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Differences in *Lsh* Gene Control over Systemic *Leishmania major* and *Leishmania donovani* or *Leishmania mexicana mexicana* Infections Are Caused by Differential Targeting to Infiltrating and Resident Liver Macrophage Populations

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Earlier studies had shown that the viscerotropic NIH 173 strain of cutaneous *Leishmania major* fails to come under *Lsh* gene control. Visceral *Leishmania donovani* LV9 and another viscerotropic cutaneous strain, *Leishmania mexicana mexicana* LV4, are controlled by *Lsh*. The results of double-infection experiments presented here show that expression of *Lsh* resistance against *L. mexicana mexicana* was enhanced in the presence of *L. donovani*, whereas *L. major* still failed to come under *Lsh* gene control, even in the presence of *L. donovani*. Prior irradiation (850 rads) of mice showed that in the absence of infiltrating monocytes, *Lsh* did exert some influence over *L. major*. The presence of a higher infiltrate of fresh monocytes after *L. major* infection was confirmed in liver macrophage populations isolated from mice after infection in vivo and in liver cryosections immunostained with monoclonal antibody M1/70 directed against the type 3 complement receptor CR3. The results support the hypothesis that *Lsh* is expressed maximally in the resident tissue macrophages and poorly in the immature macrophages preferentially infected by *L. major* amastigotes.

Recent studies reported by Mock and co-workers (20, 21) demonstrated that the NIH 173 viscerotropic strain of *Leishmania major* fails to come under *Lsh* gene control when injected intravenously (i.v.) into congenic B10.L-*Lsh*^{r} or C.D2-*Idh-1*,*Pep-3* (*Lsh*^{r}) mice. This was surprising considering the broad action of the *Lsh*/*Ity*/*Bcg* gene in controlling such phylogenetically unrelated macrophage pathogens as *Leishmania donovani* (5, 6), *Salmonella typhimurium* (24, 25), *Mycobacterium bovis* Montreal (15), *Mycobacterium lepraemurium* (8, 26), and *Mycobacterium intracellulare* (14). Significant differences in liver parasite loads 15 days after i.v. inoculation of another viscerotropic cutaneous *Leishmania* species, *L. mexicana mexicana* LV4, into congenic C57BL/10ScSn or B10 (*Lsh*^{s}) and (B10 × B10.L-*Lsh*^{r})F_1 mice (3) suggest that this species does come under *Lsh* gene control. Several explanations for the results obtained with *L. major* NIH 173 were possible. It may either fail to induce *Lsh* gene expression or be insensitive to the *Lsh*-gene-controlled resistance mechanism. Alternatively, this strain of *L. major* may target to a different macrophage population which fails to express *Lsh* gene activity in vivo. The results of experiments presented here provide evidence that *Lsh* gene control of *L. major* NIH 173 is, in fact, masked by preferential infection of infiltrating monocytes in which the gene is poorly expressed.

MATERIALS AND METHODS

Mice. CBA/Ca (*Lsh*^{r}) and C57BL/10ScSn or B10 (*Lsh*^{s}) mice were purchased from Harlan Olac Ltd. (B10 × B10.L-*Lsh*^{r})F_1 mice were bred at the London School of Hygiene and Tropical Medicine. The congenic B10.L-*Lsh*^{r} parental strain was produced by backcrossing the *Lsh*^{r} allele from C57L mice onto the B10 genetic background as previously described (3). In vivo *Lsh* typing with *L. donovani* LV9 confirmed that F_1 mice are phenotypically as resistant as homozygous B10.L-*Lsh*^{r} mice. Male or female mice between 8 and 12 weeks old were sex and age matched (±1 week) within each experiment as indicated.

Parasites. Amastigotes of *L. donovani* LV9 (7), *L. major* NIH 173 (22), or *L. mexicana mexicana* LV4 (1) were isolated from hamster spleen, mouse footpads, or mouse rump lesions as described. Parasites (10^7 or 2×10^7 per mouse) were injected i.v., and infection was measured as the number of Leishman-Donovan units (LDU) in the liver (27).

Double-infection protocol. Male B10 (*Lsh*^{s}) and (B10 × B10.L-*Lsh*^{r})F_1 mice were injected i.v. with either a single dose (10^7 amastigotes) of *L. donovani* LV9, *L. major* NIH 173, or *L. mexicana mexicana* LV4 or with 5×10^6 amastigotes of each of two *Leishmania* strains (LV9 plus LV4 or LV9 plus NIH 173) simultaneously. Mice (three per group) were sacrificed after 15 days of infection, and liver LDU were scored from duplicate impression smears taken from two separate liver sections for each mouse. On impression smears from doubly infected mice it was possible to score each *Leishmania* species separately on the basis of morphological differences. *L. major* and *L. mexicana mexicana* are each much larger than *L. donovani* in the amastigote form.

Lethal irradiation before infection. Female B10 (*Lsh*^{s}) and CBA/Ca (*Lsh*^{r}) mice were lethally irradiated (850 rads) 2 h before infection with 2×10^7 amastigotes of *L. donovani* or *L. major*. Control and irradiated mice (four per group per day) were sacrificed for liver impression smears on days 0, 1, 2, 4, 6, 8, and 10 after infection. Additional control mice were examined on days 15 and 22 postinfection. During the first 10 days of infection all mice received antibiotics (20 μg

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of neomycin per liter and 200 U of polymyxin per liter) in their drinking water.

Isolation of liver macrophages. Male B10 (*Lsh*^{s}) and (B10 × B10.L-*Lsh*^{r})F_1 mice were injected i.v. with 10^7 amastigotes of *L. donovani* or *L. major*. Control mice received 0.2 ml of medium alone i.v. On days 1, 3, and 7 after injection, liver macrophage populations were prepared (two mice per group per day) by collagenase digestion as described previously (9). Liver macrophages were cultured (37°C, 5% CO_2-95% air) on cover slips overnight before being fixed in methanol and stained with Giemsa solution for parasite enumeration, stained for peroxidase activity (23), or immunoperoxidase stained with M1/70 or F4/80 monoclonal antibodies (11), using Vectastain Kit PK4004 (Sera-Labs Ltd). Peroxidase- and immunoperoxidase-stained preparations were counterstained to a count of 5 in hematoxylin.

Immunostaining of liver cryosections. Male B10 (*Lsh*^{s}) and (B10 × B10.L-*Lsh*^{r})F_1 mice were injected i.v. with 2×10^7 amastigotes of *L. donovani* or *L. major*. Mice (two per group per day) were sacrificed on days 1, 3, 7, and 15 after infection. Impression smears were prepared for parasite enumeration. Segments of liver were mounted in Tissue-Tek O.C.T. Compound 5483 (Miles Scientific, Div. Miles Laboratories, Inc.), snap frozen in cold isopentane, and stored at −70°C ready for cryosectioning. Sections (4 μm) were cut and mounted on slides for immunoperoxidase staining with F4/80 and M1/70. The immunostaining procedure was essentially the same as that for cover-slip cultures except that a preincubation step with avidin was included to block natural avidin-binding sites as described by Kaye (18). Sections were counterstained with hematoxylin.

Monoclonal antibodies. Hybridoma supernatants for the panmacrophage marker F4/80 (1a) were generously provided by S. Gordon, Sir William Dunn School of Pathology, Oxford University, Oxford, United Kingdom. Hybridoma supernatants for monoclonal antibody M1/70, directed against the iC3b binding site of the macrophage type 3 complement receptor (CR3) (2), were prepared from cells originally obtained by S. Gordon from T. Springer, Harvard Medical School, Boston, Mass.

RESULTS

***Lsh* gene expression in doubly infected mice.** Day-15 liver parasite loads (Fig. 1) in mice receiving single infections confirmed results obtained previously (3, 20), i.e., significantly lower parasite loads were observed in (B10 × B10.L-*Lsh*^{r})F_1 mice than in B10 (*Lsh*^{s}) mice for both *L. donovani* and *L. mexicana mexicana* but not for *L. major*. For mice doubly infected with *L. donovani* and *L. mexicana mexicana*, F_1 mice had significantly lower parasite loads for both species, the expression of *Lsh* resistance against *L. mexicana mexicana* being enhanced by the presence of *L. donovani*. For mice doubly infected with *L. donovani* and *L. major*, F_1 mice had lower *L. donovani* and higher *L. major* loads than did B10 (*Lsh*^{s}) mice. Hence, even in the presence of *L. donovani*, which would be expected to stimulate expression of the resistant phenotype, *Lsh* failed to exert any measurable influence over *L. major* infection. In fact, *Lsh* resistance against *L. donovani* was diminished in the presence of *L. major*. Overall, these results suggest that *L. donovani* and *L. mexicana mexicana* share the same *Lsh*-gene-controlled macrophage environment within the liver, whereas *L. major* does not.

Effect of lethal irradiation on *Lsh* gene expression. To determine the contribution of infiltrating monocytes to the expansion of *L. donovani* and *L. major* amastigote populations in the liver, mice were lethally irradiated before infection. This treatment severely limits recruitment of monocytes to the liver after infection while leaving the radioresistant mature liver macrophage population intact. As in a previous study (9), no effect of lethal irradiation on expression of *Lsh*-gene-controlled resistance and susceptibility in CBA/Ca (*Lsh*^{r}) and B10 (*Lsh*^{s}) mice was observed after *L. donovani* infection (Fig. 2). This is also consistent with the hypothesis that the gene is expressed in the resident liver macrophage population (9, 10). For *L. major* infection, no net growth in the liver parasite population was observed in female mice of either strain. This is in agreement with the observation of Mock and co-workers (20, 21) that female mice are not normally as permissive to visceral growth of *L. major* NIH 173 as are male mice. As in the earlier experiment (Fig. 1), day-15 liver parasite loads were higher in *Lsh*^{r} (CBA/Ca) than *Lsh*^{s} (B10) control mice. No differences were observed up to 10 days after infection in these control mice. Interestingly, lethal irradiation produced a dramatic difference between B10 (*Lsh*^{s}) and CBA/Ca (*Lsh*^{r}) mice at 10 days postinfection, parasite loads in the liver of *Lsh*^{s} mice rising sharply between 6 and 10 days postinfection, whereas those in *Lsh*^{r} mice continued to fall (Fig. 2). This indicates that in the absence of a fresh monocyte influx, the *Lsh* gene may exert some control over the *L. major* liver parasite population.

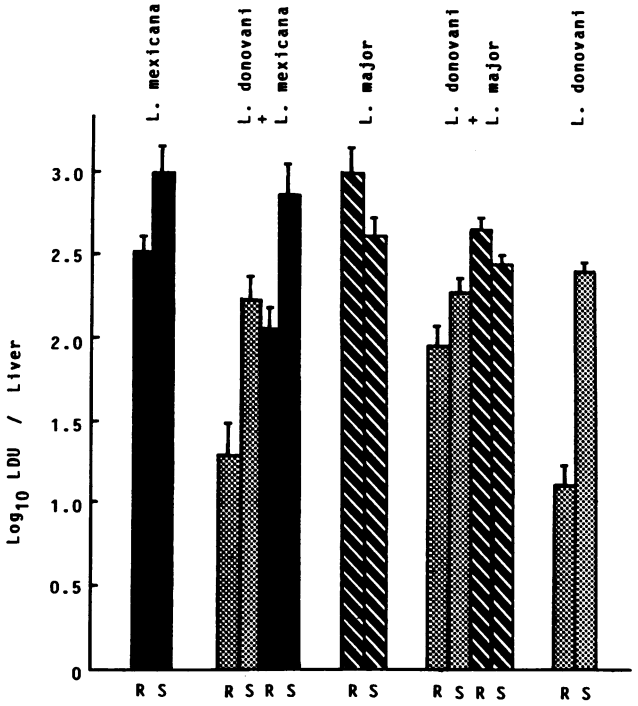

FIG. 1. Day-15 parasite loads (log_{10} LDU, mean + standard deviation, three mice per group) in the livers of B10 (*Lsh*^{s}) (S) or (B10 × B10.L-*Lsh*^{r})F_1 (R) mice infected with *L. donovani* LV9 (▨), *L. mexicana mexicana* LV4 (■), *L. major* NIH 173 (▧), or combinations thereof. Similar results were obtained in two other double-infection experiments.

Characterization of macrophage subsets infected with *L. donovani* and *L. major*. Lethal irradiation experiments provided indirect evidence that monocytes mask expression of

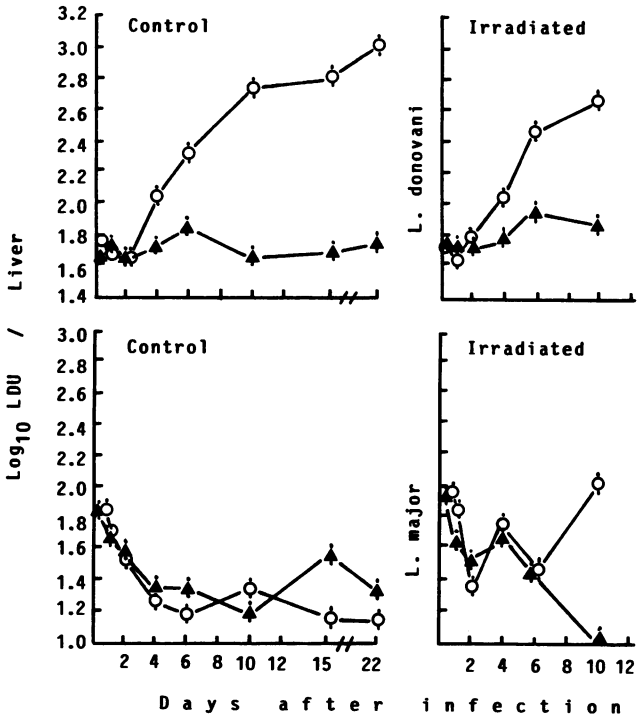

FIG. 2. Course of infection in livers (log_{10} LDU, mean ± standard deviation, four mice per group per day) of B10 (*Lsh*^{s}) (○) or CBA/Ca (*Lsh*^{r}) (▲) female mice after infection with *L. donovani* LV9 or *L. major* NIH 173. Results are shown for control mice and for mice receiving 850 rads of X-irradiation 2 h before infection with 2×10^7 amastigotes.

Lsh gene activity against *L. major* in vivo. They did not, however, provide definitive evidence that the two *Leishmania* species, *L. donovani* and *L.major*, target to different macrophage subsets after infection in vivo. Since resident liver macrophages (Kupffer cells) do not contain granular peroxidase (23) and fail to express the MAC-1 antigen (CR3) (12), peroxidase staining and M1/70 immunostaining could be used to distinguish fresh monocytes entering the liver after infection. On day 1 after infection there was a dramatic difference between *L. donovani*- and *L. major*-infected mice in the percentage of M1/70-positive cells (Fig. 3). For *L. donovani*-infected mice, the numbers of M1/70-positive cells (<15%) were not significantly different from those observed for control uninfected mice. For *L. major*-infected mice, 40 to 50% of the adherent cell population was M1/70 positive. In cryosections, M1/70-positive cells appeared as single stained cells dispersed throughout the liver section (results not shown). At this stage of infection there were no differences in the percentages of macrophages infected or the numbers of amastigotes per 100 macrophages for either strain of mouse infected with either *L. donovani* or *L. major* (Fig. 3).

Beyond day 1 of infection the two leishmanial infections took quite different courses in the two mouse strains. For *L. donovani*, parasite enumeration in Giemsa-stained preparations showed significant ($P < 0.05$) differences in amastigotes per 100 macrophages between B10 (*Lsh*^{s}) and (B10 × B10.L-*Lsh*^{r})F_1 mice by day 3 after infection. The numbers of parasites plateaued in F_1 mice but continued to increase in B10 mice, producing a very clear-cut difference ($P < 0.001$) both in amastigotes per 100 macrophages and in the percent macrophages infected by day 7 after infection (Fig. 3). This is consistent with earlier observations of Crocker et al. (9). There was a modest increase in M1/70-positive cells in adherent cell preparations from both B10 (*Lsh*^{s}) and (B10 × B10.L-*Lsh*^{r})F_1 mice; they appeared both as single stained cells and as small M1/70-positive foci in liver cryosections. Despite the presence of M1/70-positive cell foci, large numbers of *L. donovani* amastigotes were found in M1/70-negative, F4/80-positive liver Kupffer cells in B10 (*Lsh*^{s}) mice 15 days after infection (Fig. 4).

For *L. major*, numbers of parasites (amastigotes per 100 macrophages and percent macrophages infected) continued to increase in both B10 (*Lsh*^{s}) and (B10 × B10.L-*Lsh*^{r})F_1 mice (Fig. 3). M1/70-positive macrophages continued to infiltrate the liver so that by days 7 and 15 after infection many large M1/70-positive cell foci were present in the liver sections (Fig. 5). At day 7 of infection, 83% of infected macrophages from B10 mice were M1/70 positive and contained 81% of the parasite population, compared with only 23% M1/70-positive infected macrophages containing 14% of the parasite population in liver macrophages from *L. dono-*

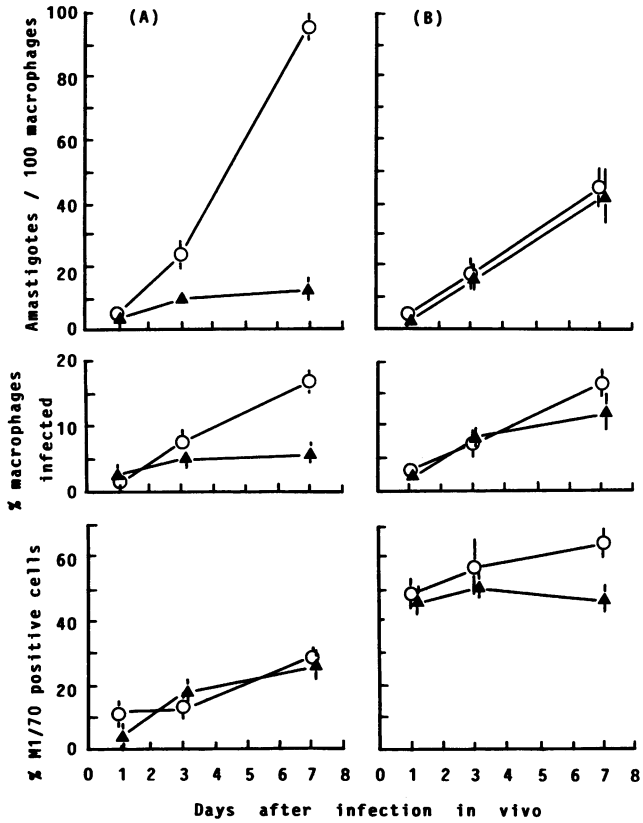

FIG. 3. Parasite enumeration (amastigotes per 100 macrophages and percent macrophages infected) in Giemsa-stained preparations and M1/70 characterizations (percent M1/70-positive cells) for adherent liver cell populations from *L. donovani* LV9 (A)- and *L. major* NIH 173 (B)-infected B10 (*Lsh*^{s}) (○) and (B10 × B10.L-*Lsh*^{r})F_1 (▲) mice. Duplicate cover slips were prepared for both Giemsa and M1/70 immunostaining of adherent liver cells from two mice per strain per time point. At least 1,000 macrophages per cover slip were scored microscopically for parasite enumerations and for percent M1/70-positive macrophages. Similar results were obtained in two other experiments. In each instance, results obtained by using peroxidase staining to characterize fresh monocytes mimicked those obtained with M1/70 staining.

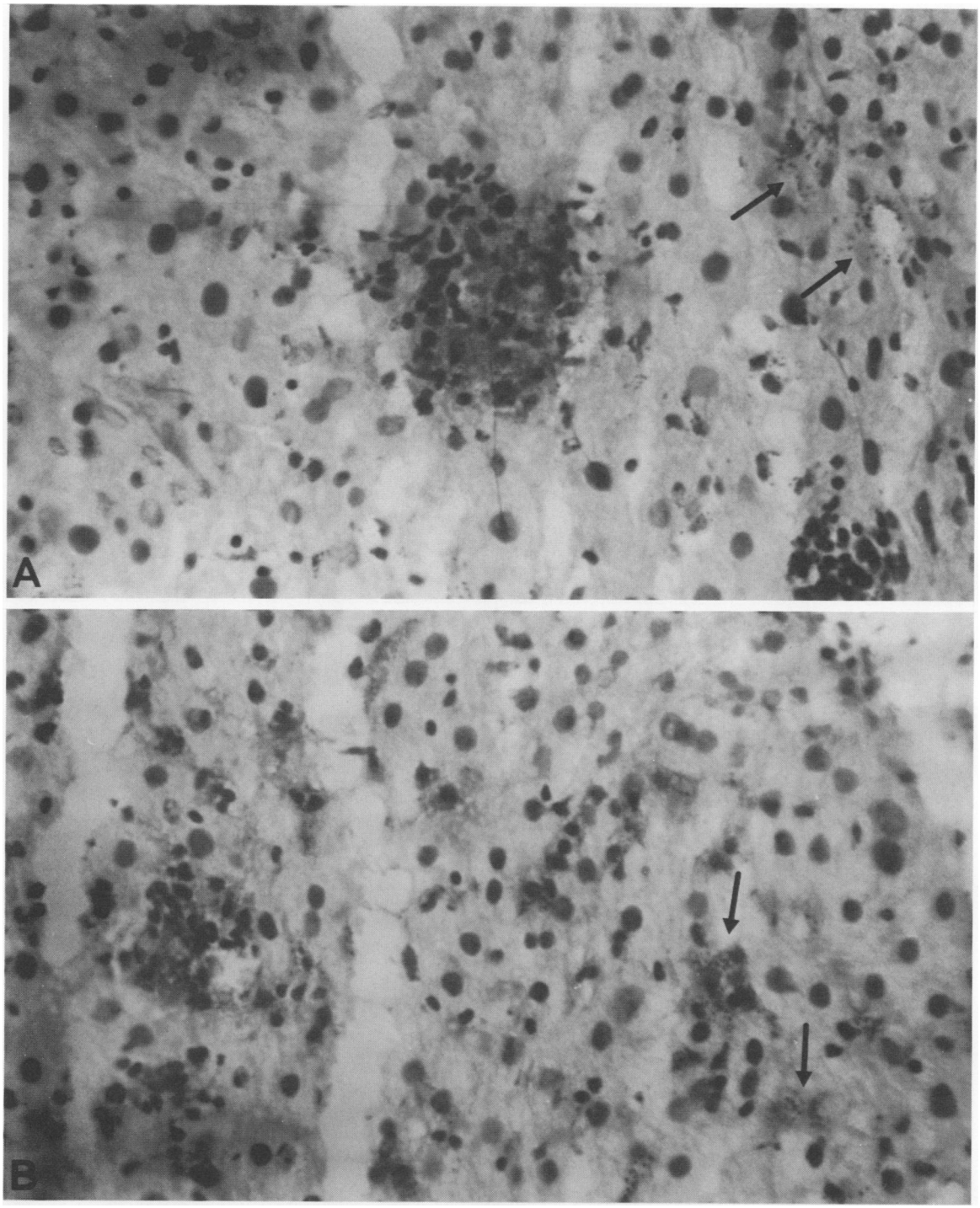

FIG. 4. M1/70 (A) and F4/80 (B) immunostaining of liver sections from B10 (Lsh^s) mice 15 days after infection with *L. donovani* LV9. Many parasites (arrows) can be seen in the M1/70-negative, F4/80-positive resident liver macrophages (Kupffer cells). The sections were counterstained with hematoxylin.

vani-infected B10 mice. Fewer M1/70-positive cells were observed in adherent liver cell populations from *L. major*-infected (B10 × B10.L-Lsh^r)F_1 mice than in B10 (Lsh^s) liver macrophages (Fig. 3), perhaps reflecting some differential growth of the parasite population in the resident liver macrophage population, leading to greater release of amastigotes and a better stimulus for infiltration of fresh M1/70-positive monocytes. This was not reflected in the total parasite count (amastigotes per 100 macrophages) but is consistent with the earlier observation (Fig. 2) of differences between Lsh^s and Lsh^r *L. major*-infected mice after lethal irradiation. At day 7 of infection, 69% of infected liver macrophages from (B10 × B10.L-Lsh^r)F_1 mice were M1/70 positive and contained 70% of the *L. major* amastigote population, compared with only

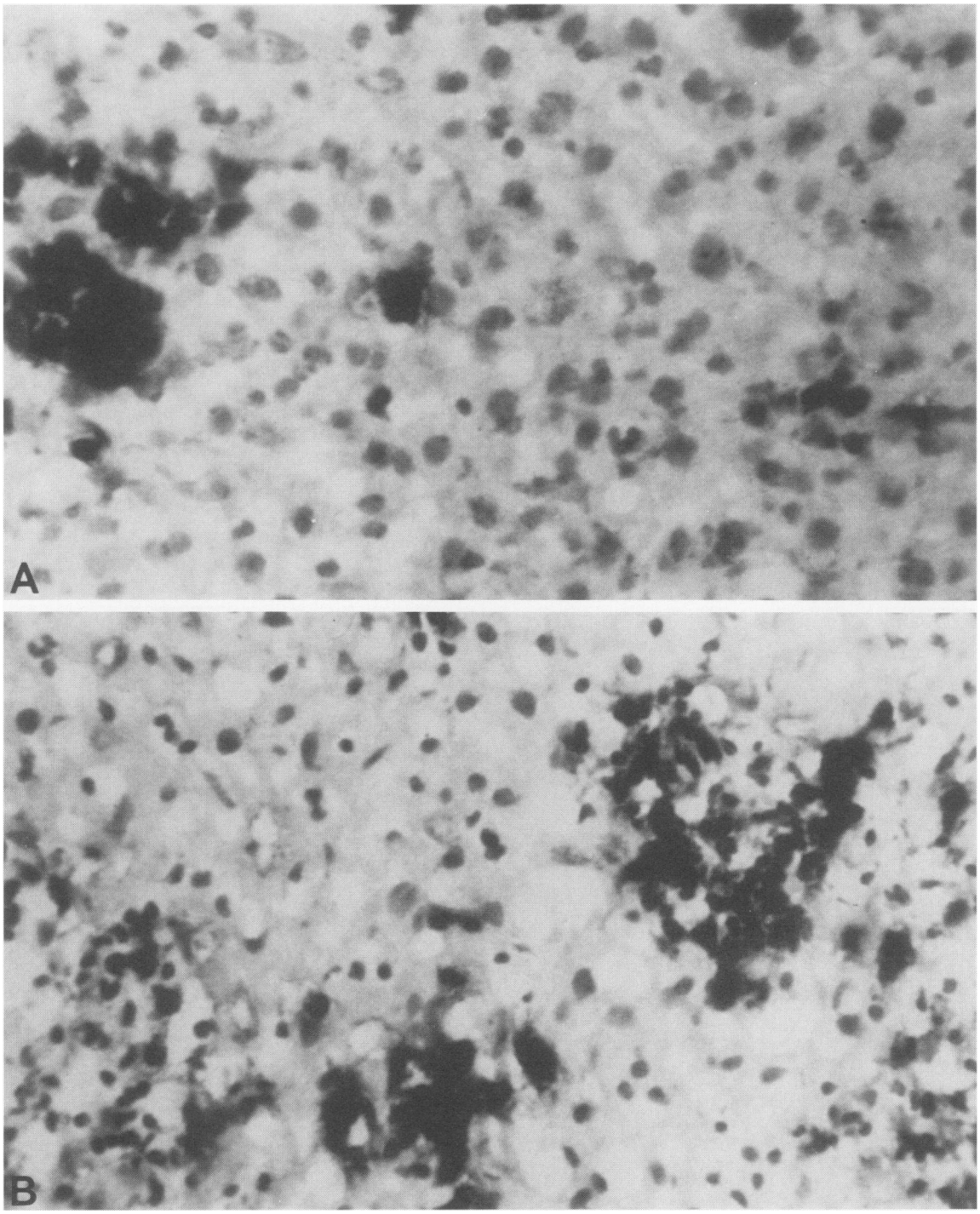

FIG. 5. M1/70-immunostained cryosections of liver from B10 (Lsh^s) mice 7 (A) and 15 (B) days after infection with *L. major* NIH 173. Many large heavily stained M1/70-positive cell foci developed as infection progressed. The same staining patterns were observed in liver sections from (B10 × B10.L-Lsh^r)F_1 mice.

6% M1/70-positive infected macrophages containing 4% of the amastigote population in liver macrophages from *L. donovani*-infected F_1 mice.

DISCUSSION

Recently, we reported (10) that expression of *Lsh*-gene-controlled resistance and susceptibility differs for different macrophage populations isolated and infected with *L. donovani* in vitro. Resident macrophages from liver and lung, as well as bone marrow-derived macrophages matured in adherent culture for 7 days, all supported growth of the parasite population and expressed some degree of *Lsh* resistance in vitro. Splenic macrophages and bone marrow macrophage lines, the latter maintained in continuous culture for 6 weeks, supported a modest increase in the parasite population but failed to express *Lsh*-gene-controlled resis-

tance in vitro. Resident peritoneal macrophages grown in adherent or suspension culture failed to support parasite growth and to express *Lsh* gene resistance. This differential expression of *Lsh*-gene-controlled resistance in, for example, the relatively homogeneous mature resident tissue macrophages of the liver versus the less-mature continuous macrophage line or splenic populations of mixed maturity and function provides a plausible explanation for our inability and that of others (20, 21) to observe *Lsh*-gene-controlled resistance against viscerotropic *L. major* NIH 173 in vivo. Results presented here demonstrate that *L. major* amastigotes both stimulate a higher influx of M1/70-positive fresh monocytes into the liver after infection and grow preferentially in this macrophage population. *L. donovani*, on the other hand, stimulates only a modest monocyte infiltration and grows preferentially in the resident (Kupffer cell) liver macrophage population, which we have shown expresses maximal *Lsh* gene regulation of parasite population growth in resistant mice (10). After lethal irradiation this radioresistant macrophage population appears able to express *Lsh*-gene-controlled resistance against *L. major*. This requires confirmation in vitro. Under normal conditions in vivo, however, *Lsh* gene control is masked by preferential infection of monocytes or young macrophages, characterized by their expression of the type 3 complement receptor, CR3.

The association of *L. major* and immature macrophages or monocytes has previously been described by Fortier et al. (13). Characterization of peritoneal exudate cells from C3H mice after exposure to *L. major* amastigotes in vitro demonstrated that young peroxidase-positive macrophages are more susceptible to infection than are more mature cells. Extraction of peritoneal cells from mice injected with inflammatory reagents also showed that *L. major* has a marked predilection to infect less-differentiated cells. More recently, work by Hoover and Nacy (17) provided evidence that cell immaturity is a major factor in defective intracellular destruction of *L. major* amastigotes by macrophages from inflammatory exudate cells. Their results suggest that the killing mechanism responsible for destruction of this parasite is not fully developed in immature cells.

Further work by Hoover et al. (16) has shown killing of *L. major* amastigotes by the complement-membrane attack complex in a sequence of reactions initiated by the components of the alternative complement pathway. This would result in production of mediators (C3a, C4a, and C5a) causing a local inflammatory response and hence an increased number of M1/70-positive cells. *L. donovani* amastigotes, on the other hand, are poor activators of the alternative complement pathway (4). Hence, they are unable to generate the local inflammatory mediators necessary to attract large numbers of M1/70 (CR3)-positive monocytes into the liver early in infection. Amastigotes of the two species of parasites also differ in their ability to gain entry into the infiltrating monocytes via CR3. Studies with *L. donovani* amastigotes (4) have shown that, unlike promastigotes, they are unable to make significant use of CR3 in gaining entry to macrophages. Equivalent studies with *L. major* NIH 173 have demonstrated up to 50% M1/70 inhibition of serum-independent binding of amastigotes to murine resident peritoneal macrophages (A. M. Cooper and J. M. Blackwell, unpublished results).

The ability of *L. major* to activate complement and elicit a fresh influx of monocytes to the site of infection may be a major adaptive strategy for survival of this species of parasite in the host. Recent studies by Mirkovich et al. (19) have shown that myelopoiesis is increased 60-fold in the spleens of susceptible BALB/c mice infected subcutaneously with *L. major*, thus generating safe targets in which the parasite may survive and multiply. Further studies are required to determine whether increased monocyte infiltration to the sites of visceral growth of *L. major* is also accompanied by an increase in myelopoiesis. Clearly, the two parasites *L. donovani* and *L. major* have adopted quite different strategies for survival and growth which may have important implications in the design of effective control measures to limit parasite growth in the host.

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