

# THE ORIGIN, KINETICS, AND CHARACTERISTICS OF THE KUPFFER CELLS IN THE NORMAL STEADY STATE

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The Kupffer cells of the liver are one of the fixed tissue macrophages of the mononuclear phagocyte system (1), and are probably the most numerous. It has been shown that the Kupffer cells are responsible for clearing the blood stream of a high proportion of injected particles or colloids and the kinetics of this clearance have been extensively studied (2).

There are two theories on the origin of the Kupffer cells in the normal steady state. The first is that they form a self-contained population of cells proliferating locally (3-5). The second is that the Kupffer cell is an end cell incapable of division in the normal steady state and originates from the bone marrow (6-11).

Under some experimental conditions Kupffer cells were derived from thoracic duct lymphocytes, (6, 7) but this was not confirmed in similar experiments by Bell and Shand (12).

In the normal steady state up to 1.5% of the Kupffer cells incorporate radioactive thymidine or are in mitosis at any one time (3, 5, 10, 13-16). When the Kupffer cells are stimulated there is evidence for an influx of cells from the bone marrow into the liver and there are indications that also local proliferation may occur depending on the agent used (3, 6-8, 11, 13).

Cytochemically the Kupffer cell has been shown to possess a diffuse cytoplasmic esterase activity (17, 18) while peroxidase activity is found in the rough endoplasmic reticulum and nuclear envelope but not in the granules (4, 18-21).

Human, guinea-pig, and rat Kupffer cells possess Fc and C3 receptors (22-24) and the lysosomes of the rat Kupffer cells contain similar quantities of acid hydrolases as the lysosomes of peritoneal macrophages (25).

Most work done so far on the Kupffer cell has used histological sections but this is a rather unsatisfactory method. Only recently have satisfactory methods of isolating Kupffer cells in suspension become available. Early attempts relied on magnetic and mechanical methods (26) which have now been superseded by enzymatic ones (23, 27-30). A combination of pronase and DNAase, first used by Bissell et al. (20) has been shown to give the best yield and to cause little damage to the Kupffer cells (18, 30, 31). This is the method used in the present study.

The aim of the work reported here was to isolate and characterize the Kupffer cells *in vitro*, to determine their origin in normal animals, to study their kinetics, and to obtain information about the turnover of Kupffer cells during the normal steady state.

## Materials and Methods

*Animals.* Specific pathogen-free (SPF)<sup>1</sup> male Swiss mice (Central Institute for the Breeding of Laboratory Animals, TNO, Bilthoven, The Netherlands) weighing between 25 and 30 g were

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<sup>1</sup> Abbreviations used in this paper: EIGM, erythrocytes coated with IgM; EIgMC, erythrocytes coated with complement; NBCS, newborn calf serum; SPF, specific pathogen free.

used in all experiments. Each result is the mean of at least four determinations.

*Mononuclear Phagocytes from the Bone Marrow, Peripheral Blood, and Peritoneal Cavity.* Bone marrow and peripheral blood monocytes were obtained, characterized, and counted as described elsewhere (32). Peritoneal macrophages were harvested and cultured as reported earlier (33).

*Isolation Method of Kupffer Cells.* The method employed has been described in detail elsewhere (18, 29, 30). Animals were anesthetized with 6 mg phenobarbital sodium (S. A. Abbott N. V., Amsterdam) intraperitoneally. The abdomen was opened and the intestines reflected to expose the superior mesenteric vein. This was cannulated with a 27 G needle on a syringe and the liver perfused with 0.2% (wt/vol) pronase E (E. Merck, Darmstadt, W. Germany) in Hanks' solution (Oxoid Ltd., England) at a flow rate of 1-2 ml/min and at a temperature of 37°C. Once perfusion was established the inferior vena cava was cut after which the liver became pale in color. The perfusion was continued for 3-4 min until the animal was completely exsanguinated.

The liver was then removed and weighed, after excision of nonhepatic tissue. Any segments that had not been perfused were removed and the liver reweighed. The liver was chopped into small pieces and washed three times in Hanks' solution.

The fragments were digested for 1 h with constant stirring in 0.2% (wt/vol) pronase (10 ml/g liver) at pH 7.4 and at 37°C. The pH was monitored continuously and adjusted as necessary with 0.1 normal sodium hydroxide. At 20 and 40 min after the start of the incubation 0.5 mg DNAase (E. Merck) in 1 ml Hanks' solution was added to digest cellular debris. Sterilized and siliconized glassware was used throughout the procedure.

At the end of the incubation the cell suspension was filtered through two layers of gauze, centrifuged for 4 min at 1,500 rpm, and washed three times in Hanks' solution. The total number of nonparenchymal cells in the final suspension was counted in a Bürker hemocytometer. The viability of the cells was determined by incubating the cells with 0.1% trypan blue in saline for 30 s at room temperature and counting the number of cells which excluded the dye. At this stage cytocentrifuge preparations of the suspension were prepared for morphological, cytochemical, and functional studies or the suspension was cultured on coverslips to study the glass-adherent cells.

*Cell Culture.* About  $1-1.5 \times 10^6$  nonparenchymal cells were cultured in Leighton tubes with flying coverslips. The culture medium was medium 199 (Microbiological Associates, Walkersville, Md.) with 20% heat inactivated newborn calf serum (NBCS) (Grand Island Biological Co., Grand Island, N.Y.), 2,000 U/ml sodium penicillin G and 20 µg/ml gentamicin. The tubes were gassed with 5% CO<sub>2</sub> in air and incubated at 37°C for 24 h. The supernate was then removed and kept to determine, when necessary, the number, identity, and characteristics of the nonadherent cells. The coverslips were washed three to five times with medium 199 and then dried, fixed, and stained. If longer incubation periods were required fresh culture medium was added every 24 h.

*Cell Morphology.* The morphology of the cells in the nonparenchymal liver cell suspension was studied in cytocentrifuge preparations and that of the glass-adherent cells on coverslips. The preparations were dried rapidly in air, fixed in methanol for 10 min, and stained with Giemsa stain for 7 min.

*Enzyme Cytochemistry.* Peroxidase activity was determined with Kaplow's (34) method by using benzidine dihydrochloride (Fluka A. G., Buchs, Switzerland) as substrate at pH 6.0 and 0.02% (vol/vol) hydrogen peroxide.

Esterase activity was determined by the method described by Ornstein et al. (35) with  $\alpha$ -naphthyl butyrate (Sigma Chemical Co., St. Louis, Mo.) as substrate at pH 6.0. The incubation time was shortened from 25 to 10 min in some experiments due to the very strongly positive reaction obtained after 25 min. In some experiments the enzyme inhibitor, sodium fluoride, in a concentration of 1.5 mg/ml was added to the substrate solution to determine its effect on the staining reaction.

*Phagocytosis and Pinocytosis.* In vivo phagocytosis by nonparenchymal cells and Kupffer cells was assessed after an intravenous injection of 10<sup>9</sup> latex particles (0.81 µm diameter; Difco Laboratories, Detroit, Mich.) or of 1 mg zymosan (kindly provided by Dr. L. Berrens, Utrecht) suspended in 0.2 ml saline. 2 h after the injection of latex and 12 h after the injection of zymosan the liver was removed and a suspension of nonparenchymatous cells made, from which cytocentrifuge preparations were prepared and 24-h cultures set up so that the percentage of total nonparenchymal cells and of adherent Kupffer cells which were phagocytic could be determined.

In vitro phagocytosis was investigated by incubating the nonparenchymal cell suspension with a suspension of *Staphylococcus epidermidis* (bacteria-to-cell ratio of 1) in medium 199 with 10%

new born calf serum with slow rotation (4 rpm) for 1 h at 37°C. The suspension was then washed with medium 199, centrifuged at 100 g, and cytocentrifuge preparations were made, fixed, and stained with Giemsa stain. The phagocytosis of glass-adherent cells was studied by replacing the culture medium of 24 or 48-h cultures with medium 199 containing 10% newborn calf serum and  $5 \times 10^6$  *Staphylococcus epidermidis* followed by incubation for 1 h at 37°C. The coverslip was then washed five times with medium 199, dried, fixed, and stained with Giemsa stain.

Pinocytosis was measured by incubating the glass-adherent cells with 10  $\mu$ g dextran sulphate (mol wt 500,000; Pharmacia Fine Chemicals Inc., Uppsala, Sweden) in 1 ml culture medium for 24 or 48 h at 37°C. The cultures were fixed in absolute methanol for 10 min and stained for 8 min with 2.5% (vol/vol) Giemsa stain in water, which stains the endocytosed dextran sulphate metachromatically.

*Fc and C3 Receptors.* The presence of Fc receptors was detected by using IgG-coated sheep erythrocytes. Washed sheep erythrocytes were made up into a 5% solution in buffered saline. 0.2 ml of this preparation was added to 0.05 ml of heat-inactivated mouse anti-sheep erythrocyte serum (prepared by repeated intravenous immunization of mice with sheep erythrocytes; hemagglutination titer of the antiserum 1:1,500) and the volume made up to 1 ml with medium 199. This suspension was incubated for 30 min at 37°C after which the erythrocytes were washed twice in buffered saline and resuspended in medium 199 to a final concentration of 0.2%. 1 ml of this suspension was added to each culture and incubated for 1 h at 37°C to allow phagocytosis. At the end of the incubation the culture was thoroughly washed with medium 199, dried, fixed, and stained with Giemsa.

The presence of complement (C3) receptors was detected by using sheep erythrocytes coated with IgM and C. The erythrocytes were first coated with IgM by incubating 0.2 ml of a 5% sheep erythrocytes suspension with an equal volume of an IgM fraction of rabbit anti-sheep erythrocyte serum for 15 min at 37°C, and then washed twice with buffered saline. Equal volumes of 2.5% IgM-coated erythrocytes in medium 199 and fresh mouse serum as a source of complement were incubated for 10 min at 37°C, washed twice with saline, and resuspended in medium 199 to a final concentration of 0.2%. C receptors were identified by using the procedure described for Fc receptors.

Rabbit IgM anti-sheep erythrocytes antibody was prepared from rabbit serum obtained 3 days after a single intravenous injection of sheep erythrocytes by passing it twice through a (Sephadex G-200) column. After concentration of the appropriate fractions by freeze-drying, possible IgG contamination was assessed by immunodiffusion by using anti-rabbit IgG antiserum (Dakopatts, Brostex A/S, Copenhagen) and by assaying the ingestion of sheep erythrocytes coated with IgM (EIgM) or with IgM and complement (EIgMC) by 24-h cultures of peritoneal macrophages. IgM was used in a dilution that when EIgM were prepared no rosettes or ingestion of coated erythrocytes by peritoneal macrophages was found and when used for the preparation of EIgMC showed rosettes on 100% of the macrophages and no more than 5% ingestion.

*Histological Preparations.* The liver was removed, cut in pieces, and fixed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 48 h. The fixed tissues were washed for 72 h in running tapwater to remove the excess of formaldehyde. After a final rinse in bi-distilled water for 6 h, the tissue was dehydrated and embedded in paraffin. 4- $\mu$ m thick sections were mounted on slides and dipped in the film emulsion.

*Labeling.* This was carried out with tritiated methyl-thymidine (sp act 6.7 Ci/mmol, New England Nuclear Corp., Boston, Mass.).

For in vitro labeling 0.1  $\mu$ Ci/ml [ $^3$ H]thymidine was added to all solutions used from the start of the removal of the liver. For in vivo labeling two schedules were used: in one a single injection of 25  $\mu$ Ci [ $^3$ H]thymidine per mouse was given intravenously, and in the other the mice received four intramuscular injections of 25  $\mu$ Ci [ $^3$ H]thymidine over 12 h. At least four animals were used for each time point.

*Autoradiography.* This was performed with Ilford Nuclear Research Emulsion K5 in gel form (Ilford Ltd., Essex, England) as described by Van Furth and Cohn (33). The slides were incubated for 3 wk at room temperature. The emulsion was developed with Kodak Developer D-19 and stained after fixation. Preparations of cultured cells were stained for 20 min with Giemsa diluted 1 in 20 at pH 5.75; histological preparations were stained with hematoxylin and eosin. 800-1,000 cells were counted for each animal. Cells with three or more grains over the nucleus were considered positive.

*Inflammation.* An inflammatory reaction was provoked by a single intravenous injection of 1 mg zymosan in 0.2 ml saline.

*Glucocorticosteroids.* The preparation used was hydrocortisone acetate (kindly donated by Merck, Sharp & Dohme, Haarlem, The Netherlands) in a dose of 15 mg subcutaneously.

*X-Irradiation.* Mice were exposed to total-body irradiation of 650 rads with both hind limbs and a part of the pelvis shielded, as described earlier (33).

*Nomenclature.* Cells isolated from the liver by pronase digestion are called nonparenchymatous cells. This population consists of mononuclear phagocytes, endothelial cells, lymphoid cells, and a group of unidentifiable cells (18).

Macrophages residing in the liver of normal animals are called Kupffer cells; the morphological, cytochemical, and functional characteristics of these cells are given under Results. The mononuclear phagocytes isolated from the liver during an inflammatory response, which include resident macrophages (Kupffer cells) and mononuclear phagocytes which have migrated into the liver from the circulation, are called liver macrophages. The morphological criteria for the identification of monocytes are described elsewhere (33, 36).

## Results

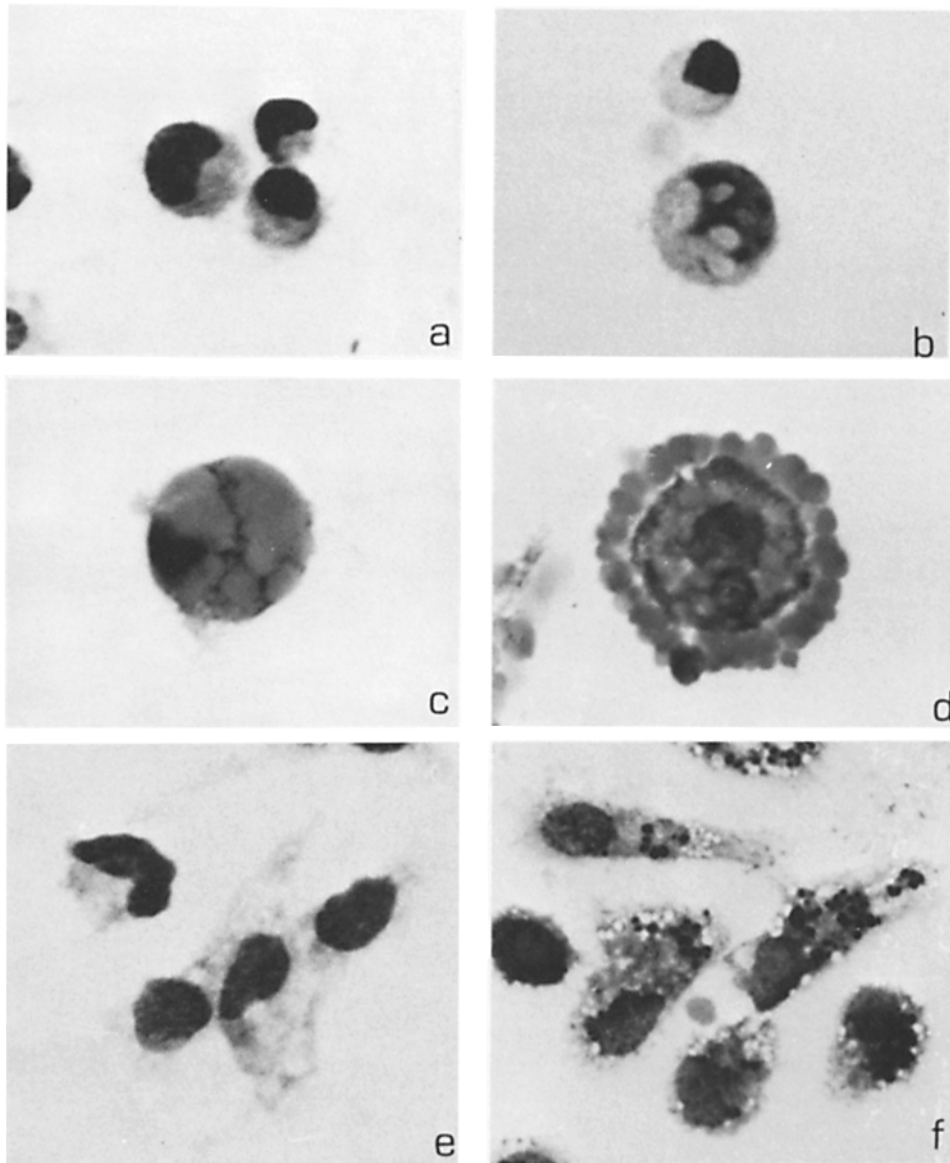
*Yield of Nonparenchymal Liver Cells.* The mean liver weight of 5-6 wk old mice (25-30 g) was 1.5 ( $\pm$  0.24 SD) g. The average yield of nonparenchymal cells obtained by pronase-DNAase digestion was 2.3 ( $\pm$  0.51 SD)  $\times$  10<sup>7</sup> cells/g liver which gives a mean of 3.4  $\times$  10<sup>7</sup> cells/liver. This suspension contained only 1-2% parenchymal cells which were either dead or very badly damaged. The nonparenchymal cells were usually more than 90% viable as judged by trypan blue exclusion.

*Morphology and Characteristics of Kupffer Cells in Suspension.* In cytocentrifuge preparations the Kupffer cells were morphologically heterogeneous in size and shape as other workers have noted (6, 7, 20, 37). In Giemsa-stained preparations the cell size ranged from 7  $\times$  7 to 15  $\times$  14  $\mu$ m; the cells had a distinct basophilic cytoplasm and the nucleus was eccentrically located and was usually oval or indented (Fig. 1).

The cytochemical and functional characteristics of the Kupffer cells in suspension are shown in Table I. The Kupffer cells were positive for esterase and this reaction was inhibited by sodium fluoride. In vitro incubation of the suspension of nonparenchymal cells with opsonized bacteria showed that only 1.3% of the Kupffer cells ingested bacteria. 2 h after an intravenous injection of latex 18.0% of the nonparenchymal cells and, among them, 88.6% of the Kupffer cells had ingested these particles; 12 h after a similar injection of zymosan the phagocytic indices for nonparenchymal cells and Kupffer cells were 13.9 and 66.0%, respectively (Fig. 1).

The percentage of Kupffer cells with Fc receptors was lower than in freshly isolated peritoneal macrophages and C receptors could not be detected (Table I), possibly because of the effect of the pronase on the cell membrane.

The effect of pronase on the membrane receptors of peritoneal macrophages was therefore investigated. These cells were first incubated in medium 199 with 20% NBCS for 2 h to allow them to adhere to the coverslips and then in medium 199 with 0.2% pronase for 1 h. After pronase treatment only 13.8% of the attached peritoneal macrophages had Fc receptors while C receptors could not be detected. When a suspension of peritoneal macrophages was treated with 0.2% pronase for 1 h 90% had an Fc receptor and 51% a C receptor. After 24 h in



**FIG. 1.** Mouse Kupffer cells. a. Giemsa-stained cytocentrifuge preparation of a suspension of Kupffer cells ( $\times 630$ ). b. Cytocentrifuge preparation showing a Kupffer cell which has ingested intravenously injected zymosan particles and a nonphagocytic cell ( $\times 630$ ). c. 24-h culture of Kupffer cell that has ingested IgG-coated sheep erythrocytes ( $\times 1,000$ ). d. 24-h culture of Kupffer cell with a rosette of sheep erythrocytes coated with IgM and complement. EIGMC are not ingested; the cytoplasm of the cell is vacuolated ( $\times 1,000$ ). e. Giemsa-stained preparation of Kupffer cells cultured for 24 h ( $\times 1,000$ ). f. Giemsa-stained preparation of Kupffer cells that after 48 h of culture have phagocytized staphylococci ( $\times 1,000$ ).

culture 97.5% of the pronase-treated peritoneal macrophages had Fc receptors like the untreated macrophages (Table I) but only 69.3% had recovered their C receptors. Only after 48 h incubation were C receptors found on 94.3% of the macrophages.

TABLE I  
 Characteristics of Macrophages

	Time of incubation					
	Kupffer cells			Peritoneal macrophages		
	0 h	24 h	48 h	0 h	24 h	48 h
		%			%	
Esterase*	95.4	99.0	100.0	85.9	99.3	99.5
Peroxidase‡	0.0	0.0	0.0	1.0	0.4	0.1
Phagocytosis§	1.3	81.5	89.0	38.0	98.0	99.0
Pinocytosis	80.6	90.4	97.0	98.0	99.0	100.0
Fc Receptors¶	66.5	84.3	94.5	99.8	100.0	100.0
C Receptors	0.0	37.5	54.0	98.5	100.0	100.0
In vitro labeling**		0.8	0.8		4.1	
Pulse labeling‡‡	1.0			2.4		

\* Diffuse cytoplasmic staining;  $\alpha$ -naphthyl butyrate as substrate.

‡ Located in granules; determined by the Kaplow method.

§ Ingestion of opsonized *Staphylococcus epidermidis* at 0 h determined in cell suspensions under slow rotation and at 24 and 48 h in macrophages attached to a glass surface.

|| Dextran sulphate.

¶ Cells with ingested erythrocytes and rosettes.

\*\* Incubated in medium with 0.1  $\mu$ Ci/ml [ $^3$ H]thymidine.

‡‡ 1 h after 1  $\mu$ Ci/g [ $^3$ H]thymidine intravenously.

On the basis of morphology an average of 26.9% of the nonparenchymal cells were Kupffer cells. This gives an overall yield of  $0.61 (\pm 0.16 \text{ SD}) \times 10^7$  Kupffer cells/g liver which gives a mean of  $0.91 \times 10^7$  Kupffer cells/liver.

The other cell types were endothelial cells (about 34%), lymphocytes (about 20%), granulocytes (about 6%), and immature cells (about 2%), while about 8% were not identifiable.

**Adherence of Cells.** It was found that 24 h incubation was required to obtain maximum adherence of cells to glass. Under these conditions 65.3% of the cells in suspension were adherent. Of the adherent cells 57.1% were Kupffer cells as judged by morphology and esterase staining. Over 70% of the nonadherent cells were not viable at this stage as judged by the uptake of trypan blue. After 48 or 72 h of incubation over 90% of the adherent cells were Kupffer cells (Fig. 1).

**Morphology and Characteristics of Adherent Kupffer Cells.** The glass-adherent Kupffer cells were as heterogeneous in size and shape as when freshly isolated. They did not spread out like peritoneal macrophages but tended to maintain a round or oval shape although occasionally they were elongated. In Giemsa-stained preparations they had a darkly stained eccentric nucleus, usually oval, or indented in shape. The cytoplasm was basophilic and vacuolated (Fig. 1).

The functional and cytochemical characteristics of the Kupffer cell are listed in Table I and compared with those of normal peritoneal macrophages. The Kupffer cells showed strong diffuse esterase staining; peroxidase-positive granules were not observed. This pattern of cytochemical characteristics is similar to that of peritoneal macrophages except that in the latter cells the esterase activity is usually weaker. Both the phagocytic and the pinocytic index of the

Kupffer cells were lower after 24 h incubation than after 48 h (Fig. 1). The percentage of cells with Fc and C receptors also increased during incubation of the Kupffer cells but remained lower than in the peritoneal macrophages (Fig. 1). This may all reflect membrane damage due to the pronase digestion (see above).

The other types of adherent cell included a cell which had a slightly irregular or oval nucleus and pale staining cytoplasm in Giemsa preparations. It was weakly positive for esterase, negative for peroxidase, pinocytosed only a little, and did not phagocytose bacteria. IgG-coated sheep erythrocytes attached to the surface of these cells but were not phagocytosed in contrast with Kupffer cells and peritoneal macrophages. This cell, which is most probably an endothelial cell (18), had the same morphological characteristics in freshly isolated suspensions of nonparenchymal liver cells and in cultured preparations, except that in cultures the cells were more spread out. A small number of fibroblasts were also present on the coverslips: these cells tended to overgrow the cultures when they were incubated for longer periods.

*In Vitro and Pulse Labeling with [<sup>3</sup>H]Thymidine.* The percentage of Kupffer cells taking up [<sup>3</sup>H]thymidine in vitro after incubation in a [<sup>3</sup>H]thymidine-containing medium and the labeling index measured 1 h after the intravenous injection of 25  $\mu$ Ci [<sup>3</sup>H]thymidine are given in Table I. These values are 1% or lower and did not increase during longer incubation with [<sup>3</sup>H]thymidine in vitro. This means that the mitotic activity of the Kupffer cells in the normal steady state is very low.

*In Vitro Labeling of Kupffer Cells with [<sup>3</sup>H]Thymidine during Treatment with Hydrocortisone.* A subcutaneous injection of 15 mg of hydrocortisone acetate, which forms a depot, causes a severe monocytopenia in mice with a monocyte count less than 10% of normal within 6 h (38). It also arrests the influx of mononuclear phagocytes from the peripheral blood into the tissues (38), but does not affect the DNA-synthesis of mononuclear phagocytes either in vitro or in vivo (38, 39). When Kupffer cells were obtained from mice given 15 mg hydrocortisone acetate and incubated in a medium with 0.1  $\mu$ Ci/ml [<sup>3</sup>H]thymidine a decrease in the labeling index to 0.2% at 24 h was found (Fig. 2).

These results demonstrate that in normal animals the DNA-synthesizing Kupffer cells are derived from the circulation, which normally contains a small percentage (less than 2.5%) of DNA-synthesizing monocytes (33). The rapid disappearance of labeled Kupffer cells during glucocorticosteroid treatment implies, furthermore, that once arrived in the liver, mononuclear phagocytes retain their capacity to synthesize DNA for only a short time.

*The Effect of an Intravenous Injection of Zymosan on in Vitro Labeling of Liver Macrophages.* 24 h after an intravenous injection of 1 mg zymosan the labeling index of the liver macrophages was 12.8% which is 16 times higher than in normal mice (Fig. 3). The percentage of labeled macrophages decreased thereafter to 1.0% at 120 h as in normal animals. These results could indicate that at the beginning of the inflammatory response mononuclear phagocytes divide in the liver.

*The Effect of Hydrocortisone Acetate on the in Vitro Labeling of Liver Macrophages in Zymosan-Treated Mice.* When 1 mg zymosan was injected

## ORIGIN OF THE KUPFFER CELL

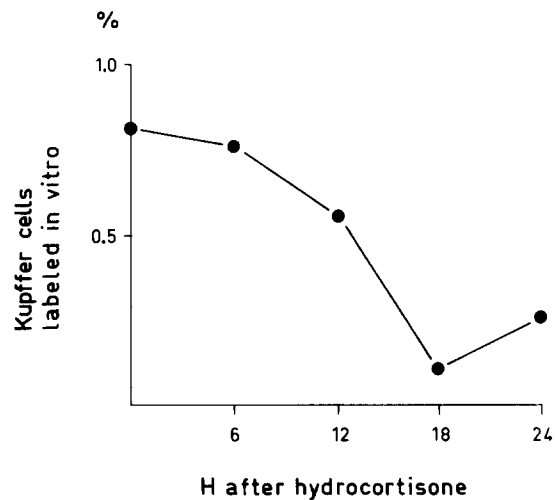


FIG. 2. The effect of 15 mg hydrocortisone acetate on the in vitro labeling of Kupffer cells.

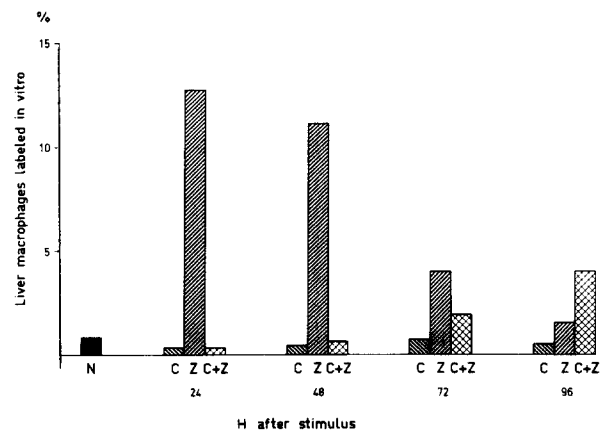


FIG. 3. In vitro labeling of liver macrophages in normal animals (N); at various time points after a subcutaneous injection of 15 mg hydrocortisone acetate (C); at various time points after an intravenous injection of 1 mg zymosan (Z) in normal mice; and at various time points after an intravenous injection of 1 mg zymosan in mice which had received hydrocortisone acetate 48 h earlier (C + Z).

into mice which had been injected 48 h earlier with 15 mg hydrocortisone acetate the in vitro labeling index of the liver macrophages was 0.33% at 24 h (Fig. 3). The percentage of labeled macrophages did not start to increase until 72 h after the injection of zymosan and was still only 4.0% at 96 h (Fig. 3).

When hydrocortisone acetate was injected 24 or 48 h after the intravenous injection of zymosan the in vitro labeling of the liver macrophages, determined 24 h after hydrocortisone administration, was 0.6% instead of about 12% in mice treated with zymosan alone.

From the results of these experiments it may be concluded that the increase of the in vitro labeling index of liver macrophages after an intravenous injection of zymosan is due to the recruitment of immature DNA-synthesizing cells, conceivably from the bone marrow via the circulation into the liver. DNA-



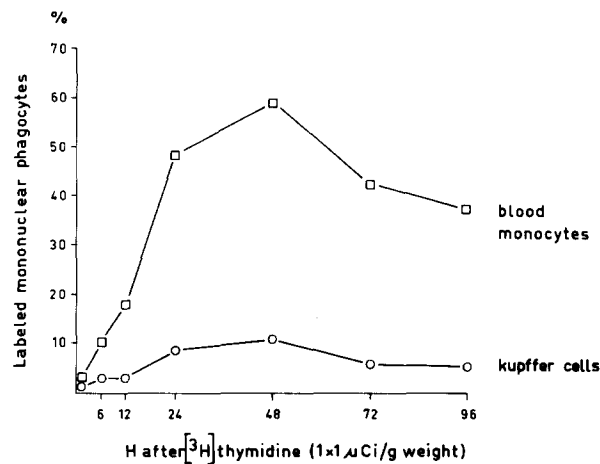


FIG. 4. Labeling index of Kupffer cells after a single intravenous injection of  $[^3\text{H}]$ thymidine. The labeling indices of blood monocytes are taken from reference 48.

synthesizing mononuclear phagocytes are not found when the circulation is depleted of circulating mononuclear phagocytes by hydrocortisone treatment. The possibility that zymosan stimulates Kupffer cells to divide, but that this activity is suppressed by glucocorticosteroids, is very unlikely since DNA-synthesis of mononuclear phagocytes is not affected by hydrocortisone (39). The rapid decline of the labeling index of liver macrophages (from about 12 to 0.6%) when hydrocortisone was given after zymosan demonstrates that immature DNA-synthesizing mononuclear phagocytes when recruited into the liver retain their DNA-synthesizing capacity for only a short period.

*In Vivo Labeling of Kupffer Cells with  $[^3\text{H}]$ Thymidine during the Normal Steady State.* After one intravenous injection of 25  $\mu\text{Ci}$   $[^3\text{H}]$ thymidine the labeling index of the Kupffer cells reached a peak of 10.4% at 48 h and thereafter declined slowly. The percentage of labeled blood monocytes was also maximal at 48 h (Fig. 4).

12 h after the last of four intramuscular injections of 25  $\mu\text{Ci}$   $[^3\text{H}]$ thymidine the labeling index of the Kupffer cells was 13.7% and reached a maximum of 24.1% 60 h after the last injection (Fig. 5).

The curves of the labeled Kupffer cells after one and four injections of  $[^3\text{H}]$ thymidine are similar to those for peritoneal macrophages (33).

*The Effect of Hydrocortisone Acetate on the Kinetics of Labeled Kupffer Cells in Normal Mice.* When 15 mg hydrocortisone acetate was injected 3 h after the last of four  $[^3\text{H}]$ thymidine injections 6.5% of the Kupffer cells were labeled 12 h after the last thymidine injection. Thereafter, almost no further increase in the percentage of labeled Kupffer cells was found in contrast to normal animals (Fig. 5). In the hydrocortisone-treated mice the total number of Kupffer cells decreased slightly from  $0.91 \times 10^7$  to  $0.67 \times 10^7$  cells at 108 h.

*Effect of X-Irradiation and Bone Marrow Shielding on the in Vivo Labeling with  $[^3\text{H}]$ Thymidine.* Mice were X-irradiated with shielding of a small portion of the bone marrow and 24 h later received four injections of 25  $\mu\text{Ci}$   $[^3\text{H}]$ thymidine. In these mice the labeling index of the Kupffer cells was reduced to very low levels but there was still a small peak of 0.39% 60 h after the last

## ORIGIN OF THE KUPFFER CELL

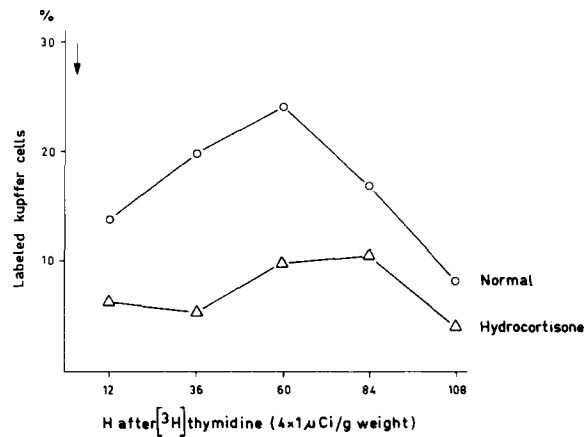


FIG. 5. Labeling index of Kupffer cells after four intramuscular injections of  $[^3\text{H}]$ -thymidine and the effect of 15 mg hydrocortisone acetate, given 3 h after the last of four injections of  $[^3\text{H}]$ thymidine, on the labeling of Kupffer cells.

TABLE II  
*Effect of X-Irradiation with Shielding of both Hind Limbs on the Labeling of Kupffer Cells*

Time after $[^3\text{H}]$ thymidine*	Labeled Kupffer cells‡	
	Normal	X-Irradiation§ + shielding
<i>h</i>	%	%
12	1.4	0.06
36	2.0	0.13
60	4.0	0.39
84	6.4	0.26
108	5.7	0.11
132	4.1	0.09
156	1.9	0.12

\*  $4 \times 25 \mu\text{Ci}$   $[^3\text{H}]$ Thymidine intramuscularly given 24–36 h after X-irradiation.

‡ Counted in tissue sections.

§ 650 Rads.

injection of  $[^3\text{H}]$ thymidine while in unirradiated controls a peak of 6.4% 84 h after the last injection was found (Table II). Since in this experiment the liver was X-irradiated these results show that the Kupffer cells labeled *in vivo* were derived from the bone marrow.

*In Vivo Labeling of Bone Marrow and Peripheral Blood Monocytes.* To calculate the turnover of the labeled Kupffer cells it was necessary to have more recent information on the production of monocytes in the bone marrow and the turnover of monocytes in the circulation, since the condition of the mice (40) had changed since our previous study (32). The mice received a single intravenous injection of  $25 \mu\text{Ci}$   $[^3\text{H}]$ thymidine and the labeling index and total number of monocytes in the bone marrow were determined as described earlier (32). The results are given in Table III. The half-time of the monocytes in the circulation

TABLE III  
*Percentage and Total Number of Labeled Bone Marrow and  
 Peripheral Blood Monocytes Per Mouse*

Time after [ <sup>3</sup> H]thymidine*	Bone marrow monocytes		Peripheral blood mono- cytes	
	Labeling index	Total num- ber labeled cells	Labeling index	Total num- ber labeled cells
<i>h</i>	%	$\times 10^5$	%	$\times 10^5$
6	10.9	3.19	3.0	0.85
12	23.8	6.97	15.3	4.31
24	43.3	12.68	30.0	8.46
48	48.9	14.32	38.7	10.91

\* A single intravenous injection of 25  $\mu$ Ci [<sup>3</sup>H]thymidine.

determined on the basis of the disappearance of the cohorts of the most heavily labeled peripheral blood monocytes (33) was 15 h.

With these values the total production of labeled monocytes was calculated according to the equation described elsewhere (32) and was  $1.04 \times 10^5$  cells per h during the first 24 h after labeling. This value is 60% higher than found for SPF mice in the normal steady state 5 yr earlier (32).

### Discussion

The results of the present study show that Kupffer cells have the characteristics of mature macrophages, that in the normal steady state there is no local proliferation of Kupffer cells, and that there is a continuous turnover of Kupffer cells, these cells being replaced by circulating blood monocytes.

Since this kinetic study was performed with isolated cells cultured on glass it was necessary to recover the maximum number of Kupffer cells from the liver without selection. Of all the various methods of preparing isolated Kupffer cells pronase digestion seems to give the best results in terms of structure and function and freedom from hepatocyte contamination (18, 20, 30, 31).

Our total yield of  $2.3 \times 10^7$  nonparenchymal cells per g liver compares very favorably with those of other workers using similar methods who obtained in mice, rats, etc., yields varying from 0.58 to  $2.2 \times 10^7$  nonparenchymal cells per g liver (18, 20, 23, 28, 31, 41). The percentage of Kupffer cells in the cell suspension of our mice averaged 26.9%, giving a total of  $0.91 \times 10^7$  Kupffer cells per liver, which is similar or a little lower than that obtained by others in normal rats by using the same method (18, 20) or other methods (23). Other workers have used enzymatic methods with albumin gradients to obtain virtually pure preparations of Kupffer cells (29, 37, 42), so their results are not comparable with ours.

It is of importance to know what proportion of the total Kupffer cell population is recovered by our isolation procedure because this will have implications for the calculation of the turnover of the Kupffer cells. In a recent study done in SPF mice, also using pronase treatment, a value of  $0.83 \times 10^7$  Kupffer cells per g liver was found (31), which agrees well with our observations. Unfortunately no reliable data on the absolute number of Kupffer cells in

the mouse liver are available. In a morphometric study of the rat liver it has been found that 33.3% of the nonparenchymal cells were Kupffer cells (43) which is in accordance with the percentage of Kupffer cells in suspensions of nonparenchymal liver cells of rats and mice.

After 24 h of incubation an average of 34.7% of the total number of nonparenchymal cells were in the supernate. Less than 30% of these cells were alive and 32.9% of these cells were Kupffer cells as judged by morphology. This means that only 3.5% of the living Kupffer cells did not attach to the glass surface during incubation and therefore indicates that the majority of the undamaged Kupffer cells adhered to the coverslip.

The characteristics of the Kupffer cell are the same as those of a mature peritoneal macrophage. The esterase activity of the Kupffer cell, which is usually strongly positive, is not such a reliable criterion for the characterization of these cells in nonparenchymatous liver cell suspensions since the endothelial cell of the liver is weakly esterase positive as found by Emeis and Planqué using light and electron microscopy (18). Peroxidase activity could not be demonstrated in Kupffer cells at the light microscope level using the Kaplow method. Other workers (18-21) using electronmicroscopy and modifications of Graham and Karnovsky's technique (44) have shown peroxidase activity in the nuclear membrane and endoplasmic reticulum.

C receptors cannot be demonstrated on freshly isolated Kupffer cells, which was also reported by Munthe-Kaas (24). This is probably due to the treatment with pronase which removes these receptors from the cell membrane as was demonstrated in the control experiments with peritoneal macrophages. After incubation the receptors reappear on the peritoneal macrophages and Kupffer cells.

The removal of receptors by pronase treatment also explains why freshly isolated Kupffer cells hardly ingest any opsonized bacteria, while after incubation on glass the majority of the Kupffer cells phagocytose normally. Pinocytosis, on the contrary, is not affected by the enzyme digestion.

Until this study the origin and kinetics of Kupffer cells in normal steady state animals have not been studied. The use of tissue sections has probably hampered a detailed approach to this question. The only data available on the steady state was the labeling index 1 or 2 h after a pulse of [<sup>3</sup>H]thymidine, which was 1.5% or less (5, 13-15). This result was interpreted as an indication that Kupffer cells are self-replicating at a very low rate or that they virtually do not divide and that cell renewal occurs from the circulation.

The present study provides evidence for the latter view. The rapid decline of the in vitro labeling index in normal mice treated with hydrocortisone acetate demonstrates that the Kupffer cells which label in vitro are newly recruited from the bone marrow, since this drug hardly affects the mitotic activity of dividing promonocytes, virtually stops the release of the newly formed monocytes from the bone marrow and causes a profound monocytopenia (38, 39).

The marked increase of the in vitro labeling index of liver macrophages induced by zymosan could lead to the conclusion that Kupffer cells were stimulated to proliferate locally. However, the experiments in which zymosan was given in hydrocortisone-treated mice proved that the increased number of

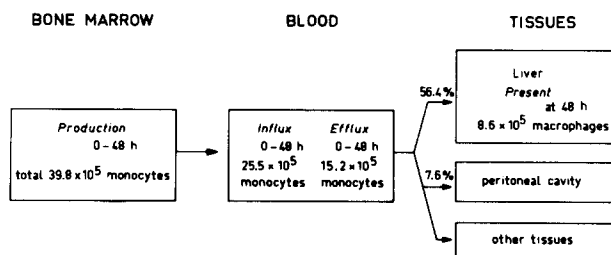


FIG. 6. Total production of labeled monocytes in the bone marrow, kinetics of monocytes in the blood compartment, and migration of these cells into the liver during the normal steady state. For comparison the influx into the peritoneal cavity is given (from reference 32).

liver macrophages that label in vitro are mononuclear phagocytes recently recruited from the bone marrow into the liver. Warr and Sljivić (11) came to the same conclusion for a number of inflammatory stimuli using the techniques of whole and part-body X-irradiation and in vitro  $^3\text{H}$ thymidine incorporation, but concluded that in addition zymosan could induce proliferation of the pre-existing cell population.

Furthermore the labeling of the Kupffer cells after one or four injections of  $^3\text{H}$ thymidine can only be attributed to labeled monocytes migrating from the peripheral blood into the liver since 1 h after a pulse of  $^3\text{H}$ thymidine the labeling index of the Kupffer cells is 1% which means that at any given time only 1% of these cells divide. This small number of dividing Kupffer cells could never account for the labeling indices found in this study.

Additional evidence that Kupffer cells are replenished by blood monocytes is provided by the course of labeled cells in hydrocortisone-treated mice. In these mice with severe monocytopenia the increase in labeled Kupffer cells is arrested; if Kupffer cells could renew themselves the labeling index would still increase in these animals. Further proof for the bone marrow origin of Kupffer cells comes from the experiments with X-irradiation with the liver exposed and a part of the bone marrow shielded. The results show that there are a small number of labeled Kupffer cells which must be derived from the shielded part of the bone marrow in which promonocytes can still form monocytes. Souhami and co-workers (10, 45) have demonstrated with X-irradiation with or without bone marrow shielding or with cytotoxic drugs along with  $^3\text{H}$ thymidine labeling that recovery of the blockade of the Kupffer cells, induced with carbon or dextran sulphate, is caused by the influx of newly formed mononuclear phagocytes from the bone marrow.

In recent experiments with parabiotic rats no evidence for the monocytic origin of Kupffer cells and peritoneal macrophages was obtained (5). In this study parabiosis was maintained by a large pedicle so that the two circulations were joined by capillaries only. The absence of labeled peritoneal macrophages and Kupffer cells in the recipients could be due to the small numbers of labeled monocytes circulating in these animals. In another more recent chimera study, however, labeled peritoneal macrophages and Kupffer cells were found in recipient rats which had been connected via the carotid artery and jugular vein to the labeled donor rats (four intravenous injections of  $0.75 \mu\text{Ci/g}$

[<sup>3</sup>H]thymidine over 7 days) for 30 min 4 days after the last injection (M. R. Parwaresch, personal communication). Their data agree with our studies on peritoneal macrophages (32) and Kupffer cells and are in complete contrast with those of Volkman (5) whose negative results cannot easily be explained.

There is also evidence in humans for the monocytic origin of Kupffer cells since analysis of the sex of the endothelial and Kupffer cells in long-surviving hepatic (male to female) homografts has shown that 3<sup>1</sup>/<sub>2</sub> mo after transplantation the Kupffer cells are of host origin, while the endothelial cells remained male in all instances (46, 47).

The input of monocytes required to maintain a constant population of Kupffer cells during the normal steady state can be calculated as described elsewhere (32). The total production of labeled monocytes using data obtained after a single [<sup>3</sup>H]thymidine injection (see under Results) is calculated for the first 48 h because thereafter no further production of labeled monocytes can be detected (32, 48). The area under the curve of the total number of Kupffer cells (see under Results) corrected for the 1 h labeling index (1%), is the total number of Kupffer cells which have arrived from the circulation. The calculated data, given in Fig. 6, show that 64% of the total number of labeled monocytes produced in 48 h leave the bone marrow and become blood monocytes and that at least more than half of the monocytes (56.4%) which leave the circulation become Kupffer cells. The other monocytes leaving the blood stream migrate to other tissues. If we consider the population of Kupffer cells as kinetically homogeneous an influx of  $8.6 \times 10^5$  cells in 48 h will give a mean turnover of the Kupffer cells, i.e. the time required for the replacement of the total population of cells, of 21 days.

### Summary

Enzymatic digestion with pronase and DNAase was used to isolate Kupffer cells from mouse liver. The characteristics of these cells were found to be similar to those of peritoneal macrophages, except that in the initial suspension the percentage of Kupffer cells with Fc receptors was low, C receptors were absent and the ingestion of opsonized bacteria was very poor, because of the effect of pronase on the cell membrane. After 24 h incubation in vitro all these characteristics return.

The in vitro and 1 h-pulse [<sup>3</sup>H]thymidine labeling of the Kupffer cells is low (0.8 and 1%, respectively) indicating that in essence these cells do not divide. It was also shown that the small percentage of in vitro labeled Kupffer cells was recently derived from the circulation. After an intravenous injection of zymosan the in vitro labeling index of the Kupffer cells increased 16-fold, but it was proven that these dividing cells were immature mononuclear phagocytes very recently recruited from the bone marrow.

The labeling of Kupffer cells after one or four injections of [<sup>3</sup>H]thymidine reached a peak of 10.4% at 48 h or 24.1% at 60 h, respectively, indicating that these cells are derived from labeled monocytes. Further evidence for this conclusion was obtained by the absence of an increase of labeled Kupffer cells during treatment with hydrocortisone, which causes a monocytopenia during which no circulating monocytes are available to migrate to the tissues. Labeling

studies in animals X-irradiated with hind-limb shielding gave a Kupffer cell labeling index of 5-10% of the normal values, which confirms their bone marrow origin.

A quantitative study on the production of labeled monocytes in the bone marrow and their transit through the circulation showed that in the normal steady state at least 56.4% of the monocytes leaving the circulation become Kupffer cells. Considering the Kupffer cells as kinetically homogeneous this gives a mean turnover time of the total population of Kupffer cells of 21 days.

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