donovani* in resident peritoneal Mϕ were observed (Table 1). The mean number of promastigotes ($2{\cdot}0 \pm 0{\cdot}3$) per infected Mϕ was significantly lower than the mean number of amastigotes ($3{\cdot}5 \pm 0{\cdot}3$) per infected Mϕ even though the amastigote:Mϕ ratio was usually lower than the promastigote:Mϕ ratio. The percentage Mϕ infected was highly variable for different promastigote preparations but within each experiment was always lower than the percent of Mϕ infected with amastigotes. Differences in RB activity elicited by the two forms of the parasite were reflected in both the percentage of Mϕ triggered and the percentage of parasites triggering. To take account of small day to day variations in parasite binding and RB response for different promastigote cultures and for different amastigote isolates, results for experimental treatments in some of the studies presented below are expressed as a percent of the relevant control promastigote or amastigote infection carried out on the same day.

Since EM studies confirmed the absence of contaminating host membrane around amastigotes this provides an unlikely explanation for the reduced RB activity elicited by amastigotes. Nor was it due to any

Table 1. A summary of binding (attached + internalized parasites) and RB data for duplicate coverslips from each of seven different experiments in which the response of resident peritoneal Mϕ to promastigotes (5–7 days; 7th–11th subcultures) and amastigotes of *L. donovani* was examined. Parasites were incubated with Mϕ for 30 min in m199 (without FCS) containing 1 mg/ml NBT. AO/EB was added 10 min prior to scoring the cultures using combined fluorescent and transmitted light microscopy. Between 200 and 500 Mϕ were scored/coverslip

Parameter	Promastigotes		Amastigotes	
	Range	$\bar{x}\pm$SD	Range	$\bar{x}\pm$SD
% Mϕ infected	28–72	55±13	60–87	74±10
Parasite/100 Mϕ	39–147	112±42	190–328	261±47
Parasites/infected Mϕ	1·5–2·2	2·0±0·3	3·0–4·2	3·5±0·3
% Mϕ triggered	71–95	86±8	20–57	38±13
% Parasites triggering	67–90	79±8	11–43	25±11

effect of the Percoll used in the preparation of the amastigotes. Promastigotes treated in the same way did not differ significantly in their ability to induce RB activity from appropriate control promastigotes (washed the same number of times as Percoll treated promastigotes) examined on the same day (data not shown).

Triggering response in the presence of azide

To examine the effect of parasite SOD on the triggering response we took advantage of the recent observation of Meshnick & Eaton (1981) that *Leishmania* has a prokaryote-type NaN_3-sensitive Fe-containing SOD whereas the host cell has the typical eukaryote-type NaN_3-insensitive CuZn-containing SOD. Mϕ RB activity in response to NaN_3-treated amastigotes measured in the presence of 20 mM NaN_3 was approximately 40% (Mϕ triggered) to 80% (parasites triggering) higher than in control amastigote infections (Table 2). For promastigotes a 10% reduction in both measures of RB activity was observed. Independent spectrophotometric assays confirmed that the level of NaN_3 employed was sufficient to inactivate the parasite SOD.

Triggering response to heat-killed and glutaraldehyde-fixed parasites

In promastigote experiments heat-killing and glutaraldehyde-fixation caused little or no change in the percentage of Mϕ triggered or parasites triggering (Table 2). When amastigotes were examined, however, both pretreatments caused increases (Table 2) in the percentages of Mϕ triggered and parasites triggering. This increase in RB activity was of the same order of magnitude as that observed for NaN_3-treated amastigotes. The results suggest that the amastigote must be viable for a proportion of the decreased triggering response to be observed.

Triggering response to transformation intermediates

Table 3 shows binding of and RB activity in response to the various transformation intermediates between amastigotes and promastigotes. In this experiment the intense formazan deposition characteristic of promastigote triggering was only observed with fully transformed promastigotes or with late stage I3 intermediates with more than just a stumpy flagellum. The increase in the percentage parasites triggering between 0 and 72 hr of transformation correlated with an increase in the proportion of these two stages in the cultures. A very striking feature of the formazan deposition was that it was often most intense along the flagellum and in some cases was observed only along the flagellum and not the parasite body.

Effect of amastigotes on promastigote triggering

Results of experiments where promastigotes and amastigotes were incubated together with Mϕ in the

Table 2. The effects of parasite pretreatments (NaN_3, heat-killing and glutaraldehyde-fixation) on binding (% Mϕ infected and parasites/100 Mϕ) and RB activity (% Mϕ triggered and % parasites triggering) in resident peritoneal Mϕ infected with promastigotes (5–7 days; 9th–11th subcultures) and amastigotes of *L. donovani*. Data are summarized ($\bar{x} \pm$ SE) over three different experiments for 5–8 single coverslip comparisons. Within each experiment results for experimental coverslips were expressed as a % of control coverslips for each parameter scored

	Promastigotes				Amastigotes			
	Binding		Triggering		Binding		Triggering	
Treatment	% Mϕ inf	p/100 Mϕ	% Mϕ	% parasites	%Mϕ inf	p/100 Mϕ	% Mϕ	% parasites
NaN_3	67±6	69±10	91±3	88±4	72±3	50±5	138±9	183±25
Heat-killed	128±9	122±13	99±2	91±5	28±1	16±1	152±15	187±22
Glutaraldehyde	65±13	58±13	98±3	96±3	61±5	61±4	144±13	167±10

Table 3. A summary of binding (parasite/infected Mϕ) and RB (% parasites triggering) data ($\bar{x} \pm$ SD from duplicate coverslips) for one of two experiments in which the response of resident peritoneal Mϕ to cultures of *L. donovani* at various stages of transformation (I1 to I3) from amastigotes (A) to promastigotes (P) was examined. At each time point (0–72 hr) transforming cultures were examined using FDA/EB and scored for viability and for the proportions of the various transformation intermediates. Control promastigotes were used from 5 to 8 days after the 14th subculture. Parasites were incubated with Mϕ for 60 min in m199 (without FCS) containing 1 mg/ml NBT. AO/EB was added 10 min prior to scoring the Mϕ cultures using combined fluorescent and transmitted light microscopy. Similar results were obtained in the second experiment

			Transformation stages (%)					Binding and triggering	
Culture	Time (hr)	Viability (%)	A	I1	I2	I3	P	Parasite/inf Mϕ	% Parasites triggering
Control promastigotes	0	94	—	—	—	—	100	1·2±0·2	83·5±0·7
	24	99	—	—	—	—	100	1·4±0·1	87·7±3·2
	48	96	—	—	—	—	100	1·3±0·1	89·0±15·6
	72	90	—	—	—	—	100	1·3±0·3	91·0±2·8
Transforming cultures	0	99	100	—	—	—	—	4·7±0·8	39·5±6·4
	24	99	3	26	42	24	5	5·8±0·6	52·0±3·5
	48	98	2	8	18	30	40	6·8±1·6	53·8±9·1
	72	98	4	6	16	19	55	3·6±0·8	61·0±6·2

presence of NBT (80·0±2·8%), or where Mϕ were incubated with amastigotes, washed and then incubated with promastigotes and NBT (78·5±13·4%), showed no significant change in the percentage of formazan positive promastigotes compared to the control promastigote infection (83·0±1·4%). Amastigotes do not, therefore, appear to be causing a generalized inhibition of RB activity.

Competitive binding studies

Results of the competitive binding assays with monosaccharides (Fig. 1) showed a dose-dependent inhibition of promastigote and amastigote binding for D-glucose, L-fucose, N-acetyl-D-glucosamine, D-galactose and D-fucose with 50% inhibition occurring between 50 and 100 mM. For D-mannose a similar dose-dependent inhibition of promastigote binding was observed but for amastigotes binding was selectively stimulated in the presence of 100 mM D-mannose in some experiments. At the highest monosaccharide concentration, the rank order for effectiveness in inhibiting promastigote binding was N-acetyl-D-glucosamine > D-galactose = L-fucose = D-glucose > D-fucose = D-mannose. For amastigotes the order was N-acetyl-D-glucosamine > D-galactose = L-fucose = D-glucose = D-fucose > D-mannose. The presence of 200 mM monosaccharides had no effect on the

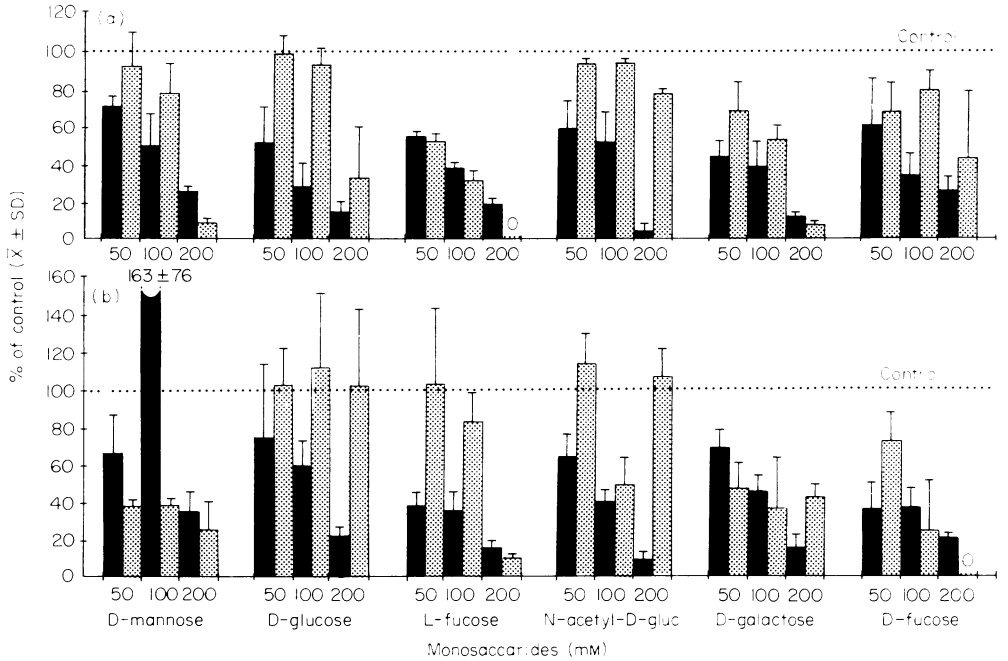

Figure 1. Effects of monosaccharides (50, 100 and 200 mM) on binding (■; parasites/100 Mϕ) and RB activity (□; % parasites triggering) for (a) promastigotes (days 5–7; 7th–10th subcultures) and (b) amastigotes of *L. donovani* in mouse resident peritoneal Mϕ. Data are summarized over several experiments for 4–12 individual coverslip comparisons per treatment for binding and 3–4 individual coverslip comparisons per treatment for RB activity. Within each experiment, results for experimental coverslips were expressed as a percentage of control coverslips. Between 200 and 500 Mϕ were scored per coverslip on AO/EB or Giemsa stained preparations.

dramatic increase in binding we observed for opsonized promastigotes (data not shown). No clear pattern emerged for the effect of the monosaccharides on the proportion of bound parasites eliciting RB activity (Fig. 1).

In examining the results obtained in competitive binding experiments using yeast mannan (Fig. 2), dramatic differences were observed for promastigotes and amastigotes. For the promastigotes a very clear dose-dependent inhibition of parasite binding and a dose-dependent decrease in the proportion of formazan positive bound parasites relative to control coverslips was obtained. For amastigotes tested at 2·5 and 10 mg/ml mannan, no significant reduction in parasite binding was observed although there was a decrease in the proportion of formazan positive bound parasites at both concentrations of mannan.

Results of the control experiment examining the RB response to serial double dilutions of promastigotes (Fig. 3) demonstrated that the percentage of both Mϕ triggered and parasites triggering remains constant irrespective of the infection rate. Hence, the reduction in percentage parasites triggering observed in the competitive binding assays does appear to represent diversion to 'non-triggering' receptors and is not simply an artefact of the overall reduction in binding.

DISCUSSION

Results presented in this study show dramatic differences in RB activity elicited by promastigotes and amastigotes of an Ethiopian strain of *L. donovani* during their interaction with resident peritoneal Mϕ from C57BL/10ScSn mice. Expressed as the percentage of infected Mϕ containing at least one formazan positive parasite in NBT preparations our results for promastigotes ($86 \pm 8\%$) and amastigotes ($38 \pm 13\%$) essentially confirm those of Murray (1982a) for a Sudanese strain of *L. donovani*. The difference in RB

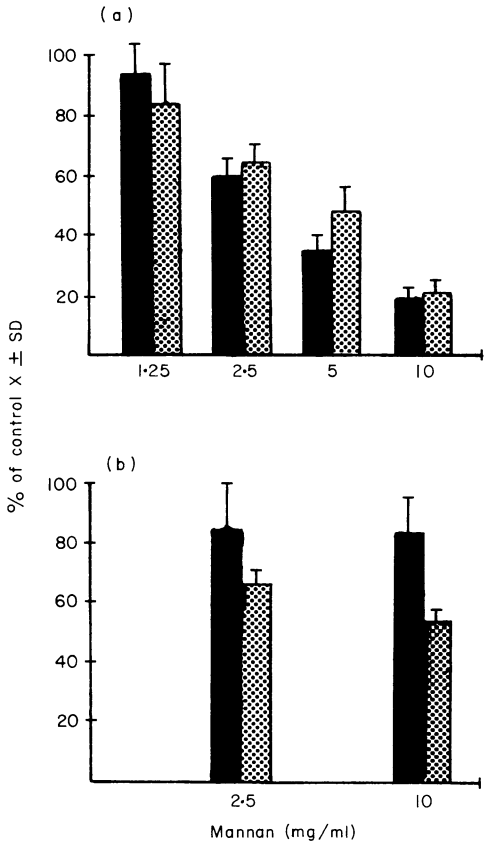

Figure 2. Effect of mannan (1·25, 2·5, 5 and 10 mg/ml) on binding (■; parasites/100 Mϕ) and RB activity (▨; % parasites triggering) for (a) promastigotes (days 6–7; 8th subculture) and (b) amastigotes of *L. donovani* in mouse resident peritoneal Mϕ. Data are summarized over two experiments for 7–10 individual coverslip comparisons per treatment for binding and 3–6 individual coverslip comparisons per treatment for RB activity. Within each experiment, results for experimental coverslips were expressed as a percentage of control coverslips. Between 200 and 500 Mϕ were scored per coverslip on AO/EB or Giemsa stained preparations.

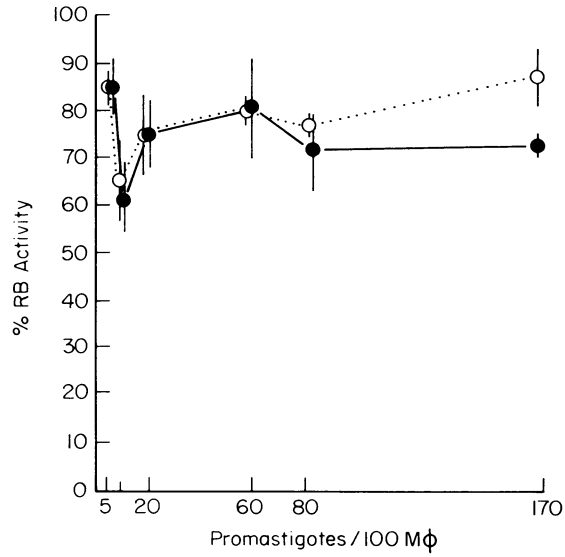

Figure 3. Results of an experiment in which serial double dilutions of promastigotes (day 5; 7th subculture) were incubated with mouse resident peritoneal Mϕ for 30 min in m199 containing 1 mg/ml NBT. RB activity (●—●% promastigotes triggering; o . . . o% Mϕ triggered) was scored on AO/EB preparations. At the lower parasite dilutions at least 1000 Mϕ were scored so that a minimum of 50 promastigotes were counted per coverslip. Results are $\bar{x} \pm$ SD for duplicate coverslips at each parasite dilution.

activity elicited by the two forms of the parasite is accentuated when our data are expressed as the percentage of individual formazan positive Mϕ-associated parasites (=% parasites triggering, Table 1). This is because binding of the two forms of the parasite also differs quite dramatically. For the amastigote form especially, individual Mϕ containing several parasites may show a localized deposition of formazan around only one or two parasites. We considered the second method of scoring RB activity to be more accurate.

Results obtained with NaN_3-treated parasites suggested that the increased RB activity elicited by treated amastigotes may have been due to inactivation of parasite SOD which might otherwise compete with NBT for O_2^-. Increased RB activity obtained using heat-killed and glutaraldehyde-fixed amastigotes was of the same order of magnitude and may also have been due to inactivation of the enzyme. Although amastigotes have higher SOD activity than promastigotes per mg soluble protein (Murray, 1982a) the difference in size between the two forms of the parasite means that, on an individual parasite basis, the SOD of promastigotes would be equally effective in competing for O_2^-. No evidence for this was obtained in our experiments. Since the RB product which mediates Mϕ leishmanicidal activity is hydrogen peroxide and not O_2^- (Murray, 1981, 1982a) it is, in any case, unlikely that SOD activity in either form of the parasite is protective.

An alternative explanation for the effect of the three pretreatments on amastigote triggering is that the amastigote possesses some other form of NaN_3-sensitive mechanism for inactivating the RB response. In our experiments, concurrent or prior infection with amastigotes had no effect on RB activity elicited by promastigotes. Similarly, Murray (1982a) observed that the presence of intracellular amastigotes in resident peritoneal Mϕ did not alter their RB activity in response to zymosan. Hence, this inactivation clearly does not take the form of a generalized desensitisation of the RB response as has been observed with a non-particulate challenge, phorbol myristate acetate (Murray, 1982b). The results do not, however, preclude the possibility that amastigotes cause a localized inhibition of the RB response.

Although the three pretreatments caused an increase in RB activity in response to amastigotes, treated amastigotes never elicited RB activity of the same order of magnitude as that observed for control and treated promastigotes. In the transformation experiment the characteristic RB response of promastigotes was not observed until fully transformed promastigotes or I3 intermediates appeared in the cultures. It was interesting that the localized deposition of formazan was frequently most intense along the promastigote flagellum and was sometimes, as Murray (1981) observed, only present on the flagellum. This suggests that there may be a differential distribution around the promastigote surface of ligands which bind to Mϕ receptors capable of transmitting the triggering signal to the NADPH oxidase. The dramatic differences observed in the binding of promastigotes and amastigotes to resident peritoneal Mϕ also suggests that there may be qualitative and quantitative differences in the distribution of surface ligands on the two forms of the parasite.

One approach which has been taken by other workers in determining the nature of receptor/ligand binding of protozoa to host cells has been to assess the competitive inhibition of various monosaccharides (Chang, 1981; Crane & Dvorak, 1982; Zehavi *et al.*, 1983). In each case, the monosaccharides which act as competitive inhibitors of binding have also been shown by independent lectin binding studies to occur as terminal residues of glycoproteins on the parasite surface (Dwyer, 1977; Katzin & Colli, 1983; Zehavi *et al.*, 1983). When these residues are absent from the parasite surface (e.g., *Toxoplasma gondii*, Sethi *et al.*, 1977; Hoshino-Shimizu, Mineo & Camargo, 1980), the monosaccharides fail to act as inhibitors and may even stimulate entry into the host cell (Crane & Dvorak, 1982). In the present study a dose-dependent inhibition of binding of *L. donovani* promastigotes was obtained for all the monosaccharides matching Dwyer's (1977) lectin binding studies. For amastigotes, a similar dose-dependent inhibition of binding was demonstrated for all monosaccharides except D-mannose, where a selective stimulation of amastigote binding was sometimes observed at 100 mM. This may relate to the findings of Hernandez (1982) for *L. braziliensis* where the number of con A receptor sites per unit of cell surface area was reduced by half during transformation of promastigotes to amastigotes.

The correlation between the presence of particular residues on the surface of protozoa and the ability of the matching monosaccharides to act as inhibitors is reminiscent of the findings of Weir and co-workers (Ogmundsdottir & Weir, 1976; Weir, 1980; Glass, Stewart & Weir, 1981) for binding of a range of different bacteria to mononuclear phagocytes. In this system it was possible to test monosaccharide inhibition against mutant bacteria which were deficient in particular sugar residues in their outer core. For *Salmonella typhimurium* and *Klebsiella aerogenes*, for example, galactose fails to inhibit binding of galactose-deficient outer core mutants. Similarly, glucose fails to inhibit glucose-deficient outer core mutants. These results led Weir (1980) to postulate that blocking of a receptor by one sugar may be sufficient to prevent binding by other sugars by steric hindrance. The prominent role played by galactose and glucose in inhibition of bacterial binding also led Weir and his co-workers (Weir, 1980; Glass *et al.*, 1981) to the belief that the lectin-like receptor on mouse peritoneal Mϕ might be a galactose/glucose-specific receptor.

Inhibition with derivatives of D-galactose and, more specifically, with the disaccharide lactose [Gal-β(1-4)-Glc] was also used by Zehavi *et al.* (1983) as evidence for a galactose-specific receptor involved in the binding of *L. tropica* promastigotes to mouse peritoneal Mϕ. The monosaccharide concentrations (300–500 mM) required to obtain up to 50% inhibition of *L. tropica* promastigote binding were far in excess of the concentrations with which the nonspecific inhibition of *L. donovani* promastigote and amastigote binding was obtained in the present study. Although galactose-specific receptors have been demonstrated on rat peritoneal (Nagamura & Kolb, 1980) and liver (Teradaira *et al.*, 1983) Mϕ, Stahl *et al.* (1978) were unable to demonstrate uptake of ^{125}I-gal-BSA by mouse alveolar Mϕ. It is possible that the observations

of Weir and coworkers (Weir, 1980; Glass *et al.*, 1981) and of Zehavi *et al.* (1983) may relate more to the surface characteristics of the micro-organisms being investigated than to the saccharide-specificity of the Mϕ lectin-like receptor.

The only lectin-like receptor on mouse peritoneal Mϕ which has been well-characterized is the mannose/fucose receptor (MFR). The range of monosaccharides used in the present study was originally chosen to include those specific for the MFR (L-fucose, D-mannose, N-acetyl-D-glucosamine and D-glucose) as well as D-galactose and D-fucose which are known to bind poorly to this receptor (Stahl *et al.*, 1978; Shepherd *et al.*, 1981). The non-specific inhibition of parasite binding obtained with all these monosaccharides meant, however, that monosaccharide inhibition data could not be used as evidence for MFR-mediated parasite binding. Nor was there any indication that the rank order for effectiveness of the different monosaccharides as inhibitors matched that reported for their relative affinities for the MFR (Shepherd *et al.*, 1981). In order to obtain more direct evidence for the role of the MFR in parasite binding we also examined the effect of competitive binding with yeast mannan. Other workers have shown that mannan selectively binds the MFR (Sung, Nelson & Silverstein, 1983). In our experiments a strong dose-dependent inhibition of promastigote binding and RB response was obtained at concentrations of *S. cerevisiae* yeast mannan ranging from 1·25 to 10 mg/ml, with 50% inhibition occurring between 2·5 and 5 mg/ml. This is comparable to the results of Sung *et al.* (1983) where 5 mg/ml of wild type *S. cerevisiae* mannan was required to obtain 50% reduction in phagocytosis of zymosan. Our results suggest, therefore, that binding of promastigotes may be strongly dependent on the MFR. The effect of the mannan on the reduced RB response could be explained by the fact that mannan has been reported to scavenge O_2^- (Ezekowitz *et al.*, 1984). If this was the sole reason for the reduced RB response we would have expected the weaker RB response of amastigotes to have been completely ablated in the presence of mannan. Presumably, therefore, NBT competes more efficiently for the O_2^- than mannan. The more likely explanation is that, as for zymosan (Berton & Gordon, 1983), the RB response to promastigotes is also mediated by the MFR and that, in the presence of yeast mannan, binding may be diverted to other non-triggering receptors of lower affinity. With amastigotes we failed to show any consistent reduction in parasite binding in the presence of yeast mannan although a 30–50% reduction in RB response was observed. Hence, the amastigote appears to be less dependent upon entry via the MFR and may enter via other receptors of equal affinity. Current experiments examining interactions of the parasite in Mϕ populations where different receptors have been selectively modulated onto coated coverslips should allow us to dissect out more precisely the receptors involved in the binding of and RB response elicited by promastigotes and amastigotes of *L. donovani*.

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