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Intracellular Replication of *Leishmania tropica* in Mouse Peritoneal Macrophages: Amastigote Infection of Resident Cells and Inflammatory Exudate Macrophages

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C3HeB/FeJ peritoneal exudate cells elicited by a variety of sterile inflammatory agents were exposed to *Leishmania tropica* amastigotes in vitro. Cytochemical characterization of cells that contained intracellular parasites suggested that young, peroxidase-positive macrophages were more susceptible to infection by amastigotes than more mature cells. Replication of the parasite in these younger cells, however, was similar to that observed in resident peritoneal macrophages.

Murine resident peritoneal macrophages maintained as nonadherent cultures support continuous replication of *Leishmania tropica* amastigotes over 4 to 5 days (8). Treatment of these macrophages with soluble products of antigen or mitogen-stimulated spleen cells (lymphokines), however, induces two potent macrophage antimicrobial activities: (i) increased resistance of activated macrophage populations to infection with the parasite, and (ii) increased intracellular destruction of the parasite by infected cells (9). Macrophages harvested from mice chronically infected with certain microorganisms also have enhanced nonspecific microbicidal activities: in vivo activated macrophages develop both antileishmanial activities that were documented in vitro with lymphokine-treated cells (M. G. Pappas, C. N. Oster, and C. A. Nacy *in* T. K. Eisenstein and H. Friedman, ed., *Host Defenses Against Intracellular Pathogens*, in press). Although the mechanism(s) of killing *L. tropica* by activated macrophages is unknown, a variety of factors influence both survival and replication of obligate intracellular parasites. Alteration in the physiology of the macrophage by simple adherence of cells to a solid matrix markedly suppresses replication of *L. tropica* in vitro (8). Inflammatory macrophages, derived from circulating monocytes that migrate to sites of inflammation or immune reactions, share many morphological and biochemical characteristics with immunologically activated macrophages: enhanced spreading on glass, increased pinocytosis, receptor-mediated phagocytosis, superoxide anion production, and secretion of a variety of enzymes (1, 2, 5–7, 11, 13). To determine whether antileishmanial activities of activated macrophages are associated with cellular alterations induced by inflammation itself, we elicited macrophage populations with sterile inflammatory agents, and compared infection and intracellular replication of *L. tropica* amastigotes in these cells with resident peritoneal macrophages.

Resident peritoneal and inflammatory exudate cells from C3HeB/FeJ mice (18 to 22 g; Jackson Laboratories, Bar Harbor, Maine) were harvested by methods published previously (10). Inflammatory macrophage populations were induced by intraperitoneal inoculation of mice with 1 ml of 4% thioglycollate broth (Difco Laboratories, Detroit, Mich.) (7 days); 2% colloidal suspension of starch (Sigma Chemical Co., St. Louis, Mo.) (5 days); 10^6 0.8-μm latex beads in saline, (Difco) (3 days); fetal bovine serum (FBS; Flow Laboratories, Rockville, Md.) (1 day); or phosphate-buffered saline (PBS; GIBCO Laboratories, Grand Island, N.Y.) (1 day). Numbers in parentheses refer to time before macrophage harvest that mice were inoculated with inflammatory agents. Peritoneal cells were adjusted to 10^6 macrophages per ml and 0.5-ml portions were placed in polypropylene tubes (no. 2063, 12 by 75 mm; Falcon Plastics, Oxnard, Calif.). Macrophages were exposed to amastigotes of *L. tropica* NIH strain 173 obtained from infected footpads of BALB/c mice (ratio of 1 amastigote per macrophage) or 0.8-μm latex beads (ratio of 1 bead per macrophage) for 1 h at 37°C. Cultures were washed and incubated at 37°C in 5% CO_2 in humidified air for up to 72 h. For microscopic analysis of the percentage of infected macrophages and numbers of intracellular amastigotes, we prepared cell smears by cytocentrifugation (Shandon Southern Instruments, Sewelicky, Pa.) and stained the smears with a modified Wright-Giemsa (Diff-Quick, Dade Diagnostics, Aquado, P.R.). Certain slides were also stained for peroxidase (14). Peroxidase in granules is a cytochem-

TABLE 1. Infection of resident and inflammatory macrophages with amastigotes of *L. tropica*[a]

Expt	Macrophage population	5′-Nucleotidase activity[b]	% Macrophages with peroxidase granules	% Infected macrophages in			χ^2 (*P*)
				Total population	300 peroxidase-positive macrophages	300 peroxidase-negative macrophages	
1	Resident cells	ND	8 ± 1	29 ± 2	32	25	3.96 (<0.05)
	Cells elicited by:						
	2% starch	ND	17 ± 2	26 ± 2	34	24	6.32 (<0.05)
2	Resident cells	331	1 ± 1	34 ± 6	50	28	29.60 (<0.001)
	Cells elicited by:						
	PBS	146	14 ± 2	32 ± 2	36	28	4.05 (<0.05)
	FBS	91	23 ± 2	24 ± 4	28	20	4.83 (<0.05)
	Latex beads	228	15 ± 2	29 ± 3	37	23	13.34 (<0.001)
	2% Starch	10	22 ± 1	29 ± 2	35	24	8.21 (<0.001)
	4% Thioglycollate	7	3 ± 1	28 ± 2	36	22	13.61 (<0.001)
3	Resident cells	400	6 ± 1	23 ± 1	40	25	14.71 (<0.001)
	Cells elicited by:						
	PBS	210	59 ± 3	31 ± 2	31	20	8.98 (<0.01)
	FBS	100	22 ± 4	27 ± 2	32	18	14.94 (<0.001)
	Latex beads	240	18 ± 2	28 ± 2	34	24	6.32 (<0.05)
	2% Starch	180	25 ± 1	32 ± 2	37	23	13.34 (<0.001)
	4% Thioglycollate	8	19 ± 1	25 ± 1	41	19	33.53 (<0.001)

[a] Macrophages were exposed to amastigotes of *L. tropica* for 1 h, washed, and samples were removed for cytocentrifugation. Cell smears were stained for peroxidase and counter stained with a modified Wright-Giemsa stain. A total of 200 to 300 macrophages were observed for duplicate cell smears of each sample. Results are expressed as mean percent infected cells ± standard error of the mean for triplicate samples. Significance was determined by chi-square analysis with Yates correction, 1 degree of freedom.

[b] 5′-Nucleotidase activity was expressed as nmol 5′-AMP hydrolyzed per 10^7 cells per 30 min; ND, not determined.

ical marker for monocytes and young inflammatory macrophages. Ingestion of amastigotes or latex particles by macrophages during a 1-h exposure to the phagocytic particles in vitro did not alter the percentage of peroxidase-positive cells. To further characterize these macrophage populations, we tested aliquots of resident and exudate cells for the ectoenzyme 5′-nucleotidase (EC 3.1.3.5; Sigma) (4). This enzyme is detectable on resident cells, but is reduced or absent on monocytes and the inflammatory macrophage populations used in this study (3). The cytochemical and biochemical profile of resident and inflammatory exudate cells is presented in Table 1.

After 1 h of exposure to amastigotes in vitro, similar numbers of macrophages were infected in resident and inflammatory cell populations (Table 1). Statistical analysis of these data suggested that minor variations in the percentage of infected macrophages among populations was within 95% confidence limits (2 standard deviations) of the mean percent infected cells in resident macrophage populations. Results of the microscopic analysis of cell smears stained for peroxidase activity, however, were startling: more peroxidase-positive (young) inflammatory macrophages were infected with *L. tropica* than one would predict by the percentage of these cells in the total population. For example, in a macrophage population that contained 17% peroxidase-positive macrophages (Table 1, experiment 1; starch-elicited cells) one would expect that 17% of infected cells would be peroxidase positive, if infection is a random event; yet 34% of infected macrophages contained peroxidase granules, twice as many as predicted by numbers in the total population (data not shown). In fact, this observation was true even in resident macrophage populations that had only 5 ± 3% peroxidase-positive cells. Analysis of the number of infected cells in peroxidase-positive and peroxidase-negative macrophage populations of both resident and inflammatory cells suggested that there was a marked and significant predilection of the parasite to enter less differentiated macrophages (Table 1). Peroxidase activity in granules is, unfortunately, a transient marker for

TABLE 2. Ingestion of amastigotes or latex beads by resident cells or inflammatory macrophages[a]

Macrophage population	% Peroxidase-positive cells	% Cells with intracellular particles after exposure to:				
		Amastigotes	Latex beads	Amastigotes and latex beads		
				Amastigotes	Latex beads	Both
Resident cells	1	22 (1.8)	26 (1.8)	21 (1.8)	23 (1.8)	2
Elicited cells	78	26 (1.7)	24 (1.9)	20 (1.7)	19 (1.9)	5
Mixture of resident plus elicited	34	22 (1.9)	26 (1.9)	17 (1.9)	22 (1.9)	4

[a] Macrophages (resident cells, inflammatory macrophages, and a 1:1 mixture of the two populations) were exposed to amastigotes of *L. tropica* or latex beads at a 1:1 multiplicity, alone or in combination. Results are expressed as mean percent cells containing intracellular particles after 1 h exposure in vitro, obtained by microscopic examination of 400 to 800 macrophages in each of duplicate samples. Numbers in parentheses refer to the average number of particles per cell. Standard errors of the means did not exceed 2% for any of the populations.

the young macrophage: as cells phagocytose the inflammatory stimulus (latex, thioglycollate, starch), they degranulate peroxidase into the phagosome and thus lose this cytochemical marker. That peroxidase activity in granules underestimates the number of young macrophages in certain of the inflammatory cells was underscored by 5'-nucleotidase activity in these populations (Table 1). Yields of cell populations elicited by 4% thioglycollate and 2% starch were 5 to 10 times that obtained from uninoculated mice; these inflammatory cells were 80 to 90% macrophages, and had little detectable 5'-nucleotidase activity (a marker enzyme for resident cells). Peroxidase activity in granules, then, underestimated the number of young macrophages in the chronically inflamed peritoneum. Even with this underestimation, however, the observation that a large percentage of the youngest cells (peroxidase positive) contained *L. tropica* amastigotes was also true for chronic inflammatory exudates (Table 1). To assess whether leishmanial infection of peroxidase-positive macrophages was secondary to more efficient phagocytosis by younger cells, we analyzed the ingestion of amastigotes and latex beads (another nonspecific phagocytic particle) in resident cells, in cells from an acute inflammatory reaction (2% starch administered 1 day before macrophage harvest), and in a 1:1 mixture of the two populations (Table 2). Despite the wide range in number of peroxidase-positive macrophages in these populations (1% in resident cells, 78% in elicited cells, and 34% in the mixture), similar numbers of cells ingested amastigotes or latex beads, whether each phagocytic particle was added separately or whether they were added in combination. Few cells exposed to amastigotes and latex in the same inoculum ingested both particles (5%). Moreover, similar numbers of particles were present in each cell that contained latex or amastigotes (Table 2). Nonetheless, when the three cell populations were segregated into peroxidase-positive and -negative cells, we again observed highly significant differences in the infection of these cells by *L. tropica* amastigotes. Two to three times as many peroxidase-positive cells were infected with amastigotes than peroxidase-negative cells, regardless of the percentage of

TABLE 3. Ingestion of amastigotes or latex beads by peroxidase-positive and peroxidase-negative macrophages

Macrophage population	No. of cells with amastigotes in:		χ^2 (*P*)	No. of cells with latex beads in:		χ^2 (*P*)
	400 Peroxidase-positive macrophages	400 Peroxidase-negative macrophages		400 Peroxidase-positive macrophages	400 Peroxidase-negative macrophages	
Resident cells	120	73	14.45 (0.001)	65	76	0.86 (ns)
Elicited cells	80	32	22.99 (0.001)	80	76	0.40 (ns)
Mixture of resident plus elicited:	80	44	11.69 (0.001)	64	88	4.4 (0.05)

[a] Resident peritoneal macrophages, inflammatory macrophages elicited by injection of starch 1 day before harvest, or a 1:1 mixture of the two populations were examined microscopically to determine the ingestion of amastigotes or latex particles by peroxidase-positive and -negative cells. Results are expressed as number of cells that contained either amastigote or latex beads in 400 cells observed. Significance was determined by chi-square analysis with Yates correction, 1 degree of freedom; ns, not significant.

TABLE 4. Intracellular replication of *L. tropica* amastigotes in resident and inflammatory macrophages

Expt	Macrophage population	Macrophages analyzed at 1 h		Macrophages analyzed at 72 h		Fold increase in intracellular amastigotes over 72 h
		% Infected macrophages	Mean amastigotes per infected macrophage	% Infected macrophages	Mean amastigotes per infected macrophage	
1	Resident cells	19 ± 2	1.3	41 ± 1	3.9	6
	Cells elicited by:					
	PBS	16 ± 1	1.1	40 ± 1	3.8	8
	FBS	24 ± 2	1.3	54 ± 1	3.6	6
	2% Starch	17 ± 1	1.3	44 ± 4	3.4	7
2	Resident cells	32 ± 4	1.5	76 ± 1	5.5	8
	Cells elicited by:					
	FBS	44 ± 4	1.8	88 ± 1	7.5	8
	2% Starch	42 ± 6	1.6	61 ± 1	3.5	4
	4% Thioglycollate	35 ± 3	1.5	61 ± 4	3.7	5
3	Resident cells	23 ± 1	1.4	36 ± 1	2.3	3
	Cells elicited by:					
	PBS	31 ± 2	1.4	73 ± 2	4.2	7
	FBS	27 ± 2	1.4	64 ± 2	3.5	6
	Latex beads	28 ± 2	1.4	55 ± 1	3.1	4
	2% Starch	32 ± 2	1.5	55 ± 2	3.8	4
	4% Thioglycollate	25 ± 1	1.4	48 ± 1	3.1	4

[a] Macrophages were exposed to amastigotes of *L. tropica* for 1 h, washed, and samples were removed at 1 and 72 h for cytocentrifugation. Cell smears were stained with a modified Wright-Giemsa stain and microscopically observed for percent infected macrophages and mean number of intracellular amastigotes per infected cell. A total of 600 to 800 macrophages were observed for duplicate cell smears of each sample. Results are expressed as mean percent infected cells ± standard error of the mean for triplicate samples.

peroxidase-positive cells in the population. Ingestion of latex particles by peroxidase-positive and peroxidase-negative cells, in contrast, was not different in two of the three cell populations (Table 3); results of the mixed cell population suggested that peroxidase-negative cells were slightly more phagocytic for latex particles than were peroxidase-positive cells. The results of this experiment suggest that increased phagocytic activity of peroxidase-positive cells under these culture conditions is not a factor in analysis of infection by *L. tropica*.

Although macrophages at different stages of maturation were infected with *L. tropica* in resident and inflammatory cell populations, the parasite replicated in all infected cells (Table 4). The percentage of infected macrophages and the number of intracellular amastigotes were occasionally higher in inflammatory cell populations at 72 h than in resident cells; variation among these inflammatory populations, however, was not significant. In all cases, macrophages from populations segregated by peroxidase staining (peroxidase-negative and peroxidase-positive cells) sustained a similar rate of replication of the parasite over 48 h that could not be distinguished from the population as a whole (data not shown). After 48 h, however, peroxidase positive cells had completely degranulated, and could no longer be distinguished from cells that were peroxidase negative at the outset of the experiment. Table 3 depicts three typical experiments with inflammatory macrophages. In the large number of experiments ($N = 10$) performed in these studies and in related studies with inflammatory macrophages (manuscript in preparation), the most inconsistent rate of replication of the parasite occurred in starch- and thioglycollate-elicited macrophages (Table 3). Intracellular amastigotes increased three- to eightfold in these cells, whereas the range of replication was six- to eightfold in cells elicited by other inflammatory agents.

The percentage of young cells present in the peritoneum is influenced by both the stimulus and duration of inflammation; it is intuitively obvious, however, that even in an acute inflammatory exudate there is a spectrum of cells of different maturational stages. The ability to mark macrophages by staining peroxidase gran-

ules enabled us to observe the parasite interaction with the youngest (least differentiated) of these cells. Characterization of the intermediate stages of macrophage maturation at the single cell level is not feasible; despite considerable interest and effort on the part of a number of investigators, reliable differentiation markers have not been identified. Within the current limits of technology, we detected in this study a subpopulation of macrophages that is susceptible to infection with *L. tropica*; the parasite entered the younger, peroxidase granule-containing macrophages of both resident and inflammatory populations in numbers disproportionate to those expected for random entry into cells. The contribution of heterogeneity within the parasite population in any of these studies is, at present, unknown.

Although inflammatory macrophages are more responsive to lymphokines that induce certain effector activities of activated macrophages (12), macrophages that respond to lymphokines that induce intracellular destruction of amastigotes of *L. tropica* are the more differentiated resident cells (9; D. L. Hoover, C. A. Nacy, Fed. Proc. **41**:768, 1982). The implications of preferential parasite entry into newly arrived inflammatory cells in the natural history of the developing leishmanial lesion are provocative; inflammation may actually contribute to the pathogenesis of leishmanial disease by supplying susceptible host cells that sequester the parasite from developing immune responses.

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