THE MITOTIC POTENTIAL OF FIXED PHAGOCYTES IN THE LIVER AS REVEALED DURING THE DEVELOPMENT OF CELLULAR IMMUNITY*

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There is convincing evidence that immunity to facultative intracellular parasites depends on the emergence during infection of a population of host macrophages with enhanced bactericidal ability (1, 2). It is not known, however, whether the macrophages responsible for immunity are new cells with different properties, or preexisting macrophages which have undergone metabolic modification.

If new macrophages are needed for successful immunity against intracellular parasites, they may originate in two ways: by maturation in infective foci of emigrant bone marrow–derived blood monocytes (3, 4), or by the local division of mature tissue macrophages. Recent studies on mice infected with *Listeria monocytogenes* or with *Bacillus* Calmette-Guérin (BCG) (5) have shown that the host response to these organisms includes a vigorous proliferation of mature resident macrophages in the peritoneal cavity. Furthermore, the period of peritoneal macrophage proliferation showed a constant temporal relationship to other components of the host response, and to the onset of antibacterial immunity. It was predicted, therefore, that during an infection with either BCG or *L. monocytogenes*, fixed macrophages in the liver should also proliferate in concert with those of the peritoneal cavity.

This study represents the first of a series of investigations on the role of macrophage division in the development of cell-mediated immunity. It will show that during an infection with *L. monocytogenes* fixed macrophages in the liver divide in large numbers, and that the act of division immediately precedes the onset of an effective level of antimicrobial immunity.

Materials and Methods

Animals and Bacteria.—The mice used were the same as those described in a preceding study (5). The preparation of *L. monocytogenes* for injection and the method used for enumerating it in the livers of infected mice have also been described (5). Sublethal infections were initiated by injecting approximately 2×10^3 viable organisms intravenously.

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Test for Bactericidal Ability of Liver Macrophages.—Mice were infected with *L. monocytogenes*, and on each day of the infection five mice were challenged intravenously with approximately 10^6 viable *Salmonella typhimurium*. This organism was taken from log phase cultures in Trypticase soy broth and diluted appropriately in 0.9% sodium chloride solution. The number of organisms in the liver after 30 min, 1 hr, and 2½ hr was determined by plating serial dilutions of liver homogenates. Preliminary experiments showed that *S. typhimurium* was effectively cleared from the blood within 10 min. Hence, the difference between the number of *S. typhimurium* in the liver at 30 min and 2½ hr was taken as an indicator of the bactericidal ability of liver macrophages.

Proporiton of Macrophages Synthesizing DNA.—On each day of a primary *Listeria* infection, four mice were each injected with 20 μc of tritiated thymidine (^{3}H-TdR). 30 min later their livers were removed, cut into 2–4 mm pieces, and fixed for 18 hr in 10% formaldehyde solution neutralized with 0.1 M sodium cacodylate. In later experiments the livers were perfused with fixative via the portal vein before being diced and fixed as above. The fixed tissue was washed for 8 hr in tap water, dehydrated in ethanol, and embedded in glycol methacrylate (6). Sections 1 μ thick were cut on a Porter-Blum microtome with glass knives, and the sections were radioautographed for light microscopy (7). They were exposed for 2 wk; after processing they were stained with 1% toluidine blue made alkaline with 1% Tris buffer, and counterstained with 1% basic fuchsin or 1% crystal violet. The proportion of labeled cells lining the liver sinusoids was determined by counts made with an oil immersion objective. Only those cell profiles containing a nucleus were counted.

Estimating Macrophage Mitosis with an Antimitotic Drug.—Mice were injected at different stages of a *Listeria* infection with 100 μg of the *Vinca* alkaloid vinblastine sulfate (Velban, Eli Lilly and Co., Indianapolis, Ind.) dissolved in 0.9% sodium chloride solution. This drug irreversibly blocks mitosis at metaphase (8). 3 hr after the drug was administered intravenously the mice were killed, and their livers were fixed, embedded, and sectioned as described above. Counts were made of the number of mitotic figures in liver sinusoids per microscopic field, using a 40× objective. Eight fields were counted for each mouse, and four mice were used for each time point studied.

Use of a Physiological Marker to Identify Macrophages.—Individuals of a large group of mice were injected with 0.2 ml of 10% India ink (Pelikan C11/1431a, Gunther Wagner, West Germany) suspended in 0.9% sodium chloride solution. 3 wk later they were infected with 2×10^3 *L. monocytogenes*, and on the second day of the infection were pulsed with 20 μc of ^{3}H-TdR or given 10 μc of ^{3}H-TdR every 2 hr for 8 hr. The livers were fixed 30 min after the last injection of ^{3}H-TdR, and 1 μ sections were prepared and radioautographed. The sections were examined to determine whether cells which contained carbon also incorporated ^{3}H-TdR into their nuclei.

Mice which had been injected with carbon were also used to determine whether cells arrested in mitosis carried the physiological marker.

Radiometric Measurement of DNA Synthesis in Liver.—A group of mice were infected with *L. monocytogenes*, and on each day of the infection four mice were each injected with 20 μc of ^{3}H-TdR. 30 min later part of the liver was removed, weighed, and homogenized in 5 ml of 5% ice-cold trichloroacetic acid (TCA). The homogenate was washed twice in ice-cold TCA over a period of 60 min and then subjected to two hot extractions (90°C) with TCA. The two hot extracts were pooled and diluted in 1,4-dioxane containing 0.5% 2,5-diphenyloxazole and 10% naphthalene. The ^{3}H-DNA content was counted in a Beckman LS-100 liquid scintillation counter programed to count to a 5% error. The results were expressed as counts per minute per gram of liver, wet weight.

RESULTS

DNA Synthesis by Cells in Liver Sinusoids during a Listeria Infection.—An infection with *L. monocytogenes* caused large numbers of cells lining the sinusoids

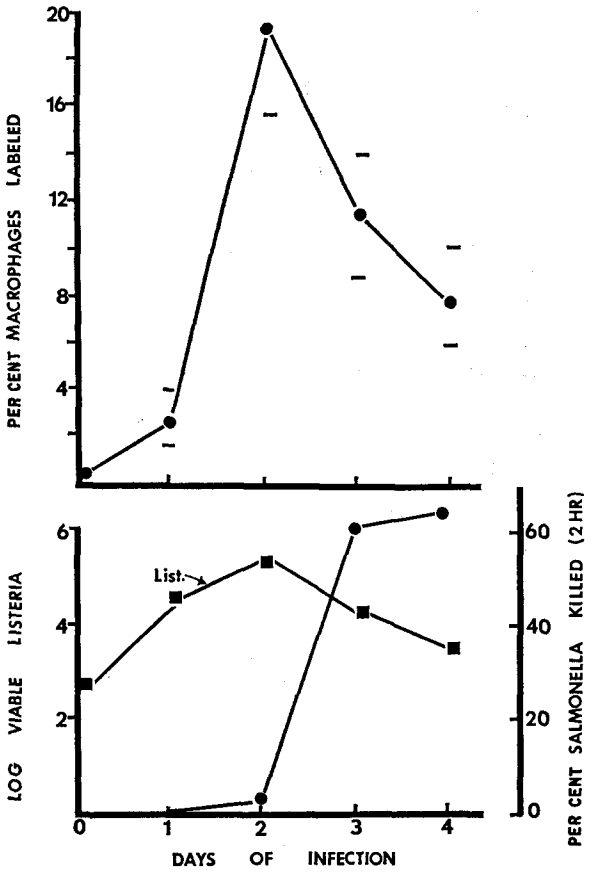

FIG. 1. Percentage of sinusoid-lining cells which incorporate ^{3}H-TdR from a single pulse on different days of a *Listeria* infection. The bottom graph shows both the growth curve of the infecting organism and changes in the ability of liver phagocytes to kill *S. typhimurium* over a 2½ hr period. The experiments were carried out concurrently.

of the liver to incorporate ^{3}H-TdR. Practically none of these cells could be labeled by a pulse of ^{3}H-TdR given at the beginning of the infection (Fig. 1), but by 24 hr the numbers which incorporated label rose to 2%, and by 48 hr an average of 18% of sinusoid-lining cells were labeled by a single pulse of ^{3}H-

TdR. The proportion which labeled then declined progressively until the 4th day of the infection, when the experiment was terminated.

The cells which incorporated ^{3}H-TdR marginated cross-sectional profiles of the liver sinusoids. They were flat cells and were less conspicuous than the larger parenchymal cells, which rarely incorporated label. A description of the

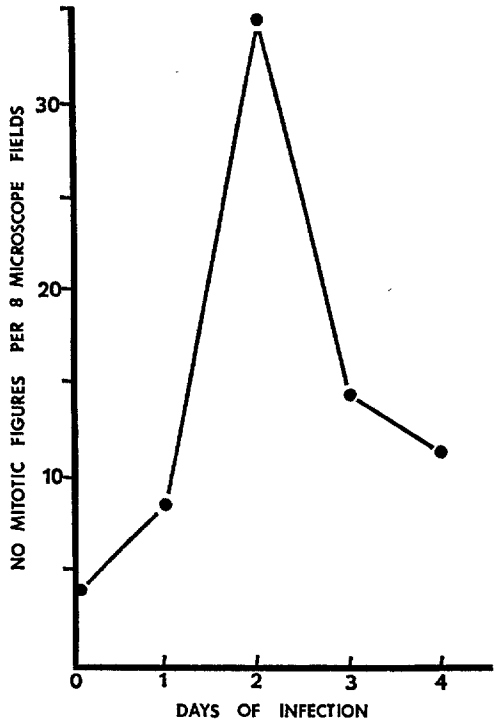

FIG. 2. Number of mitotic figures in liver sections counted in eight microscopic fields. Mice were injected with 100 μg vinblastine 4 hr before sacrifice. All mitotic figures were in liver sinusoids.

labeled cells will be given below, where it will be shown that they are the fixed phagocytes, or Kupffer cells.

Temporal Relation between DNA Synthesis by Cells in the Liver and the Onset of Cellular Resistance.—It will be seen in Fig. 1 that the peak labeling of cells lining the liver sinusoids was closely followed by the active elimination of *L. monocytogenes* from the liver. Fig. 1 also shows that the ability of phagocytic cells in the liver to kill a challenge dose of *S. typhimurium* increased between the 2nd and 3rd days of the *Listeria* infection, i.e. soon after the peak labeling of cells lining liver sinusoids.

Development of Mitotic Figures in Liver Sinusoids during Infection.—Listeria-

infected mice which were injected with vinblastine at different stages of the infection and sacrificed 4 hr later showed that the infection resulted in an increase in the number of mitotic figures per unit area of liver sections. This can be seen in Fig. 2, which shows that the number of mitotic figures per microscopic field increased 7-fold over the first 48 hr. The graph showing changes in the number of mitotic figures almost parallels the graph showing changes in the proportion of cells which incorporated ^{3}H-TdR (Fig. 1). More than 99.9% of the mitotic figures were in sinusoids.

Detection of the Macrophage Mitotic Response with a DNA Extract of Liver.—The above results showed that during a *Listeria* infection large numbers of liver macrophages synthesized DNA. The results showed also that there was practically no DNA synthesis on the part of parenchymal cells. It was anticipated, therefore, that the mitotic response of liver macrophages could be more easily monitored by radiometric measurement of ^{3}H-DNA in a DNA extract of liver.

Changes in the relative amounts of ^{3}H-TdR incorporated into DNA in liver at different stages of the infection are shown in Fig. 3. It can be seen that the rise and fall in the amount of ^{3}H-TdR incorporated into total DNA during the infection almost paralleled the rise and fall in the proportion of macrophages labeled in radioautographs of liver sections from the same animals, thus confirming the impression that the mitotic response in the liver is confined to cells of the liver sinusoids.

Evidence That the Cells Which Synthesize DNA are Fixed Phagocytes.—When mice were injected intravenously with colloidal carbon, many of the cells lining the liver sinusoids contained ingested carbon 3 wk later (Figs. 4 and 5). They were, by definition, fixed phagocytes. When these mice were later infected with *L. monocytogenes*, at least 69% of the cells in the liver which incorporated ^{3}H-TdR into their nuclei on the 2nd day of the infection also contained carbon (Table I). In addition, those radioactively labeled cells which did not contain carbon had the same characteristics as those that did. Furthermore, an examination of smears of blood taken from mice at progressive stages of the infection showed no evidence of carbon-containing cells in the circulation. It is reasonable to conclude, therefore, that the cells in the liver which incorporated ^{3}H-TdR were fixed phagocytes which were present in the liver before the infection was initiated. DNA synthesis by these cells was not due to the presence in them of ingested carbon, because carbon-containing cells in the liver of uninfected mice did not incorporate label (Table I). In order to interpret the results presented in Table I, it should be noted that only those cell profiles containing a nucleus were included in the calculations. In fact, many more than 37% of the macrophages contained carbon. Another consequence of the use of sections was that many of the radioactively labeled macrophages which did not show ingested carbon probably did contain it in a portion of the cytoplasm lying beyond the plane of section.

The above conclusions were reinforced by the finding that mice which had been first injected with carbon and then infected with *L. monocytogenes* showed many carbon-containing cells in mitosis in the liver on the 2nd day of the

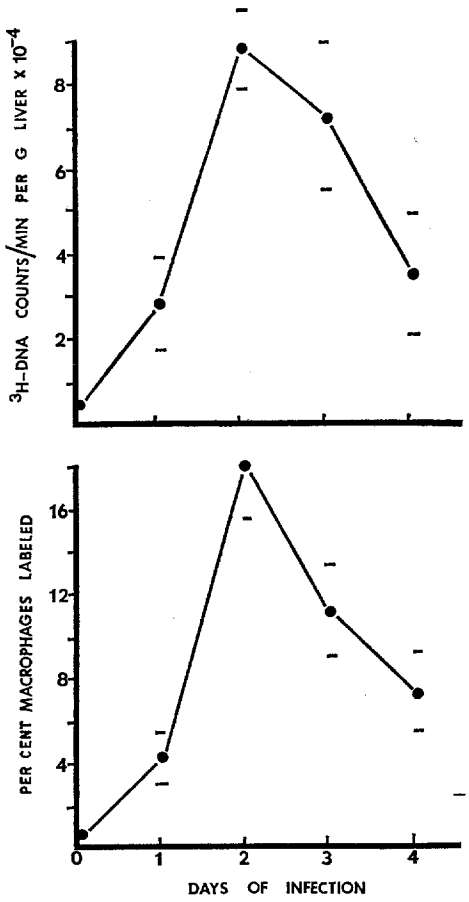

FIG. 3. Changes in the amount of ^{3}H-TdR incorporated into total liver DNA at different stages of a *Listeria* infection. The bottom graph shows changes in the proportion of sinusoid-lining cells labeled in the same livers. The mice were given a single pulse of ^{3}H-TdR.

infection. The number of carbon-containing cells in the act of mitosis was increased substantially in animals injected with vinblastine.

Ingested carbon was very easy to see and was easily distinguished from silver grains over nuclei which were in a different focal plane. The carbon was always segregated in large phagosomes, which showed a yellow-brown color in transmitted light. This was probably due to the interspersion of carbon with lysosomal protein.

It should be pointed out that the fixed phagocytes in the liver were larger in mice which were first injected with carbon and then infected with *L. monocytogenes*. Part of the increase in size was due to the carbon injection alone, because uninfected control mice also had larger than normal fixed phagocytes. However, cell size also increased as a consequence of infection, because it occurred in the cells of infected mice which had not been injected with carbon.

It should be mentioned that the mitosis of liver macrophages was accompanied by a rounding-up of these cells. Rounding-up did not occur during

TABLE I

*Percentage of Carbon-Containing Cells in Liver Sinusoids Labeled by Four Injections of ^{3}H-TdR on the 2nd Day of a Listeria Infection**

Animals (carbon injected)	Sinus cells containing		
	Cytoplasmic marker	Nuclear label	Cytoplasmic marker + nuclear label
	% ± SD	% ± SD	% ± SD
4 infected mice	37 ± 6.3	29 ± 3.2	20 ± 1.4
4 uninfected mice	38 ± 5.3	1.4	1.2

* Carbon was injected 3 wk before infection with *L. monocytogenes*.

prophase, but many cells in metaphase and anaphase had a spherical appearance. Nevertheless, such cells always marginated the sinusoids and were opposed to the parenchyma cells by a flat side.

DISCUSSION

This study, like that published from another laboratory (9), shows unequivocally that the fixed macrophage population of the liver is capable of vigorous proliferative activity. It also shows that the timing of liver macrophage proliferation during a sublethal *Listeria* infection can be accurately predicted, and that peak macrophage proliferation always precedes the acquisition by the host of an effective antibacterial immunity. When the present findings are compared with those of a previous study (5), it is apparent that the timing of macrophage proliferation in the liver corresponds to the timing of macrophage proliferation in the free phagocytes of the peritoneal cavity. In both cases the cells which divide are mature macrophages which are present in these tissues before the infection is initiated. Furthermore, because a peritoneal macrophage mitotic response also occurs in mice infected with BCG, it is suggested that the simultaneous mitosis of macrophages in the liver and peritoneal cavity of *Listeria*-infected mice is not the result of a mitogenic factor released from *L. monocytogenes*, but may represent a special feature of the host's immune response to infection by organisms which can survive initially inside phagocytic cells and which are capable of provoking a state of delayed-type hypersensitiv-

ity. The latter is still a speculative view, but is supported by the observation[1] that mitosis occurs earlier in mice which have been passively sensitized by lymphoid cells from a *Listeria*-immune donor.

A causal relationship between macrophage division and the subsequent increase in the bactericidal activity found in infected mice has not been established by the present investigation. However, the large proportion of cells which can be labeled by a single pulse of ^{3}H-TdR makes it almost certain that most of the macrophages in the liver and peritoneal cavity of mice 3–4 days after infection with *L. monocytogenes* are recently divided cells. This leads to the interesting possibility that the stimulus which provides for increased bactericidal activity also causes the cell to engage in the synthesis of DNA. Alternatively, it could be imagined that macrophages which become synthetically active in preparation for division acquire enhanced microbicidal properties as a consequence of their enlarged synthetic activity.

The present evidence for a major mitotic response on the part of fixed phagocytes during a bacterial infection must be reconciled with convincing evidence (3, 4) that the mononuclear phagocytes which populate regions of sterile inflammation are emigrant bone marrow–derived blood monocytes. It may well be asked whether cells with a similar origin come to dominate the lesions developed during a *Listeria* infection. The answer to this question is important, because host resistance during a primary infection is expressed initially within lesions (10). Although blood monocytes may migrate into lesions and thereby contribute to host resistance, there is little doubt from this study that at least some of the macrophages which eventually populate an infective focus are local tissue macrophages which have replicated *in situ* and have then migrated into the lesions, for it was observed in infected animals previously injected with carbon that some of the cells which accumulated in the lesions contained carbon. Moreover, some carbon-containing cells within lesions became labeled by a 30 min pulse of ^{3}H-TdR.

The present evidence is therefore in keeping with the conclusions reached many years ago by Evans et al. (11), who observed in tuberculosis-infected rabbits that Kupffer cells divide and populate infective foci in liver sinusoids.

SUMMARY

Fixed macrophages in the liver of the mouse undergo mitosis in large numbers during an infection with *Listeria monocytogenes*. The macrophage mitotic response always precedes the expression of efficient host immunity to infection. It is suggested that macrophage proliferation is an important event in the development of host immunity to *L. monocytogenes*.

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[1] Mackaness, G. B., and R. J. North. Results to be published.

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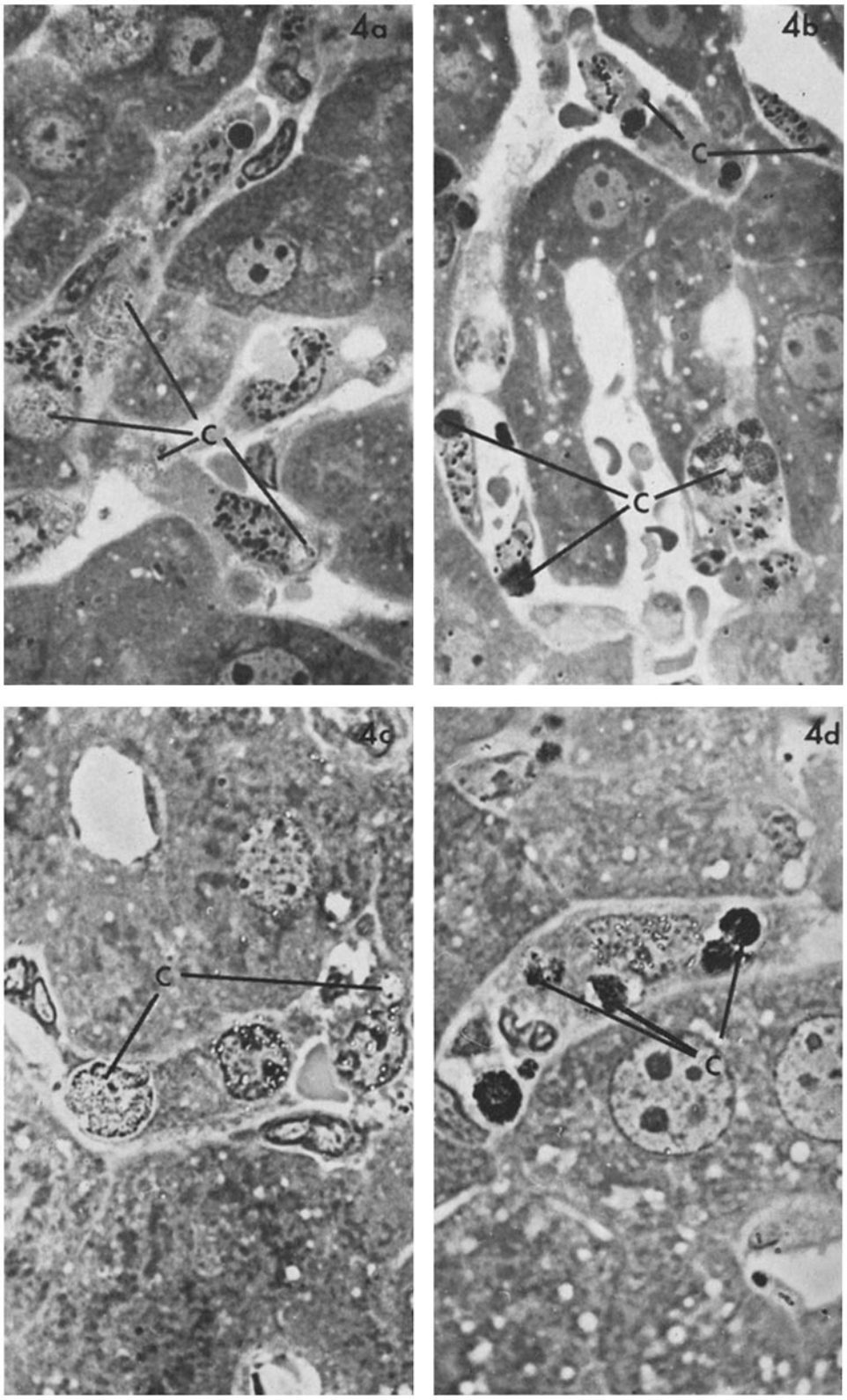
FIG. 4. Radioautographs of carbon-containing cells in liver sinusoids labeled after four injections of ^{3}H-TdR on the 2nd day of a *Listeria* infection. The carbon (*C*) is in large phagosomes and was injected into the mice 3 wk prior to infection. a and b are bright-field images. × 1240. c and d were photographed with phase contrast and show out-of-focus silver grains (white) over chromatin clumps. × 1860.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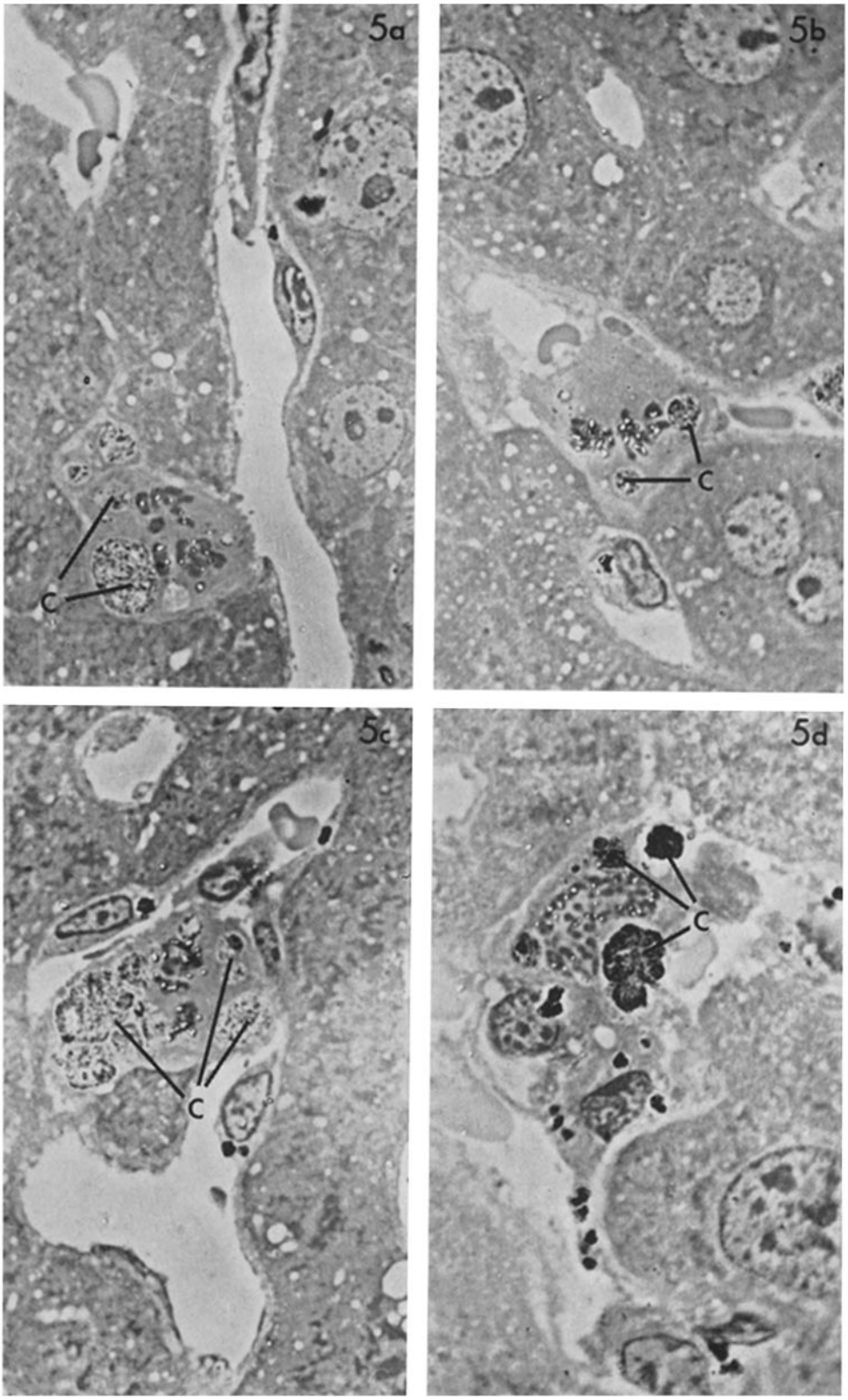

FIG. 5. Radioautographs of the same material displayed in Fig. 4, showing carbon-containing cells (*C*) in liver sinusoids at different stages of mitosis. The out-of-focus silver grains are seen as white dots directly over chromosomes. Phase contrast; × 1860.