THE RELATIVE IMPORTANCE OF BLOOD MONOCYTES AND FIXED MACROPHAGES TO THE EXPRESSION OF CELL-MEDIATED IMMUNITY TO INFECTION*

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(Received for publication 20 April 1970)

Recent publications (1, 2) have shown that the cellular response in the mouse which leads to cell-mediated immunity against infection with *Listeria monocytogenes* includes a vigorous proliferation of mature macrophages in the liver and peritoneal cavity. Since peak macrophage proliferation was found to immediately precede the onset of antimicrobial immunity in the host, it was suggested (2) that the process of division in these cells may be causally related to the increased bactericidal activity acquired by their cytoplasm. There is a good possibility, however, that fixed phagocytes do not make a major contribution to resistance during a primary *Listeria* infection. Even though dividing sinus-lining macrophages can be shown to have acquired increased bactericidal activity during infection (2), the destruction of *L. monocytogenes* is more likely to rest with those cells which accumulate at sites of bacterial implantation; and these might be expected to derive from circulating monocytes rather than from fixed phagocytes. This would mean that the massive division of Kupffer cells is essentially irrelevant to the acquistion of immunity by the liver.

The present study examines these questions and shows that the macrophages which populate infective foci in the liver during a primary *Listeria* infection are derived mainly from cells which circulate in blood; that many of the "fixed" macrophages which are found randomly distributed in the sinusoids of the infected liver have a similar origin; and that the division of fixed phagocytes which occurs most vigorously on the 2nd and 3rd days of a primary *Listeria* infection is not essential for the development of cellular immunity in the liver.

Materials and Methods

Animals.—Specific pathogen-free adult mice were employed. The colony was obtained from Charles River Breeding Laboratories, North Wilmington, Mass. It was maintained in an infection-free environment and fed on a sterile, vitamin-enriched diet.

Bacteria.—*Listeria monocytogenes* (strain EGD) was maintained in a virulent state by repeated passage in mice. It had an intravenous LD_{50} of 5×10^3. Log phase cultures were washed and diluted in 0.9% sodium chloride solution and injected intravenously. Bacteria were enumerated in the liver by plating serial dilutions of liver homogenates on nutrient agar. Mice were infected with approximately 2×10^3 viable organisms.

* This investigation was supported by Grant AI-07015 from the National Institute of Allergy and Infectious Diseases, United States Public Health Service.

Labeling of Cells in DNA Synthesis.—Tritiated thymidine (TdR-^{3}H, 3 Ci/mmole) in 0.9% sodium chloride solution was injected intravenously in the amounts indicated in individual experiments.

Radiometry and Radioautography.—A known weight (approximately 2.5 g) of liver was homogenized in 5% ice-cold trichloroacetic acid (TCA) and washed for 1 hr with two 20-ml volumes of cold TCA. It was then extracted for 1 hr with 6 ml of hot (90°C) TCA. The hot extract was diluted appropriately in 1,4-dioxane containing 0.5% diphenyloxazol and 10% napthalene for counting DNA-^{3}H in a Beckman LS-100 liquid scintillation counter.

Radioautography was carried out with Ilford K5 liquid emulsion (3) on 1–2-μ sections of formalin-fixed tissues embedded in glycol methacrylate (4). Radioautographs were exposed for 2 wk, processed in the usual fashion, and stained with a 1% solution of toluidine blue made alkaline with 1% Tris buffer.

Radiation.—Animals were irradiated with a 250 kv, 15 ma machine at a target distance of 50 cm and with added filtration by 0.5 mm of Cu and 1.0 mm of Al to give a half-value layer of 1.5 mm of Cu. When shielding was needed, it was done with molded lead sheeting of 1 mm thickness. The efficiency of shielding was measured with a thermoluminescence dosimeter using Teflon micro rods containing lithium fluoride (L1 F-7).

RESULTS

Accumulation of Migrant Macrophage Precursors in Infective Foci and in Liver Sinusoids.—Mice were injected intravenously with either 20 μCi or 40 μCi of TdR-^{3}H given as two doses 1 hr apart. 1 hr later they were infected intravenously with a standard dose of *L. monocytogenes*. Five mice were killed, 15 min after infection and at 24-hr intervals thereafter. Pieces of liver were removed for DNA extraction and for radioautography.

The results plotted in Fig. 1 show that infection resulted in a progressive increase over days 2 and 3 in the level of DNA-^{3}H in the liver. No such increase occurred in the livers of uninfected controls. Since TdR-^{3}H was injected before infection and is available for labeling DNA in vivo for less than 30 min after injection, the progressive increase in DNA-^{3}H in the livers of infected mice must have resulted from implausibly efficient re-utilization of label or from an influx and accumulation of cells which were labeled outside the liver at a much earlier time.

Radioautography of liver sections showed that label was present both in macrophages at infective foci (Figs. 5 and 6) and in macrophages lining liver sinusoids (Fig. 7). The labeled sinus-lining macrophages were morphologically indistinguishable from unlabeled Kupffer cells. Their rate of accumulation in the liver ran parallel with the rise in total liver DNA-^{3}H (Fig. 1). In one experiment, as many as 24% of sinus-lining cells were labeled at 72 hr of infection by two 20-μCi injections of TdR-^{3}H given 1 hr before infection. One result of the influx of macrophage precursors into the liver was that on days 3 and 4 of infection there was an obvious increase in the total number of sinus-lining phagocytes. The proportion of labeled macrophages in infective foci also increased during this time.

Radioautography showed that the accumulation of labeled macrophages in infective foci took place at the expense of a reduction in the number of poly-

morphonuclear leukocytes in these sites. In some experiments occasional polymorphs were labeled on the 3rd and 4th days of infection. By this stage, however, the lesions were almost completely dominated by macrophages.

In the liver, the rates of macrophage accumulation and bacterial growth were inversely related (Fig. 1). A progressive accumulation of labeled macrophages

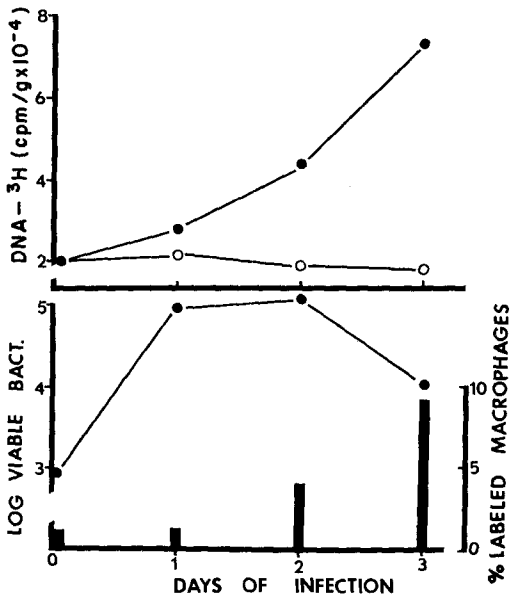

FIG. 1. Influx from blood of labeled macrophage precursors into infected liver. Two 10-μCi injections of TdR-^{3}H 1 hr prior to infection with *L. monocytogenes* resulted in an increase in the level of DNA-^{3}H in the liver during infection (top graph). No such increase took place in the livers of noninfected mice (open circles). Radioautographs showed that increased liver DNA-^{3}H corresponded to increased numbers of labeled macrophages in infective foci and in sinusoids. The progressive increase in liver DNA-^{3}H corresponded to a progressive increase in the per cent of labeled sinus-lining macrophages, as shown in the histogram in the bottom graph. It can also be seen that increased antimicrobial immunity in the liver roughly paralleled increased accumulation of labeled macrophages.

was associated with a parallel increase in the level of host resistance in this organ, as indicated by a downward trend in the bacterial growth curve. Radioautographs showed that the initial phase of rapid bacterial multiplication took place in infective foci dominated by polymorphonuclear leukocytes. The cytoplasm of many of these cells was replete with bacteria, indicating that they play no part in antimicrobial immunity. In contrast, it was difficult to find bacteria when infective foci became populated by macrophages.

Accumulation in Infective Foci and Liver Sinusoids of Migrant Macrophages

Labeled during Infection.—In the above results, the labeled migrant macrophages which accumulated in the liver during infection were generated by the host before infection was initiated. In other words, they were produced under "steady-state" conditions. The following experiments were performed in order

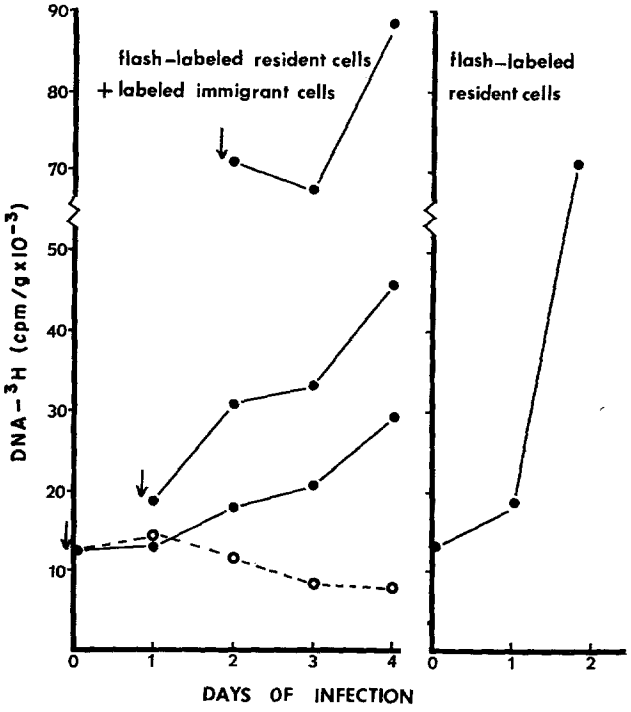

FIG. 2. Labeling of migrant macrophages during infection. A single 20 μCi injection of TdR-^{3}H either just before infection, or at 24 or 48 hr of infection (arrows), resulted in the flash labeling of cells residing in the liver at these times (right-hand graph) and also in a subsequent influx and accumulation of labeled cells from outside the liver (left-hand graph). No such labeling occurred in the liver of noninfected mice (open circles). The label was found in macrophages in infective foci and in macrophages in sinusoids.

to determine whether such cells are produced in a similar fashion during the course of infection.

Mice were infected with *L. monocytogenes* and divided into groups according to whether they received a 20 μCi injection of TdR-^{3}H either 1 hr before infection, or at 24 or 48 hr after initiating infection. Five mice were sacrificed from each group 30 min after each was injected with TdR-^{3}H, and also at 24-hr intervals thereafter. Liver samples were prepared for radiometry and radioautography.

The results plotted in Fig. 2 show that a single pulse of TdR-^{3}H given either just before infection or after 24 or 48 hr always resulted in an immediate labeling of cells already present in the liver (right-hand graph) and in a subsequent influx of labeled cells from an outside source (left-hand graph). The immediate incorporation of TdR-^{3}H from a single pulse increased markedly between 24 and 48 hr of infection. It has been shown already (2) that this represents increased proliferation of fixed phagocytes in liver sinusoids. Radioautography confirmed this conclusion; and it showed, in addition, that the subsequent increase in liver DNA-^{3}H corresponded to an observed increase in the number of labeled macrophages not only in infective foci but in liver sinusoids at large. The results indicate, therefore, that macrophage precursors are continually produced and attracted to liver lesions and sinusoids during *Listeria* infection. It will be seen in Fig. 2 that the accumulation of macrophage precursors was greatest between 72 and 96 hr of infection. This was later than in earlier experiments but not an uncommon occurrence, since the timing and intensity of macrophage accumulation in the liver is influenced by the severity of infection (unpublished results).

Preliminary Estimate of the Extent of Mobilization of New Macrophages during Infection and Their Permanency in the Liver.—The foregoing results show that the infected liver acquires new macrophages in the following two ways: by the division of fixed phagocytes which line sinusoids and by the accumulation of macrophage precursors from blood. The extent to which the infected liver acquires new macrophages is indicated by the results of the following experiment in which migrant macrophages and dividing sinus-lining phagocytes were labeled over 3 days of infection.

A large group of mice were infected with *L. monocytogenes* and injected intravenously with 20 μCi TdR-^{3}H on days 1, 2, and 3 of infection. Five mice were sacrificed on days 5, 12, 19, and 28, and their livers were processed for radiometry and radioautography. Fig. 3 shows that this procedure resulted in a high level of DNA-^{3}H in the liver on day 5 of infection. Radioautography showed that DNA-^{3}H was predominantly in fixed sinus-lining macrophages and in macrophages in infective foci. It will be seen in Fig. 3 that on day 5 about 50% of sinus-lining macrophages were labeled. In addition, counts made of cells in 10 infective foci in the liver of each of five mice showed that an average of 63.4% (range 45–85%) of macrophages were labeled.

A majority of labeled macrophages remained in the liver for only a relatively short time. It can be seen from Fig. 3 that about two-thirds of the total liver DNA-^{3}H was lost between the 5th and 12th days. Radioautography showed that this corresponded to the complete disappearance of infective foci and their complement of labeled cells and to a halving of the percentage of labeled Kupffer cells. It is apparent that this initial rapid loss of labeled phagocytes

was related to the elimination of *L. monocytogenes* from host tissues, because it is found routinely in this laboratory that organisms cannot be detected in the liver and spleen after the 9th or 10th day of infection. The loss of liver DNA-^{3}H was more gradual after the 12th day. In this case, however, loss of

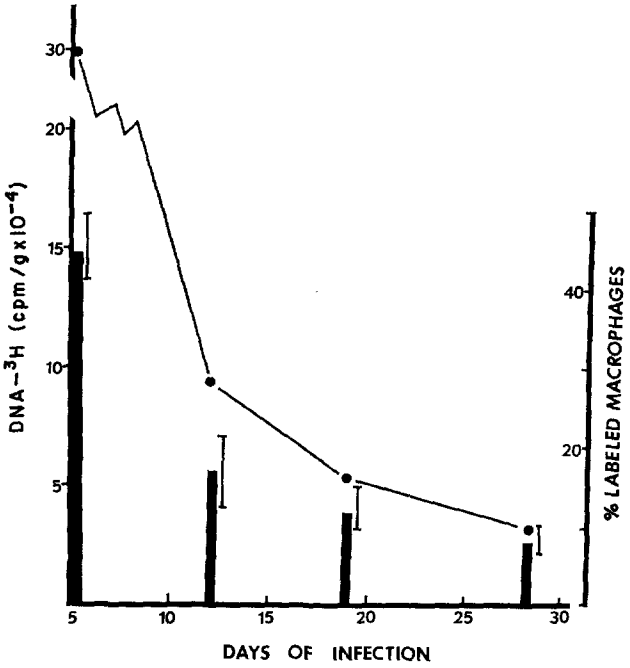

FIG. 3. Disappearance of acquired macrophages population from the liver. TdR-^{3}H was given on days 1, 2, and 3 of infection, and the disappearance of DNA-^{3}H from the liver followed from day 5 by radiometry and radioautography. The initial rapid loss of label between days 5 and 12 corresponded to the disappearance of labeled macrophages from infective foci and also to more than a 50% reduction in the per cent of labeled sinus-lining phagocytes (histogram). This loss was associated with the elimination of the parasite from the host. The slower disappearance of label which followed corresponded to a gradual loss of labeled sinus-lining macrophages.

label was due entirely to a progressive reduction in the percentage of labeled Kupffer cells (Fig. 3).

Evidence that the Division of Kupffer Cells Is Not Essential for the Expression of Cellular Immunity in the Liver.—Previous observations (2) indicated that the increased number of macrophages resulting from division of sinus-lining phagocytes may be important for the expression of cellular immunity in the liver. To test this hypothesis, advantage was taken of preliminary studies which showed that the division of fixed phagocytes in a *Listeria* infection can

be almost completely inhibited by X-irradiating the liver a few hours before infection.

Mice were completely enveloped in sheet lead except for a 2.5 × 2 cm window over the dorsal aspect of the liver. They were then X-irradiated from the dorsal aspect with 300 rads.

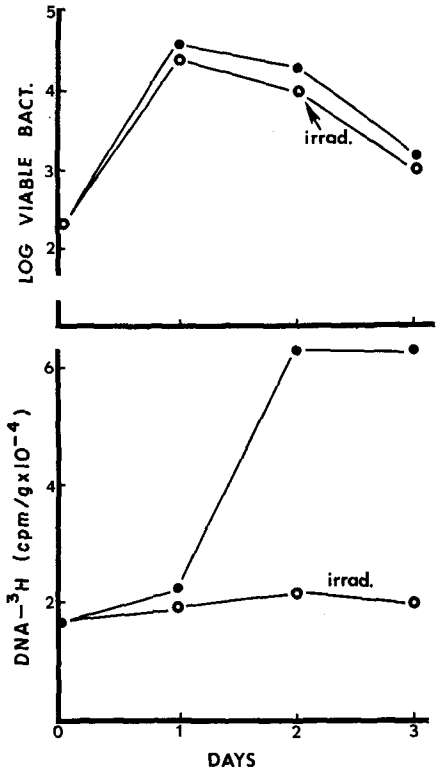

FIG. 4. X-irradiation of the livers of otherwise shielded mice blocked the division of Kupffer cells which occurs maximally on days 2 and 3 of infection as detected by flash-labeling with a single injection of TdR-^{3}H on days 1, 2, and 3 of infection (bottom graph). The top graph shows that the failure of Kupffer cells to divide had no effect on the expression of immunity in this organ.

After the shields had been removed, the animals and two groups of controls were infected intravenously with 10^3 *L. monocytogenes*. The control mice received either whole-body irradiation (300) or no irradiation. The mice of all groups had been splenectomized 2 wk previously in order to eliminate any influence that might result from irradiation of the spleen. The incidence of replicating cells was revealed by pulse labeling with TdR-^{3}H at the time of infection and at intervals of 24 hr. The growth of the *Listeria* in the liver was followed in all groups.

The results plotted in Fig. 4 show that local irradiation of the liver 1–2 hr before infection almost completely blocked the increased incorporation of

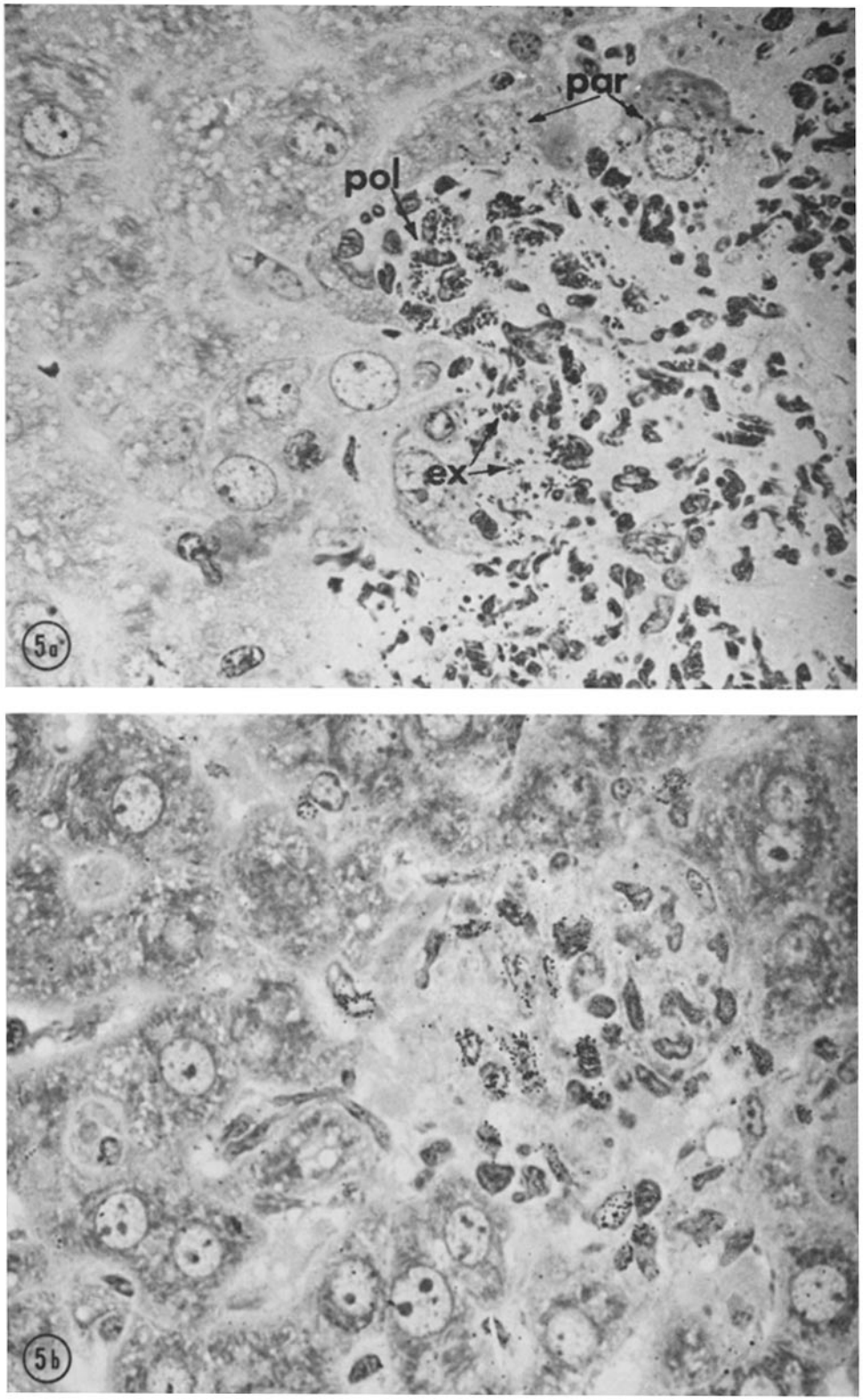

FIG. 5. Radioautographs of infective foci in the liver at (a) 24 hr and at (b) 96 hr of infection. At 24 and 48, infective foci are dominated by polymorphs and contain large numbers of visible bacteria, some of which are extracellular (ex) but most appear in the cytoplasm of polymorphs (Pol). Bacteria have also invaded surrounded parenchyma (Par). In contrast, the 96 hr lesion (b) is dominated by macrophages, many of which contain DNA-^{3}H. The labeled cells in this lesion incorporated TdR-^{3}H from two 20-μCi injections given approximately 1 hr before infection and have migrated from blood. Bacteria can no longer be seen. $\times$ 640.

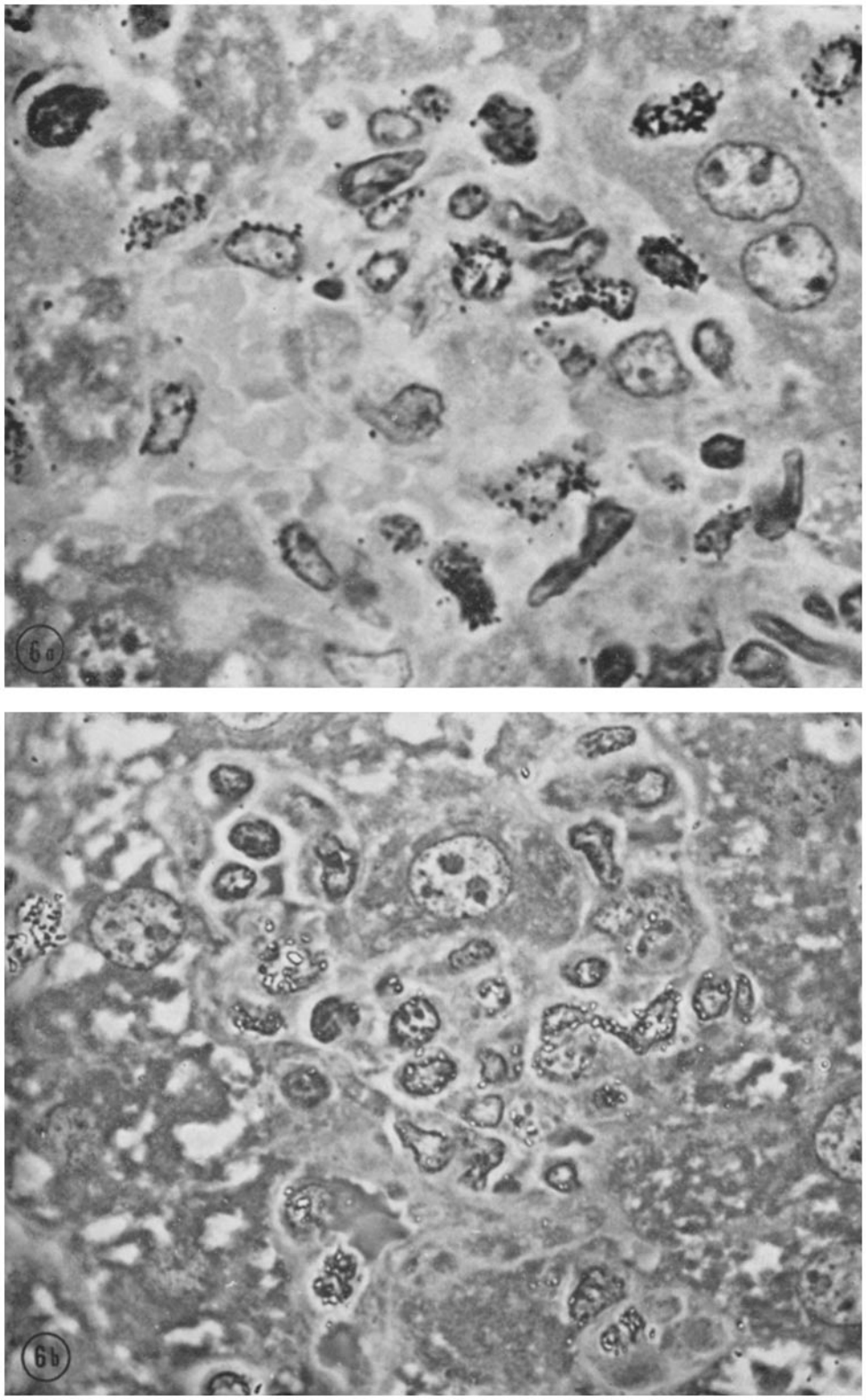

FIG. 6. Selected radioautographs of infective foci at 96 hr of infection, photographed with (a) bright-field optics and (b) phase-contrast. They illustrate the accumulation at infective foci of blood-borne macrophages whose precursors had been labeled with TdR-^{3}H *prior* to infection. The phase-contrast picture shows out-of-focus silver grains as white dots over nuclei. × 1900.

TdR-^{3}H into fixed macrophages which occurred in nonirradiated mice on days 2 and 3 of infection. It will be seen, however, that the absence of Kupffer-cell division did not reduce the capacity of the liver to develop resistance to the infection, indicating that the division of Kupffer cells is not an essential step in the development of cellular resistance to this infection.

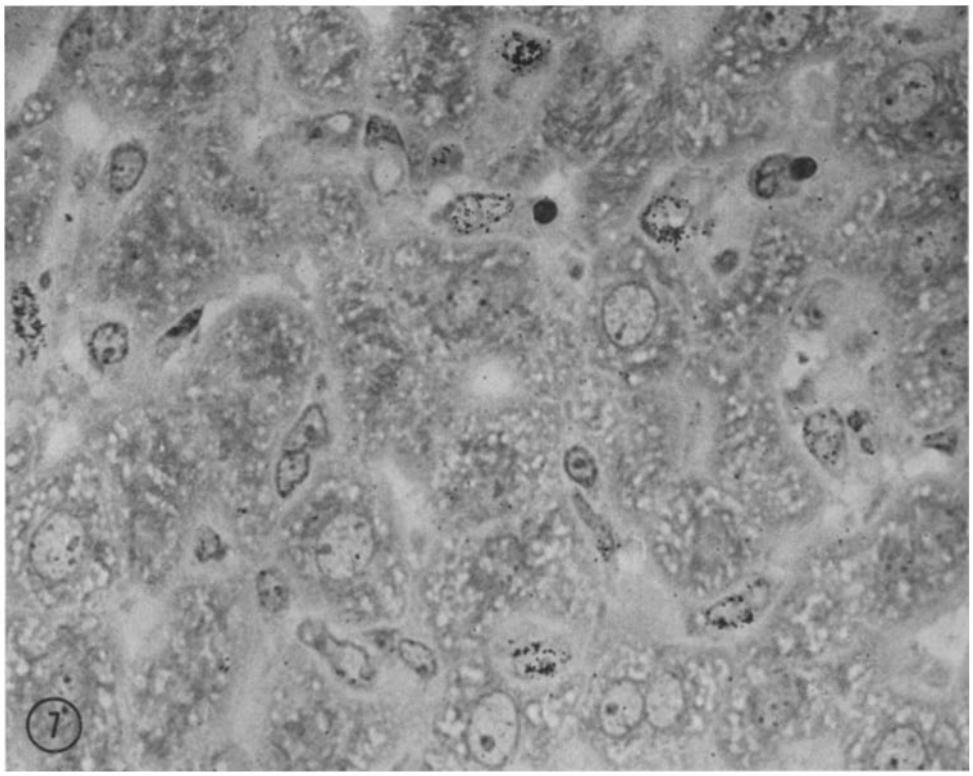

FIG. 7. Radioautograph of liver showing labeled sinus-lining macrophages on day 4 of a *Listeria* infection. They were derived from precursors which had been labeled by two 20-μCi injections of TdR-^{3}H given approximately 1 hr *before* infection. The labeled cells and an indeterminate number of unlabeled cells must have arisen outside the liver, and they have taken up residence in the sinusoids between days 2 and 4 of infection. × 640.

The foregoing experiment also showed that total-body X-irradiation completely suppressed the development of immunity in the liver, and that splenectomy does not interfere with the host's capacity to respond to a *Listeria* infection. These findings are part of a more extensive investigation of the influence of irradiation on cell-mediated immunity and will be discussed in a forthcoming report.

Evidence that X-Irradiation has a Direct Effect on the Division of Kupffer Cells.—It was possible to show that X-irradiation has a direct effect on the division of Kupffer cells. This was achieved by shielding all but a small window over the dorsal right-hand side of the liver. After receiving 300 rads of X-irradiation dorsally, the mice were infected with *L. monocytogenes*. 2 days later they were flash-labeled with 20 μCi TdR-^{3}H. The extreme right and left sides of the liver were then removed for radiometry and radioautography.

Table I shows that on day 2 of infection, the shielded side of the liver incorporated 4–5 times as much TdR-^{3}H as the irradiated side. Radioautography showed no histological differences between exposed and shielded tissue except that the latter contained an average of 16% labeled Kupffer cells, whereas ir-

TABLE I

Suppression of Kupffer Cell Division on Day 2 of Infection by X-Irradiating Liver Before Infection

Animal	DNA-^{3}H (control values subtracted)	
	Irradiated side	Shielded side
	cpm/g $\times$ 10^{-3}	
A	13.2	67.8
B	19.6	71.8
C	10.6	60.2
D	13.2	67.8

Mice were shielded except for window over dorsal right-hand side of liver. Kupffer cells in irradiated-side of the liver incorporated much less TdR-^{3}H than those in shielded liver.

radiated liver was virtually devoid of labeled cells. It is obvious, therefore, that this dose of X-ray has a direct effect on the mitotic potential of Kupffer cells.

DISCUSSION

Acquired immunity to a primary *Listeria* infection is expressed by the host at a time when macrophages replace polymorphonuclear leukocytes in infective foci in the liver and spleen (5). Coincident with the domination of infective foci by macrophages is a systemic emergence of macrophages with increased physiological and microbicidal potential (1, 2). These changes follow a wave of vigorous proliferation both of fixed phagocytes lining the sinusoids of the liver and of free resident macrophages in the peritoneal cavity (1, 2). The expression of immunity to this infection, therefore, is concomitant not only with the activation of individual macrophages (5), but also with a substantial augmentation of their number as a result of local division.

The present study shows that the infected liver also acquires a substantial number of macrophages from the blood. These accumulate in sinusoids and in infective foci. The origin of these cells was not demonstrated in the present ex-

periments, but their labeling characteristics indicate that they are blood monocytes. The latter are known to be the progeny of rapidly dividing cells in bone marrow (6, 7) and to be constantly supplied to the circulation for delivery to sites of inflammation. The rate at which labeled monocytes are released into the blood and their half-life in circulation (7) are consistent with the observed build-up of extrahepatic phagocytes in the infected liver. It seems clear, therefore, that the cells which populate infective foci during a *Listeria* infection are predominantly bone marrow-derived blood monocytes. The fact that certain cells in thoracic duct lymph can also give rise to liver macrophages (8) does not contradict this view; it merely draws attention to the fact that we may not yet be able to recognize all the morphological forms of the circulating precursors of macrophages.

The conclusion that circulating monocytes are of preeminent importance to the development of local resistance receives indirect support from the finding that proliferation of fixed phagocytes in the liver is not essential for the expression of host immunity to a primary *Listeria* infection. Suppressing the proliferation of this cell population by ionizing radiation did not disturb the development of resistance in the liver, even though their proliferation normally results in an increased number of liver macrophages with enhanced bactericidal ability (2). Presumably, then, immunity to a primary infection is expressed by the migrant macrophages which predominate in infective foci. Even so, this result does not mean that the division of Kupffer cells is without significance. On the contrary, the presence of large numbers of activated macrophages in liver sinusoids could help to explain why convalescent mice are able to resist a large challenge infection with *L. monocytogenes* and other organisms (5, 9). They are well equipped, therefore, to terminate the bacteremia which is a constant feature of the early phases of a *Listeria* infection.

The substantial accumulation of blood monocytes in liver sinusoids and their maturation into cells which are indistinguishable from Kupffer cells by light optics are further reasons for the increased number of sinus-lining macrophages observed after the 2nd day of a *Listeria* infection. It has yet to be determined whether this represents an exaggeration of the normal replacement mechanism for Kupffer cells or whether it is merely a stage in the migration of blood monocytes to infective foci.

Most of the new macrophages acquired by the liver during infection have only a relatively short half-life in this organ. Once the parasite is eradicated from the tissues of the host, there is a rapid loss of labeled macrophages both from infective foci and from sinusoids. However, a certain proportion of the newly acquired sinus macrophage population is lost at a much slower rate. Experiments are now in progress to determine whether the cells which disappear first are the monocyte-derived macrophages, and those which are lost more slowly are the ones derived by Kupffer-cell division.

The relation between massive macrophage mobilization and other components of the host response deserves comment. There is now good reason to believe that immunity to *L. monocytogenes*, as expressed by activated macrophages, is mediated by an acquired population of specifically sensitized lymphoid cells. It has been shown, for example (10), that the recipients of an infusion of sensitized lymphoid cells from *Listeria*-immune donors quickly acquire a high level of antimicrobial immunity. Evidence for the production of sensitized lymphoid cells during a primary *Listeria* infection is furnished by experiments (1) which show that the onset of acquired cellular immunity is preceded by massive lymphoid cell proliferation in the spleen and by the development of specific delayed-type hypersensitivity. It is probable, therefore, that increased mobilization of host macrophages during infection is triggered and sustained by factors produced by a specific immunological component of the host response. This subject will be discussed in a forthcoming report.

SUMMARY

Infection in mice with *Listeria monocytogenes* results in a substantial accumulation of migrant macrophages in the liver. The immigrant cells populate both the infective foci and intervening sinusoids. They have the labeling characteristics of blood monocytes, and their appearance in infective foci in the liver corresponds to the expression of a high level of antimicrobial immunity in this organ. The infected liver acquires additional new macrophages by Kupffer-cell division. The proliferation of these cells, however, is not essential for the expression of immunity in the liver. The results indicate that the macrophages which express immunity to a primary infection with *L. monocytogenes* are those derived from circulating monocytes. Most of these cells are quickly lost once the parasite is eliminated from the tissues.

I wish to thank Mr. Jon Deissler and Mrs. Sandra Lee Warner for expert technical assistance.

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