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Susceptibilities of Macrophage Populations to Infection In Vitro by *Leishmania donovani*

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Many studies have demonstrated differences in the resistance of strains of mice to infection by *Leishmania donovani*, *Salmonella typhimurium*, and *Mycobacterium bovis* BCG; this resistance/susceptibility phenotype seems to be controlled by a single gene. The present study investigated the susceptibility of liver, lung, peritoneal, and spleen macrophages to infection by *L. donovani* promastigotes in vitro; the objective was to determine if the susceptibility of animals was expressed by their macrophages when infected in vitro. This study indicated that the Lsh phenotype was only expressed by liver macrophages. The liver macrophages of the susceptible C57BL/6J strain were significantly more phagocytic than those of the resistant C57L/J strain; infection affected the phagocytic activity of the macrophage population. These results indicated that only liver macrophages can express the *Lsh* gene. Recognition of expression is in part due to its effect on the phagocytic activity of the macrophages.

Studies of infection of various inbred strains of mice by *Leishmania donovani* have demonstrated that innate resistance to this intracellular parasite seems to be controlled by a single gene (*Lsh*) which maps on chromosome 1 (4). Innate resistance to *Salmonella typhimurium* and *Mycobacterium bovis* in the macrophages of the spleen and liver also seems to be controlled by a gene located on chromosome 1 (18, 19). The genes controlling innate resistance to these three intracellular microorganisms are probably identical (17). Many in vivo studies have demonstrated that T cells are ineffective in regulating the expression of this gene (2, 11, 16, 21). Innate resistance can, however, be transferred by bone marrow cells, as demonstrated for *L. donovani* and *S. typhimurium* (6, 7, 9). Crocker et al. (10) have shown that innate resistance (Lsh^r) is expressed by resident liver macrophages after infection in vivo and cultivation in vitro. The infection in vitro of liver macrophages extracted by enzymatic digestion has been recently demonstrated (15).

In this study, we demonstrate that resident liver macrophages are the only macrophages which can express the *Lsh* phenotype after infection in vitro with *L. donovani* promastigotes. We also demonstrate that in radiation chimeras, resident liver macrophages originate from bone marrow progenitors and migrate to and mature in the liver, acquiring a capacity to express the phenotype of the donor, and that the percentage of infected macrophages in vitro is related to phagocytic activity.

MATERIALS AND METHODS

Animals. Male C57L/J (Lsh^r) and C57BL/6J (Lsh^s) inbred mice aged 6 to 8 weeks were obtained from the Jackson Laboratory (Bar Harbor, Maine). Female Syrian hamsters (LaK:LVG) were purchased from Charles River Breeding Laboratories, Inc. (Newfield, N.J.).

Parasite. The Ethiopian LV9 strain of *L. donovani* was maintained in our laboratory by passage in hamsters; this strain was provided by J. M. Blackwell of the London School of Hygiene and Tropical Medicine. The isolation and enumeration of amastigotes were done as described by Bradley and Kirkley (3). Amastigotes were transformed into promastigotes in chemically defined RE 9 medium (20) and maintained in the laboratory by weekly transfers. The promastigotes used for infection of the macrophages in vitro were from day-8 cultures (stationary phase) of passage 2 in culture. They were centrifuged from the medium at 650 × *g* for 10 min and suspended in RPMI 1640 medium (Flow Laboratories, Inc., McLean, Va.) supplemented with 10% heat-inactivated fetal calf serum and 100 μg of streptomycin and 100 U of penicillin per ml; this medium wil be referred as the complete medium. The number of promastigotes was assessed by using a hemacytometer, and their number was adjusted to 5×10^6/ml. Viability was determined with ethidium bromide-fluorescein diacetate as described by Jackson et al. (13).

Chimeras. C57L/J (Lsh^r $H\text{-}2^b$) and C57BL/6J (Lsh^s $H\text{-}2^b$) mice were exposed to a lethal dose (950 rads) of ^{60}Co gamma radiation in a Gammacell 220 (Atomic Energy of Canada, Ltd.). Following irradiation, the mice were reconstituted intravenously with 5×10^6 (Lsh^s or Lsh^r) viable bone marrow cells in 0.2 ml of the complete medium. Bone marrow cells were obtained from normal mouse donors of both strains by aseptically flushing the femurs with the complete medium through a 23-gauge needle after cutting the epiphyses; the cells were then dissociated by repeated passage through a 26-gauge needle. After reconstitution, the recipient mice were treated with commercial penicillin G (Derapen-A; Ayerst Laboratories, Montreal, Quebec, Canada) and kept in a barrier facility; the reconstituted mice were used in the experiments 3 months later.

Isolation of macrophages. Resident peritoneal macrophages (PEC) were harvested from mice by lavage of the peritoneal cavity with 5 ml of cold complete medium. Spleen macrophages were obtained by pressing the organ through a stainless steel screen and then suspended in 10 ml of cold complete medium; erythrocytes were lysed by using Tris-buffered ammonium chloride. Lung macrophages were extracted by the technique previously described by Egwang et al. (8), with the exception that 10 ml of warm medium was used for the perfusion.

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Liver macrophages were extracted by enzymatic digestion (0.05% collagenase [type IV] and 100 μg of DNase [type I] per ml; Sigma Chemical Co., St. Louis, Mo.) as previously described (6). The isolated cells were washed twice in cold medium and kept at 4°C in an ice bath until used. Differential counts were done on 300 cells from Giemsa-stained cytocentrifuge smears; cell viability, as determined by trypan blue exclusion, was consistently between 90 to 95%, and 95% of the adherent cells were esterase positive. A sample of the cells incubated at room temperature for 5 min with fluorescein diacetate (5 μg/ml) indicated that 95% of the cells in the suspension showed the characteristic green cytoplasmic fluorescence of macrophages. After cell counts were done, cell numbers were adjusted to 10^6 macrophages per ml, and 100-μl aliquots of the cell suspension were placed on 18-mm glass cover slips. Macrophages were allowed to adhere for 2 h at 37°C in humidified 5% CO_2 and were then exposed to 5×10^5 promastigotes for 2 h. Nonadherent cells and free promastigotes were removed, and the adherent cell population was cultured in 2 ml of the complete medium for 1, 2, 3, and 4 days.

On days 1, 2, 3 and 4 after exposure, three cover slips per group were removed, fixed with methanol, and stained with Giemsa stain. Preparations were examined at 100×, and the percentages of infected macrophages were determined for 300 to 500 macrophages on each cover slip. The number of intact parasites inside infected macrophages was also determined. The same numbers of adherent cells were observed on cover slips for all groups and different cell populations.

Phagocytosis assay. To assess the ability of different macrophage populations (liver macrophages and PEC of C57L/J and C57BL/6J mice) to phagocytose targets, we used the macrobead colorimetric assay kit (CK 001), purchased from Cellular Diagnostics (Potsdam, N.Y.). Macrobeads are synthetically prepared uniform spheres (0.4 μm in diameter) that contain Cr^{3+} (20% [wt/wt]). These spheres, once ingested by macrophages, can be liberated by using a specifically formulated oxidant solution which dissolves macrophages and macrobeads and converts Cr^{3+} to Cr^{6+} in a one-step reaction. Diphenylcarbazide solution was used to convert Cr^{6+} to a red-colored complex (1). The preparation of macrophages for infection and the phagocytosis assay were done as follows. Liver macrophages and PEC from C57L/J and C57BL/6J mice were recovered for this experiment as previously described. Macrophages (10^5/100 μl of cell suspension) were added to individual wells of 96-well microtiter plates (the assay was done in sextuplicate). The plates were incubated at 37°C in humidified 5% CO_2–air for 2 h. After incubation, macrophages were infected with 5×10^5 promastigotes per 100 μl for 2 h. After incubation, nonadherent cells and free promastigotes were removed, and the wells were gently washed with warm complete medium. The adherent cells were incubated in 300 μl of complete medium for 1, 2, 3, and 4 days. Uninfected macrophages were used as positive controls. Macrobeads were observed in 87% of liver adherent cells and in more than 95% of PEC adherent cells. During this experiment, macrophages cultured on cover slips were also infected with the parasite in parallel to assess the infection.

Every day following infection, the medium was removed from the wells and, after a gentle wash, 100 μl of a 1:50 dilution of macrobeads was added to each well followed by 100 μl of medium. After 1 h of incubation at 37°C, noningested macrobeads were removed by washing the wells (three times) with warm complete medium. Once noningested macrobeads were removed, microscopic examination of the wells revealed that less than 10% of the macrophages had macrobeads on their surfaces. To each well, 50 μl of oxidant was added. After 15 min at room temperature, the well contents were transferred to 2-ml glass vials and then incubated for 3 min at 100°C. The contents of each vial were then transferred to a new 96-well microtiter plate, and to each well the following solutions were added in succession: 50 μl of NH_4Br, 10 μl of concentrated sulfuric acid, 50 μl of sulfosalicylic acid diluted 1:25 with distilled water, and 100 μl of diphenylcarbazide solution.

The optical density was read at 540 nm by using a microplate reader (Titertek Multiscan; Flow Laboratories). The absorbance values were converted to micrograms of macrobeads per 0.6 absorbance units.

RESULTS

In vitro infection of different macrophage populations by *L. donovani*. The results shown in Fig. 1 indicate the numbers of macrophages obtained from the livers, spleens, peritoneal cavities, and lungs of C57BL/6J (*Lsh*^{s}) mice, C57L/J (*Lsh*^{r}) mice, and their reciprocal radiation chimeras, which became infected after inoculation in vitro with *L. donovani* promastigotes. The macrophages from the spleens, lungs, and peritoneal cavities of normal or chimeric mice showed no significant difference in their susceptibility to infection in all groups. Liver macrophages, on the other hand, clearly expressed the resistance/susceptibility phenotype when infected in vitro.

The difference observed between the percentages of infected liver macrophages in C57L/J (*Lsh*^{r}) and C57BL/6J (*Lsh*^{s}) mice is highly significant ($P < 0.01$) during all the days postinfection in the normal and chimeric mice. The resistance of macrophages isolated from the livers of C57BL/6J mice which had received bone marrow cells from C57L/J mice clearly demonstrated that recipients acquired the resistance phenotype from the bone marrow cells of the donors. Susceptibility was also transferred to the liver cells of the resistant C57L/J strain by bone marrow cells from the susceptible C57BL/6J strain. Differences in the level of infection of liver cells between the two groups of chimeric mice are also highly significant. Controls for the chimeric mice (macrophages from lethally irradiated mice which received syngeneic bone marrow cells) also showed the phenotype proper to the strain. Infected macrophages always had the same numbers of intracellular amastigotes (1.6 ± 0.5 amastigotes per cell), irrespective of the strain of mouse or population of cells.

Phagocytic activity of liver and peritoneal cavity macrophage populations infected in vitro by *L. donovani*. The phagocytic activity of liver macrophages and PEC of C57L/J (*Lsh*^{r}) and C57BL/6J (*Lsh*^{s}) mice was determined during the course of the infection in vitro (Table 1). There was no significant difference between the phagocytic activities of the PEC of either strain. In contrast, liver macrophages from C57BL/6J (*Lsh*^{s}) mice were significantly more phagocytic ($P < 0.05$) than those from C57L/J (*Lsh*^{r}) mice.

Growth conditions in culture did not preserve the phagocytic activity of the cells, since uninfected PEC and liver macrophages tested after 1 day in culture were significantly less phagocytic than those tested within 1 h of harvesting. Infection further depressed endocytosis, since the phagocytic activity of the infected cells was significantly lower than that of the uninfected cells in all groups throughout the infection. Despite this decrease, infected liver macrophages from C57BL/6J (*Lsh*^{s}) mice still demonstrated significantly

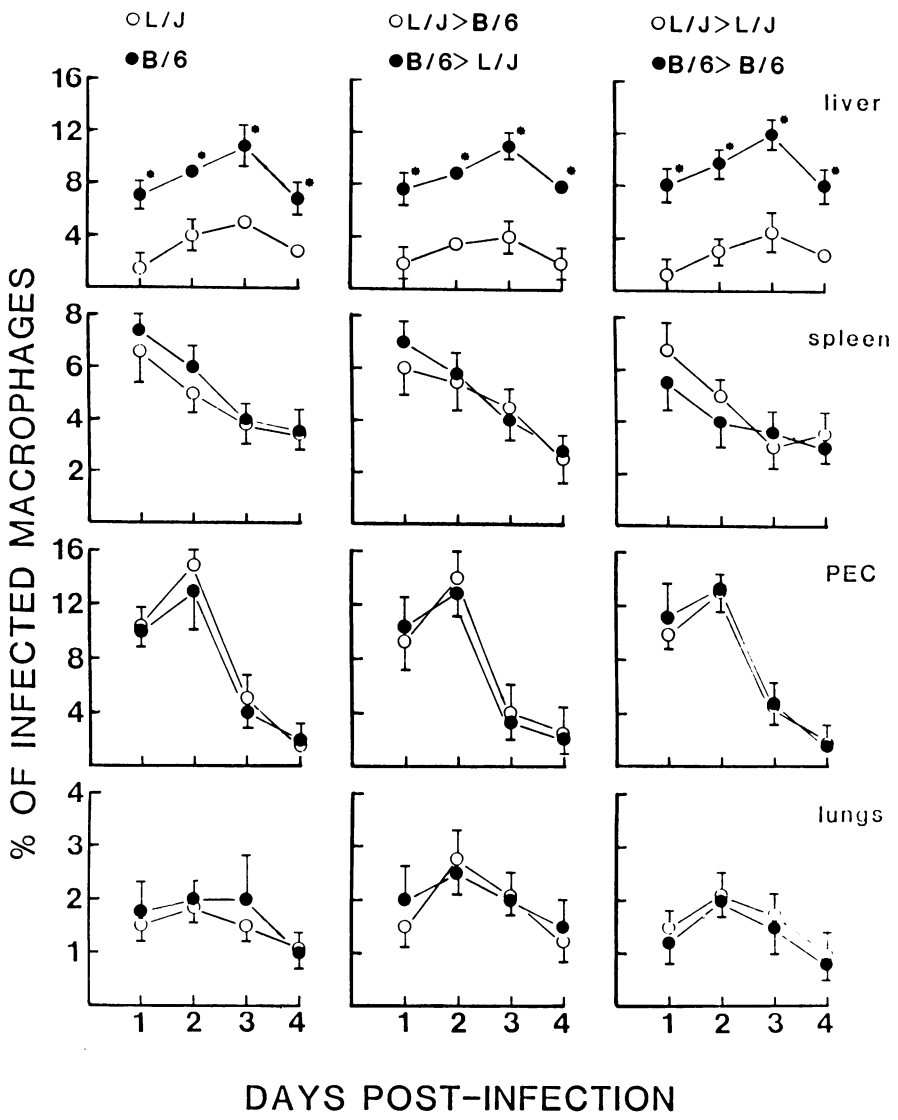

FIG. 1. In vitro infection by *L. donovani* promastigotes of PEC and liver, spleen, and lung macrophages obtained from C57L/J (L/J), C57BL/6J (B/6), chimeric (donor → recipient), and control mice. Each point represents the percentage of macrophages infected with the parasite (±standard error). For each point, 300 to 500 macrophages were counted on each cover slip. *, Significant difference ($P < 0.01$) by analysis of variance between the two groups in each panel.

more phagocytic activity than did those from C57L/J (*Lsh*^{r}) mice during the first 3 days of infection in vitro; the PEC of neither strain differed in phagocytic activity.

Although there were differences in the intensity of phagocytosis between the different groups of macrophages, microscopic examination of the cells in all the groups and at all times of incubation showed that at least 95% of the adherent cells were phagocytic. These results suggest that

TABLE 1. Phagocytic activity of macrophage populations infected in vitro by *L. donovani*

Group	μg of macrobeads/2 × 10^5 macrophages (mean ± SE) in the following mouse macrophages:			
	C57BL/6J (*Lsh*^{s})		C57L/J (*Lsh*^{r})	
	Liver	Peritoneal cavity	Liver	Peritoneal cavity
Uninfected (1 h)	0.937 ± 0.021[a]	0.833 ± 0.026	0.701 ± 0.025	0.859 ± 0.081
Uninfected (day 1)	0.832 ± 0.032[b]	0.740 ± 0.047[b]	0.622 ± 0.016[b]	0.736 ± 0.064[b]
Infected (day 1)	0.330 ± 0.003[a,c]	0.377 ± 0.004[c]	0.310 ± 0.005[c]	0.363 ± 0.015[c]
Infected (day 2)	0.352 ± 0.001[a,c]	0.441 ± 0.013[c]	0.308 ± 0.003[c]	0.452 ± 0.006[c]
Infected (day 3)	0.314 ± 0.005[a,c]	0.385 ± 0.015[c]	0.220 ± 0.001[c]	0.394 ± 0.014[c]
Infected (day 4)	0.219 ± 0.002[c]	0.351 ± 0.010[c]	0.202 ± 0.002[c]	0.335 ± 0.010[c]

[a] Significantly different from corresponding C57L/J group by analysis of variance ($P < 0.05$).
[b] Significantly different from uninfected (1 h) control by analysis of variance ($P < 0.05$).
[c] Significantly different from corresponding control by analysis of variance ($P < 0.05$).

the resistance/susceptibility phenotype of liver macrophages in vitro is related in part to their phagocytic activity and that the phagocytic activity of macrophages is affected by intracellular parasitism.

DISCUSSION

The major goal of this study was to determine if the Lsh phenotype is expressed in vitro in various macrophage populations of susceptible (C57BL/6J) and resistant (C57L/J) mice. The infection of PEC, lung, spleen, and liver macrophages in vitro with *L. donovani* promastigotes clearly demonstrated that only liver cells can express the Lsh^r phenotype (Fig. 1), corroborating the findings of Crocker et al. (6). These authors demonstrated the expression of the natural resistance gene *Lsh* in resident liver macrophages obtained from preinfected mice after 48 h of cultivation in vitro. No significant differences were obtained in our study in vitro in the susceptibilities of spleen macrophages, PEC, and lung macrophages from resistant or susceptible strains of mice; the similarities (and differences) held true during the course of the infection in vitro.

A number of studies have indicated that the transfer of bone marrow to irradiated mice can transfer resistance or susceptibility to *S. typhimurium* (9), *L. tropica* (12), and *L. donovani* (7) but not to *Listeria monocytogenes* (14). The results of this study demonstrate that the Lsh phenotype is transferrable to macrophages by bone marrow cells (Fig. 1) but that it is detectable only in liver cells. These results demonstrate clearly that the *Lsh* gene is present in cells of the hematopoietic system and that progenitors of hepatic macrophages are present in bone marrow. Bone marrow must, therefore, contain immature macrophages which have the capacity to express the Lsh phenotype when they have colonized the liver. It is most interesting that the expression of the Lsh phenotype by hematopoietic cells occurs only in the liver, since it indicates that the environment influences the susceptibility of macrophages. It is well known that the expression of the *Lsh/Ity* gene is not affected by lymphocytes (2, 16, 21), but the nature of the influence of other cells of the organ on the behavior of resident macrophages in the liver is completely unknown. It now appears clear that the natural resistance of mice to *L. donovani* may be due principally to the resistance of liver macrophages.

One reason for the susceptibility of liver macrophages to infection in vitro (Fig. 1) is (Table 1) the fact that uninfected macrophages of the livers of susceptible mice (C57BL/6J) were significantly more phagocytic than those of resistant mice (C57L/J). Factors which may influence differences in vitro in the resistance or susceptibility of macrophages include the effect of the respiratory burst, which is absent in Kupffer cells (15), or the presence, absence, or density of surface receptors recognized by the parasite (1a). Differences in resistance or susceptibility could be also due to the response of uninfected macrophages to soluble signals secreted by infected cells or to soluble factors secreted by the parasite which may stimulate or suppress phagocytic activity. The inhibition of the phagocytic activity of infected macrophages (Table 1) supports the finding of Bray et al. (5) that although 95% of macrophages heavily parasitized by *L. mexicana mexicana* took up macrobeads, their phagocytic activity was greatly decreased. Such a decrease may be due to a negative feedback from infected to uninfected cells or to the influence of factors secreted by the parasite itself. These aspects are presently under active investigation.

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