DUAL ORIGIN OF MOUSE SPLEEN MACROPHAGES

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Isolation of macrophages from the peritoneal cavity, liver, and lung of normal mice has enabled us to characterize these cells with respect to their morphology, cytochemical characteristics, and functional properties (1–3). Furthermore, their origin, proliferative behavior, and turnover have been investigated (1–6). Chimera studies have shown that macrophages derive from a precursor cell in the bone marrow (7), and kinetic studies with [³H]thymidine as cell marker have demonstrated that the immediate ancestor of the macrophage is the circulating monocyte. These kinetic studies have also provided quantitative information about the influx of monocytes into a body cavity (e.g., the peritoneal cavity) (4) or an organ (e.g., liver, lung) (5, 6).

Morphological studies have shown that the spleen contains a large number of macrophages (8, 9), but the origin and kinetics of these cells have not been studied in detail. It is highly probable that spleen macrophages too derive from circulating monocytes, but local formation of macrophages by dividing precursor cells is possible in rodents, where the spleen continues to be a hematopoietic organ in the adult animal.

The present report concerns a study on the cytochemical and functional characteristics of spleen macrophages and the origin and kinetics of these cells, using macrophage suspensions obtained by mechanical treatment and enzyme digestion.

Materials and Methods

Animals.   Specific pathogen–free male Swiss mice (The Central Institute for the Breeding of Laboratory Animals, TNO, Zeist, The Netherlands) weighing 25–30 g were used.

Isolation of Spleen Macrophages.   The animals were anesthetized with 5 mg phenobarbital (Abbott NV, Amsterdam) injected intraperitoneally. The abdomen was opened, the diaphragm exposed and pierced, and, after collapse of the lungs, a piece of the thoracic cage was excised. The heart was punctured with a 22-gauge needle on a 10-ml syringe containing 0.6 mM EDTA in phosphate-buffered saline. ~4 ml was injected slowly while the abdominal aorta was clamped; next, the aorta and vena cava in the abdomen were cut and the remaining EDTA-saline solution was injected slowly; during this perfusion the spleen became slightly paler. The organ was then removed, weighed, washed once in medium 199 (M.A. Bioproducts, Walkersville, MD), and cut into small pieces (~1 mm³). The fragments were incubated in 10 ml 0.1% collagenase (126 U/mg; Worthington Biochemical Corp., Freehold, NJ) in Hanks' balanced salt solution (pH 7.4) for 1 h in a 37°C water bath under constant stirring. During incubation, the pH was monitored and if necessary adjusted with 0.1 N sodium hydroxide. The suspension was then drawn into and expelled from a syringe with a 19-gauge needle three times, filtered through two layers of gauze, and then centrifuged for 7 min at 400 g. The pellet was washed twice

with medium 199 and resuspended in 10 ml medium 199. Cell counts were performed in a hemocytometer with Türk's solution, and viability was determined with 0.1% trypan blue. Cytocentrifuge preparations were made for Giemsa and cytochemical staining.

Cell Culture. To obtain cell cultures, a 1-ml cell suspension containing 1×10^7 cells was incubated in Leighton tubes with flying coverslips. The culture medium was composed of medium 199, 20% heat-inactivated newborn calf serum (Gibco Europe Ltd., Paisley, Scotland), 20 μg/ml gentamicin, and 1,000 U/ml sodium penicillin G. The tubes were incubated in a 5% CO_2 incubator at 37°C for 24 or 48 h, after which the cells on the coverslips were washed three times with medium 199, air-dried quickly, fixed, and stained.

Cell Morphology and Enzyme Cytochemistry. The morphology of the cells in the original suspension was studied in cytocentrifuge preparations; glass-adherent cells were studied after a 24 or 48 h incubation in a Leighton tube. For Giemsa staining, the preparations were fixed for 10 min in methanol and stained for 7 min. The staining for nonspecific esterase was done with α-naphthyl butyrate (Sigma Chemical Co., St. Louis, MO) (pH 6.0) as substrate (10). Peroxidase staining was done according to Kaplow (11) and staining for lysozyme was performed with rabbit anti-rat lysozyme as described elsewhere (10). Membrane antigens on spleen macrophages were identified by monoclonal antibodies 30.G.12 and M1/70 (National Tissue Type Collection, Rockville, MD), F4/80 (donated by Dr. S. Gordon, Dunn School of Pathology, Oxford University), and 2.4.G.2 (donated by Dr. J. Unkeless, The Rockefeller University, New York). Binding of monoclonal antibodies was detected by biotin avidin amplification of the immunoperoxidase as described in detail elsewhere (12).[1]

Receptor Studies. Receptors for Fcγ and C3b, respectively, were determined with sheep erythrocytes (SRBC)[2] coated with heat-inactivated mouse anti-SRBC serum (EIgG) and SRBC coated with the IgM fraction of rabbit anti-SRBC serum and fresh mouse serum as source of complement (EIgMC), as described elsewhere (10).

Functional Studies. The phagocytic activity of glass-adherent cells was studied with *Staphylococcus aureus* in the presence of 10% newborn calf serum, as described elsewhere (10). Noningested *S. aureus* were removed by lysostaphin treatment (13). The ingestion of EIgG or EIgMC was assessed as described elsewhere (13). Pinocytosis of glass-adherent cells was studied by incubating the cells with 50 μg/ml dextran sulphate (500,000 mol wt) for 24 h, as described elsewhere (10).

In Vitro Labeling with [^{3}H]Thymidine. The DNA synthesis of macrophages was studied in vitro by culturing spleen macrophages in the presence of 0.1 μCi/ml [^{3}H]thymidine (sp act, 6.7 Ci/mmol; New England Nuclear, Boston, MA) for 24 h at 37°C (1). In vivo, the labeling index was determined 1 h after an intravenous injection of 1 μCi [^{3}H]-thymidine per gram body weight. The labeling index of the cells was determined by evaluation of the silver grains over the nucleus after autoradiography of the preparations (1); the exposure time was 21 d.

Cell Kinetics Studies. To follow the course of spleen macrophages, we used in vivo labeling with [^{3}H]thymidine. After a single intravenous injection of 1 μCi [^{3}H]thymidine per gram body weight, the labeling index of spleen macrophages was determined at various time points in cells cultured on glass for 24 h (1).

The equations used to calculate the various parameters of spleen macrophage kinetics have been described elsewhere (6). In the steady state the population of spleen macrophages is constant; this means that the rate of monocyte influx plus the rate of local macrophage production equals the rate of disappearance of spleen macrophages, which can be described by:

$$pk_1B + \frac{M^*_{t=1}}{t_s} = k_2M \tag{1}$$

in which p is the fraction of the blood monocyte population that enters the spleen, k_1 the disappearance rate constant of blood monocytes from the circulation, B the total number

[1] Nibbering, P. H., P. C. J. Leijh, and R. van Furth. A cytochemical method for quantification of the binding of monoclonal antibodies to individual cells. Manuscript in preparation.

[2] *Abbreviations used in this paper:* SRBC, sheep erythrocytes.

of circulating monocytes, k_2 the disappearance rate constant of spleen macrophages, and M the total number of spleen macrophages. M^*_{i-1}/t_s describes the local production rate of macrophages in the spleen calculated with the number of labeled mononuclear phagocytes 1 h after a pulse of [^{3}H]thymidine (M^*_{i-1}) and the DNA synthesis time (t_s), under the assumption that all mononuclear phagocytes synthesizing DNA will divide.

The kinetics of the labeled cells after a single intravenous administration of the label can be described by the following equation:

$$pk_1 \int_0^t B^* dt + M^*_{local_t} = M^*_t + k_2 \int_0^t M^* dt \qquad (2)$$

in which $\int_0^t B^* dt$ is the area under the curve of the labeled blood monocytes from $t = 0$ to $t = t$; $M^*_{local_t}$ describes the number of locally pulse-labeled mononuclear phagocytes and their labeled descendants, reaching a maximum value of $2M^*_{i-1}$ when all locally pulse-labeled cells have divided; M^*_t is the number of labeled macrophages present in the spleen at time t, and $\int_0^t M^* dt$ is the area under the curve of the labeled spleen macrophages.

Glucocorticosteroids. Hydrocortisone acetate (Merck, Sharp & Dohme B. V., Haarlem, The Netherlands) was injected subcutaneously in a dose of 15 mg.

Results

Cell Yield. The spleen weight averaged 106.4 mg and differed considerably between mice (range, 57–174 mg). The average number of cells collected was 0.15 (range, 0.10–0.23) × 10^7 per milligram spleen and viability amounted to >90%. In the cell suspension, 2.5% (range, 1.7–4.0%) of the cells were spleen macrophages on the basis of the morphological picture, esterase staining, and staining of cytocentrifuge preparations with monoclonal antibodies (see below). The mean total number of macrophages was 39.9 × 10^5 per spleen.

Morphology of Spleen Macrophages. In cytocentrifuge preparations of freshly isolated cells, the spleen macrophages are fairly homogeneous as to size and the shape is usually round (11.6 × 11.7 μm); the round or oval nucleus is usually located excentrically. In Giemsa-stained preparations the cytoplasm is greyish blue and often contains large refractile inclusions. The macrophages are frequently surrounded by cells of other types such as lymphocytes, blast cells, and erythrocytes.

After 24 h of incubation on glass, the macrophages are more stretched, often have clearly visible refractile inclusions, probably hemosiderin, in the cytoplasm, and are usually surrounded by lymphocytes. Of the cells in the supernatant of these cultures, only 0.3% were macrophages as judged from the morphological picture and esterase staining. In 48-h cultures the cytoplasm of the macrophages contained ingested cells. Blast cells in cytocentrifuge preparations and amongst glass-adherent cells were identified but not further characterized.

Cytochemical and Functional Characteristics. In cytocentrifuge preparations of freshly isolated cells, 97% of the cells with the morphology of macrophages were esterase positive and 9% peroxidase positive. Almost all cells with the morphology of macrophages were faintly positive for lysozyme and stained positively with monoclonal antibodies to a common leukocyte antigen (30.G.12), a distinct macrophage antigen (F4/80), and C3bi receptor (M1/70), but not to the FcII receptor (2.4.G.2).

In 24- and 48-h cultures the percentages of esterase-positive macrophages were similar and only a minority of the macrophages had peroxidase-positive

granules (Table I). Staining for lysozyme showed weak positivity of all of the macrophages.

The Fcγ and C3b receptors were only studied in cultured macrophages, and most of these cells proved to be positive (Table I). Opsonized bacteria and EIgG were ingested by the majority of the macrophages cultured for 24 and 48 h, whereas EIgMC was phagocytosed by only a small proportion of the macrophages (Table I). All macrophages showed pinocytic activity (Table I).

In Vitro and In Vivo [³H]Thymidine Labeling of Spleen Macrophages.   Spleen macrophages cultured for 24 h in the presence of [³H]thymidine had a labeling index of 5.2% (range, 2.5–8.5%). After a single intravenous injection of [³H]-thymidine the labeling index of spleen macrophages rose from 3.7% at 1 h to a maximum of 11.5% at 24 h and then declined (Fig. 1).

Effect of Glucocorticosteroids on the Number of Spleen Macrophages and In Vitro [³H]Thymidine Labeling.   After a single injection of a depot of 15 mg hydrocortisone acetate, the spleen weight decreased from 125 mg (range, 89–161 mg) to 32 mg (range, 25–43 mg) between zero time and 48 h, and the total number of macrophages decreased from 28 × 10⁵ cells to 7 × 10⁵ cells at 48 h (Fig. 2). The numbers of other types of cells in the spleen decreased as well. The number of peripheral blood monocytes decreased by 95% within 12 h and remained that low during the next 120 h (data not shown). When cell suspension samples taken at various time points after hydrocortisone administration were cultured for 24 h in the presence of [³H]thymidine, the percentage of labeled macrophages increased to 13.5% at 72 h (Fig. 3). However, the total number of in vitro labeled macrophages, calculated with the values of the in vitro labeling index and the total number of macrophages under hydrocortisone treatment, showed a gradual decrease during the first 48 h (Fig. 4).

Kinetics of Spleen Macrophages.   The increase of the number of labeled macrophages in the spleen after a pulse of [³H]thymidine reflects not only the influx of labeled monocytes originating from labeled dividing promonocytes and monoblasts in the bone marrow, but also the local production of macrophages from

TABLE I
Cytochemical and Functional Characteristics of Mouse Spleen Macrophages

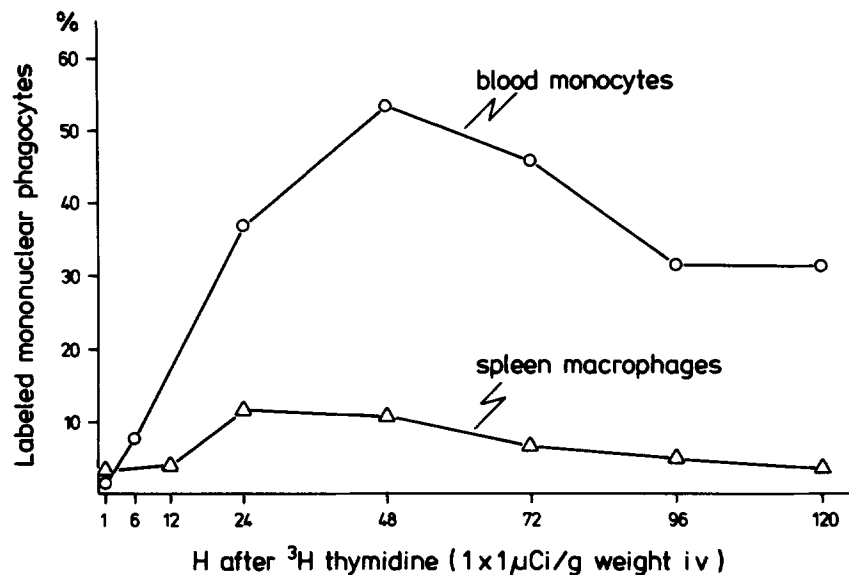|	Duration of cell culture	
	24 h	48 h
	%	
Esterase	99	99
Peroxidase	13	7
Lysozyme	100	100
Fcγ receptor	99	99
C3b receptor	84	94
Phagocytosis		
S. aureus	86	91
EIgG	91	95
EIgMC	8	15
Pinocytosis	99	99

FIGURE 1.   Labeling indices of spleen macrophages and blood monocytes (from reference 3) after a single intravenous injection of [³H]thymidine.

labeled mononuclear phagocytes. The in vitro [³H]thymidine labeling of spleen macrophages for 24 h and the in vivo labeling of these cells 1 h after a pulse of [³H]thymidine reflect the local division of mononuclear phagocytes in the spleen. In the present calculations concerning the kinetics of spleen macrophages, both the influx of monocytes that become spleen macrophages and the local production by dividing mononuclear phagocytes in the spleen were taken into account.

In the steady state the population of spleen macrophages is constant, which means that an equilibrium exists between the rate of monocyte influx into the spleen plus the rate of local macrophage production in the spleen and the rate of disappearance of macrophages from the spleen. For calculation of the kinetics of spleen macrophages, the following values were used: the total number of spleen macrophages (39.9×10^5 cells), the total number of peripheral blood monocytes per mouse (15.6×10^5 cells), the in vivo labeling indices of spleen macrophages and of labeled monocytes at various time points during the first 48-h period after a single intravenous injection of [³H]thymidine (Fig. 1), the half-time of circulating monocytes, i.e., 17.4 h (4), and the number of DNA-synthesizing macrophages in the spleen (14.7×10^4 cells) 1 h after a single injection of [³H]thymidine. For the calculation of the local production of macrophages it was assumed that each DNA-synthesizing mononuclear phagocyte contributed no more than one extra cell to the macrophage population by division (6) and that the DNA synthesis time and the cell cycle time of these cells were 12 and 16 h, respectively (4). It was also assumed that monocytes leave the circulation randomly (1, 4) and that macrophages disappear from the spleen randomly as well. With these data and the equations given in Materials and Methods, the various parameters of the kinetics of spleen macrophages were calculated (Fig. 5). The results showed that 24.7% of the circulating blood
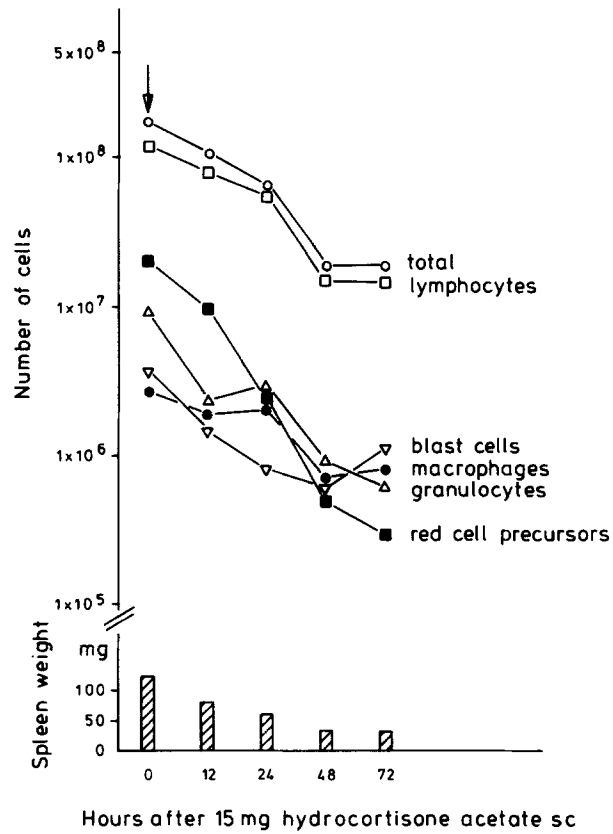
Hours after 15 mg hydrocortisone acetate sc

FIGURE 2.   The effect of hydrocortisone on the weight of the spleen and the number of cells isolated from the organ. 15 mg hydrocortisone acetate was injected subcutaneously; the spleen was then removed and the cells isolated at various time points.
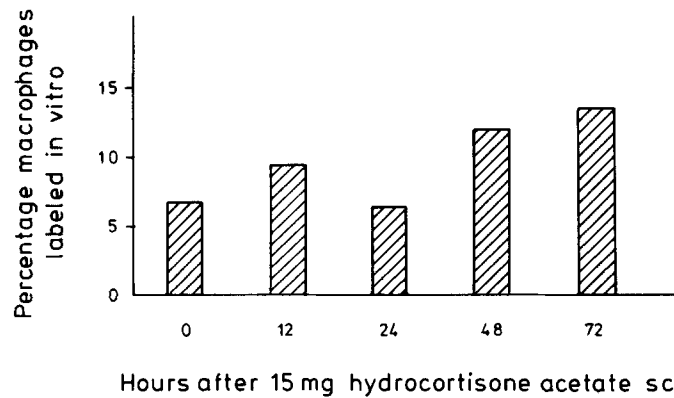

Hours after 15 mg hydrocortisone acetate sc

FIGURE 3.   Course of the percentage of in vitro labeled spleen macrophages under hydrocortisone treatment. Spleen macrophages obtained after a subcutaneous injection of 15 mg hydrocortisone acetate were incubated for 24 h in the presence of 0.1 μCi [^{3}H]thymidine.
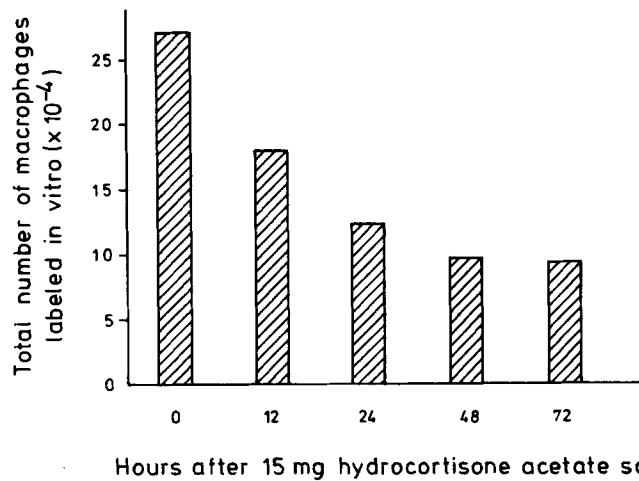
FIGURE 4. Course of the total number of spleen macrophages labeled in vitro under hydrocortisone treatment. The values were calculated with the macrophage-labeling indices shown in Fig. 3 and the numbers of macrophages in Fig. 2.
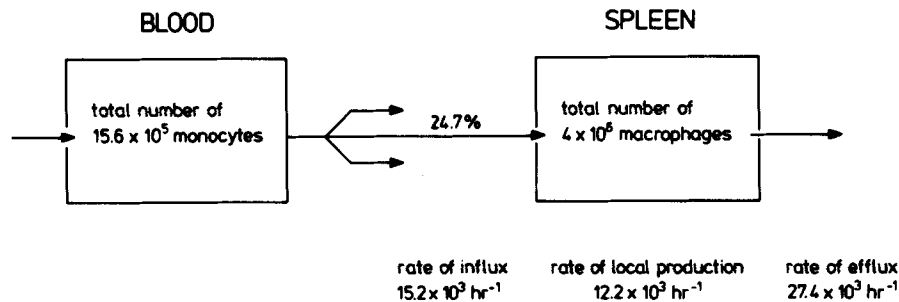

FIGURE 5. Schematic representation of the dual origin of spleen macrophages in mice in the steady state. The rate of monocyte influx into the spleen, the rate of production of spleen macrophages by locally dividing cells, and the rate of efflux of spleen macrophages are indicated. As shown, 24.7% of the blood monocytes migrate to the spleen, where they account for 55% of the macrophage population; the other arrows indicate monocyte migration to other organs. Local production accounts for the other 48% of the macrophage population.

monocytes migrate to the spleen, which amounts to an influx of 15.2×10^3 monocytes per hour, and that the local production of macrophages in the spleen amounts to 12.2×10^3 cells per hour. This means that for the maintenance of the population of spleen macrophages, 55% of the total number is provided by the influx of monocytes and 45% by local production in the steady state. The rate of efflux amounts to 27.4×10^3 macrophages per hour, which gives a half-time of 4.2 d for the disappearance of macrophages from the spleen and a mean turnover time of 6.0 d.

Discussion

In the present study, macrophages were isolated from the spleen after perfusion to remove the circulating monocytes, and this was followed by mechanical disruption of the spleen and enzyme digestion. That this procedure removes the

majority of the monocytes is shown by the low percentage of peroxidase-positive cells in the population of spleen macrophages, as compared with the >90% peroxidase-positive monocytes in the circulation (14). With respect to such characteristic features as Fcγ and C3b receptors, phagocytosis of opsonized bacteria and opsonized red cells, and pinocytic activity, spleen macrophages correspond completely with peritoneal macrophages (7), Kupffer cells (2), and pulmonary macrophages (3). The almost total absence of ingestion of EIgMC indicates that spleen macrophages are not activated, since normal macrophages very rarely ingest complement-coated red cells (15). Cells characterized as macrophages in the present study bear no morphological, cytochemical, or functional resemblance to dendritic cells (16, 17).

In initial attempts to obtain optimal recovery of macrophages, various methods for mechanical disruption and enzyme digestion with pronase, thermolysine, collagenase, or a combination of these enzymes, were evaluated as well as various times of incubation. Mechanical disruption alone gave fewer cells than when combined with enzyme treatment. With most of the enzymes used in these preliminary studies, the recovery of viable cells and the total number of macrophages after 1 h of incubation were similar to those obtained with the method used in the present study itself. In the end, collagenase was chosen instead of one of the proteolytic enzymes, because the latter affects both the C3 receptor and the receptor for IgG2a (20). Since the present method probably gives the highest number of spleen macrophages in suspension, it was considered permissible to use these values to calculate the kinetics of the cells. It is possible, however, that the value used for the total number of spleen macrophages is an underestimation. Estimation of the number of spleen macrophages on the basis of α-naphthylesterase staining of suspended cells originating from collagenase-treated spleens of BALB/c mice (mean weight, 129 ± 26 mg) gave an average total macrophage population of 4.5–5.6 × 10^6 cells (21), and the number of macrophages recovered from CBA-mouse spleens amounted to ~2.1 × 10^6 cells (22). A roughly similar number of total spleen macrophages of C57BL/6 mice was calculated on the basis of the binding of F4/80 monoclonal antibody (S. H. Lee and S. Gordon, personal communication).

For spleen macrophages, the in vitro [^{3}H]thymidine labeling index and the in vivo labeling index 1 h after a pulse of [^{3}H]thymidine were rather low, and the values agreed rather well with each other. These labeled cells represent the locally dividing mononuclear phagocytes.

The local production of mononuclear phagocytes might be expected to contribute to the pool of circulating monocytes, but earlier studies in splenectomized mice labeled with [^{3}H]thymidine showed no difference between the percentages of labeled monocytes in splenectomized and normal mice (1).

After administration of hydrocortisone acetate, which causes monocytopenia (18) but barely affects the mitotic activity of promonocytes (19), the in vitro labeling index of spleen macrophages increased. This differs from the findings for peritoneal macrophages (18), Kupffer cells (2), and pulmonary macrophages (3), whose in vitro labeling indices decreased drastically within 24 h under hydrocortisone treatment. On these grounds we concluded that the in vitro labeled cells are immature mononuclear phagocytes that have migrated rather

recently from the bone marrow to the peritoneal cavity, liver, and lungs (2, 3, 18). The decrease of the total number of spleen macrophages and the relative increase of the in vitro labeling indices of such macrophages after the administration of hydrocortisone acetate indicate that macrophages which do not synthesize DNA disappear from the spleen faster than the DNA-synthesizing mononuclear phagocytes do. However, the total number of in vitro labeled macrophages decreased as well. This indicates that at least a proportion of the DNA-synthesizing mononuclear phagocytes in the spleen have come from the circulation and could be immature mononuclear phagocytes that have recently left the bone marrow. There are indications that other DNA-synthesizing mononuclear phagocytes in the spleen have not recently left the bone marrow and that they divide more than once in the spleen (unpublished observations). Whether these cells represent a separate compartment of the spleen is not certain from the present results, and we lack both an experimental approach and a mathematical model to obtain conclusive information on this point. Since cell suspensions were used, the unequal distribution of macrophages over the white and red pulp of the spleen (23) could not be taken into account either.

Calculation showed that in mice in the steady state, 55% of the macrophage population of the spleen is maintained by monocyte influx and 45% by local division of mononuclear phagocytes in the spleen. This means that there is a dual origin of spleen macrophages, i.e., some of the spleen macrophages derive directly from circulating monocytes and others derive from locally dividing mononuclear phagocytes, some of which have probably left the bone marrow recently. If we calculate the kinetic parameters for a 1½ times larger number of spleen macrophages, to cover the possibility that not all of the macrophages were collected with the present method, we find that ~36% of the monocytes migrate to the spleen, but the relative contribution of the monocyte influx and local production to the total number of spleen macrophages, as well as the mean turnover time, are similar to those calculated for the number of spleen macrophages found experimentally and used in the present study.

For both pulmonary macrophages (6) and Kupffer cells (5) also, it was recently shown that under steady-state conditions, local division of mononuclear phagocytes contributes to the macrophage population. The new mathematical approach used in those two studies and here provides a bridge between the views of those who argue that the macrophage population in tissues and serous cavities is maintained solely by local division (24, 25) and our earlier view that monocyte influx was solely responsible (1-4, 18), a conclusion reached before quantitative assessment of local division became possible.

The combination of a constant influx of monocytes into the spleen and the local formation of macrophages implies a steady rate of local cell death and/or a steady efflux of macrophages from the spleen. Otherwise, macrophages would accumulate in the spleen, which would be in conflict with the steady-state principle. The calculated mean turnover time of spleen macrophages amounts to 6.0 d. Recent findings have shown that the mean turnover times of macrophages in the liver and lungs (5, 6) and in the peritoneal cavity too (unpublished observation) are much more rapid than had been thought (1, 3, 4). This implies that under steady-state conditions the macrophage population is renewed rather

rapidly, and this in turn unquestionably has implications for the interpretation of the functional contribution of macrophages under many different conditions.

Summary

The present study concerns the isolation, characterization, origin, and kinetics of spleen macrophages. The spleen was first perfused in situ to remove monocytes from the vascular bed and then dissected and treated with collagenase. The macrophages in the cell suspension thus obtained were characterized morphologically and cytochemically and then quantitated. The spleen cell suspension was incubated for 24 h in Leighton tubes to obtain an enriched glass-adherent population of macrophages for characterization and [^{3}H]thymidine-labeling studies. Almost all of the adhering macrophages were esterase positive, had Fc and C3b receptors, and ingested EIgG and opsonized bacteria.

In vitro labeling with [^{3}H]thymidine showed that ~5% of the mononuclear phagocytes in the spleen synthesize DNA and must be considered to be dividing cells. The course of the number of labeled monocytes and macrophages after a single injection of [^{3}H]thymidine indicates migration of monocytes into the spleen, where they become macrophages.

Calculation of the influx of monocytes into the spleen and of the local production of macrophages by DNA-synthesizing mononuclear phagocytes showed that under steady-state conditions, 55% of the population of spleen macrophages is supplied by monocyte influx and 45% by local production. This means that there is a dual origin of spleen macrophages. The mean turnover time calculated with the value for the efflux of spleen macrophages is 6.0 d.

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