ORIGIN, KINETICS, AND CHARACTERISTICS OF PULMONARY MACROPHAGES IN THE NORMAL STEADY STATE

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Macrophages are part of the mononuclear phagocyte system, the cells of which originate in the bone marrow (1). In vertebrate animals, the cells of the lung comprise many different types (2); among them, pulmonary macrophages, mostly located in the alveolar airspaces, but also found in the interstitial tissue.

Three theories have been put forward to explain the origin of the pulmonary macrophages. One theory considers them to be end cells, derived from blood monocytes and incapable of division in the steady state (3, 4). According to another theory, a bone marrow-derived cell population in the interstitium of the lung produces pulmonary alveolar macrophages by local division and maturation (5, 6). In the third theory, monocytes mature in the lung capillaries and undergo a final mitosis in the alveoli, leading to alveolar macrophages (7).

The descent of alveolar macrophages from bone marrow precursor cells was concluded first from studies with radiation chimeras, using chromosome markers to identify the origin of the cells (8, 9), but this approach only allowed the analysis of a very small number of macrophages in mitosis. Later, cytotoxic antisera (4) and enzyme markers (10, 11) were used for studies in radiation chimeras; these techniques made it possible to establish the bone marrow origin of greater numbers of pulmonary macrophages. In man, the same descent was demonstrated by fluorescent Y-body staining of alveolar cells in bone marrow transplant recipients (12).

Because most kinetic studies on macrophages in the lung are based on either free lung cells harvested by lavage (4, 10–12) or on a combination of lavage cells and histological sections (5, 7, 8), true quantitation of the total pulmonary macrophage population and quantitative study of the origin and kinetics have been impossible. Furthermore, the characteristics of the macrophages in the alveoli and those in the interstitial tissue have never been analyzed comparatively to find out whether the precursor of the alveolar macrophage is a circulating monocyte or a macrophage in the interstitium.

The aim of the work reported here was to study the characteristics, origin, and kinetics of both pulmonary alveolar and tissue macrophages in mice in the normal steady state and to establish the presence or absence of a compartment for the production of macrophages in the pulmonary capillaries, the interstitial tissue, or the alveoli.

Materials and Methods

Animals. Specific pathogen free (SPF),[1] male, Swiss mice (Central Institute for the Breeding

[1] *Abbreviations used in this paper:* HBSS, Hanks' balanced salt solution; i.m., intramuscular; i.v., intravenous; M199; Medium 199; NBCS; newborn calf serum; PAM, pulmonary alveolar macrophages; PBS, phos-

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of Laboratory Animals, TNO, Bilthoven, The Netherlands) weighing 25–30 g were used. Each result is the mean of determinations in at least four animals.

Isolation of Pulmonary Alveolar and Tissue Macrophages. The isolation procedure comprised three phases: The lung circulation was first flushed to reduce the number of monocytes in the lung vessels (13) to a minimum. Next, the free alveolar cells were harvested by bronchial lavage. Lastly, the lung tissue was digested by an enzymatic method to obtain a cell suspension. In detail, the method used is as follows:

FIRST PHASE. Animals are anesthetized with 5 mg phenobarbital (Abbott N.V., Amsterdam, The Netherlands) intraperitoneally. The abdomen is opened and the diaphragm exposed and pierced. After collapse of the lungs, a triangular piece is cut out of the thoracic cage and the exposed aorta, esophagus, and inferior caval vein are cut. The right ventricle is then punctured with a 21-gauge needle on a syringe containing 5 ml of 0.6 mM EDTA in phosphate-buffered saline (PBS), and the contents are slowly injected into the beating heart. The major blood vessels in the neck and shoulders are cut to diminish backflow to the heart. During the perfusion the pink color of the lungs changes to pale white. If part of the lung remains pink, which indicates incomplete perfusion, the animal is discarded.

SECOND PHASE. The trachea is exposed and cannulated with a 20-gauge Teflon catheter (Abbocath-T, Abbott N.V.) connected to a three-way stopcock on which a syringe containing 15 ml lavage fluid at 37°C and an empty syringe for collection of fluid from the lungs are mounted. The lavage fluid consists of 0.6 mM EDTA in PBS. Portions of 1 ml lavage fluid at a time are introduced and recovered. The cells in the pooled lavage fluid from one mouse are counted directly in a Bürker hemocytometer in duplicate and then washed once in PBS. Viability is determined by trypan blue exclusion. Cytocentrifuge preparations (14) are made for the characterization of the cells. For culture, the cells are suspended in culture medium at a concentration of about 5×10^5 viable cells/ml.

THIRD PHASE. After the lavage procedure, a heart-lung preparation is dissected en bloc. The lungs are expanded with Hanks' balanced salt solution (HBSS) containing 0.2% wt/vol pronase (E. Merck, Darmstadt, West Germany), separated from mediastinal structures, cut into pieces measuring $\cong 1$ mm^3, and collected in a total of 15 ml of the pronase solution. Finer dispersion of tissue is obtained by repeated gentle passage through a 19-gauge needle. The mixture is then incubated for 1 h under constant stirring at pH 7.4 and 37°C. The pH is monitored and adjusted if necessary with 0.1 N sodium hydroxide. After 20 and 40 min of incubation, 0.5 mg DNA-ase (E. Merck) in 1 ml HBSS is added to digest cellular debris and prevent cell clumping. Next, the cell suspension is filtered through two layers of gauze, centrifuged for 10 min at 400 *g*, washed twice in HBSS, and suspended in culture medium at a concentration of 5×10^6 viable cells/ml. For the cells in this suspension, too, counts are performed, the viability is tested, and cytocentrifuge preparations are made.

Sterile and siliconized glassware is used throughout all isolation procedures.

Cell Culture. The cell suspensions were incubated in Leighton tubes with flying coverslips for the study of glass-adherent cells. The culture medium was Medium 199 (M199) (Microbiological Associates, Walkersville, Md.) containing 20% heat-inactivated newborn calf serum (NBCS) (Grand Island Biological Co., Grand Island, N. Y.), 2,000 U/ml sodium penicillin G, and 50 μg/ml streptomycin. The tubes were gassed with 5% CO_2 in air and incubated at 37°C for 24 or 48 h. The culture medium was then removed and kept to determine, when necessary, the number, the identity, and the characteristics of the nonadherent cells.

Cell Morphology and Enzyme Cytochemistry. The morphology of the cells obtained by lavage or enzyme digestion was studied in cytocentrifuge preparations and, after glass adherence, on coverslips. The preparations were washed 3–5 times with M199, rapidly dried in air, fixed in methanol for 10 min, and stained with Giemsa stain for 7 min. Peroxidase activity was determined by a modification of Kaplow's method, as described elsewhere (15). Esterase activity was determined with α-naphthyl butyrate (Sigma Chemical Co., St. Louis, Mo.) as substrate at pH 6.0 (15); the incubation time was 2 min. Glucose-6-phosphate dehydrogenase activity was determined with the use of tetranitroblue tetrazolium (Sigma Chemical Co.) as substrate at pH 7.0 (16).

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phate-buffered saline; PTM: pulmonary tissue macrophages; s.c., subcutaneous; SPF, specific pathogen free; SRBC, sheep erythrocytes.

Phagocytosis and Pinocytosis. The phagocytic activity of glass-adherent cells was studied with *Staphylococcus epidermidis* in the presence of NBCS, as described elsewhere (15). Phagocytosis of sheep erythrocytes (SRBC) coated with antibody with or without complement was studied in the experiments for the detection of Fc receptors and C3 receptors (see below). Pinocytosis of glass-adherent cells was studied with dextran sulphate as described elsewhere (15).

Fc and C3 Receptors. The presence of Fc and C3 receptors was detected on glass-adherent cells by the use of SRBC coated with heat-inactivated mouse anti-SRBC serum, and of SRBC coated with the IgM fraction of rabbit anti-SRBC serum and complement from fresh mouse serum, as described elsewhere (15).

Histological Preparations. The lungs were removed before or after lavage, and fixed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 48 h, embedded in methacrylate, processed further, and PAS- or Giemsa-stained (17). For the histochemical demonstration of esterase activity, 2-mm-thick slices of lung tissue fixed in 4% formaldehyde buffer were incubated with α-naphthyl butyrate as substrate at pH 6.0 for 6 h, postfixed for 24 h in 4% formaldehyde buffer, embedded in methacrylate, and further processed (17).

Labeling and Autoradiography Techniques. Labeling was carried out with tritiated methylthymidine (^{3}H-thymidine) with a sp act of 6.7 Ci/mmol (New England Nuclear, Boston, Mass.). For in vitro labeling, 0.1 μCi/ml ^{3}H-thymidine was added to all solutions used after the first phase of isolation and to the culture medium. For in vivo labeling, either a single intravenous (i.v.) injection of 25 μCi ^{3}H-thymidine or four intramuscular (i.m.) injections of 25 μCi ^{3}H-thymidine at 4-h intervals were given. Autoradiography of cell preparations was performed as described elsewhere (18). More than 1,000 cells were counted per mouse; cells with 3 or more grains over the nucleus were considered positive.

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

X-Irradiation. Mice were anesthetized with 2 mg phenobarbital intramuscularly and taped to soft wooden board; both hind limbs and part of the pelvis were shielded with 4-mm Pb shields. The irradiation dose was 650 rad given by one unit (Siemens Stabilipan, Siemens, Erlangen, West Germany; 240 kV, 15 mA, source-skin distance 40 cm, half-value layer 3, 1 mm Cu, dose rate: 70 rad/min).

Nomenclature. The mononuclear phagocytes in the suspension of cells isolated from the airspace of the lungs by lavage are called pulmonary alveolar macrophages (PAM). The mononuclear phagocytes present in the cell suspension obtained by enzyme digestion of lung tissue after lavage, are called pulmonary alveolar macrophages (PAM) and pulmonary tissue macrophages (PTM), according to whether they were located in the alveolar airspace or pulmonary interstitium before isolation.

Results

Yield of Cells by Lavage and Subsequent Enzyme Digestion of Lung Tissue. Lavage was performed up to a total volume of 15 ml, since larger amounts caused progressive leakage. Experiments were discarded if more than 5 ml of the introduced fluid were lost during recovery. The average recovered volume was 12 ml. In preliminary experiments physiological saline, PBS, M199, and culture medium were compared as lavage fluid. The best cell recoveries were obtained with PBS, i.e., $\cong 1 \times 10^5$ alveolar cells per mouse. Because the presence or absence of Ca^{++} and Mg^{++} ions in lavage fluid influences the cell yield (19), a chelating agent was added to the lavage fluid: 0.6 mM EDTA in PBS increased the cell yield considerably without affecting the viability of the cells. Lavage fluid of this composition was used in all experiments reported here. The cell yield for 24 normal mice in the steady state was 1.39×10^6 cells per mouse (Table I) with a viability of >98%. After lavage, the lung tissue was subjected to enzyme digestion, which gave a yield of 21.37×10^6 cells per mouse and a viability of >94%. The total number of cells recovered from the lungs was 23.09×10^6 cells per mouse (Table I).

TABLE I
*Pulmonary Cells Isolated in the Steady State**

Isolation	Cells isolated	Macrophages among cells isolated	
	$\times 10^6$	%	$\times 10^6$
Lavage‡	1.39 ± 0.36	97.80 ± 0.72	1.37 ± 0.35
Enzyme digestion§	21.37 ± 4.0	2.73 ± 0.77	0.58 ± 0.19
Total	23.09 ± 4.0		1.95 ± 0.41

* Results are given as mean ± SD; $n = 24$.
‡ Lavage with 15 ml PBS containing 0.6 mM EDTA.
§ Incubation of fragmented lung tissue (after lavage) for 60 min at 37°C in HBSS containing 0.2% (wt/vol) pronase; 0.5 mg DNA-ase added at 20 and 40 min.

Morphology and Quantitation of Pulmonary Macrophages. In Giemsa-stained cytocentrifuge preparations of the cell suspensions obtained by lavage, the PAM were fairly homogeneous in size (8–13 × 12–17 μm) and oval or round in shape, with a basophilic cytoplasm often containing black inclusions (probably dust), and an eccentric oval or bean-shaped nucleus containing two or three darker-stained nucleoli. About 1% of the PAM contained two or more nuclei. In cytocentrifuge preparations of enzyme-digested lung tissue the macrophages (PAM and PTM) showed sizes and shapes comparable to those of the PAM isolated by lavage (Fig. 1a); they rarely contained two or three nuclei. Among the thousands of cells observed, only once a mitotic figure was seen in the cells isolated by lavage and only twice in the cells isolated by enzyme digestion.

To determine the total number of macrophages, differential cell counts were performed in the cytocentrifuge preparations of both types of suspension and the percentage of macrophages was established (Table I). The total number of pulmonary macrophages per mouse (isolated by lavage and enzyme digestion) was 1.95×10^6 (Table I). Although conditions for the identification of other cell types were not optimal, the cell suspension of enzyme-digested tissue showed 5–10% type II alveolar epithelial cells, 3–7% granulocytes, and 15–20% lymphocytes.

The isolated cells were cultured on glass for 24 h to allow maximal attachment. The number of viable pulmonary macrophages among the non-adherent cells removed after 24 h incubation was <3% and <6.5% of the original number of macrophages in the cultures of PAM and PAM plus PTM, respectively. The adherent cell population in lavage fluid was composed solely of macrophages. In cultures of the suspension obtained by enzyme digestion, most of the adherent cells were macrophages, but some granulocytes and fibroblasts were also present. The number of the latter increased during subsequent culture; these cells were recognized by their specific morphology, their inability to ingest bacteria, and their divergent cytochemical staining. No mitoses were seen in the adherent macrophages. No type II alveolar epithelial cells or bronchial epithelial cells were found among the adherent cells. After the first 24 h of culture, no spreading of macrophages was observed; but at later times, a small number of macrophages spread out.

Histology. The general structure of the lungs was well retained after flushing of the pulmonary circulation and lavage of the airways (Fig. 1b). A few erythrocytes but no monocytes were seen in the blood vessels. Esterase staining showed esterase-positive cells in the interstitial tissue, among the cells lining the airways, and in the airspace, but not in the blood vessels. Differentiation between macrophages and type II alveolar

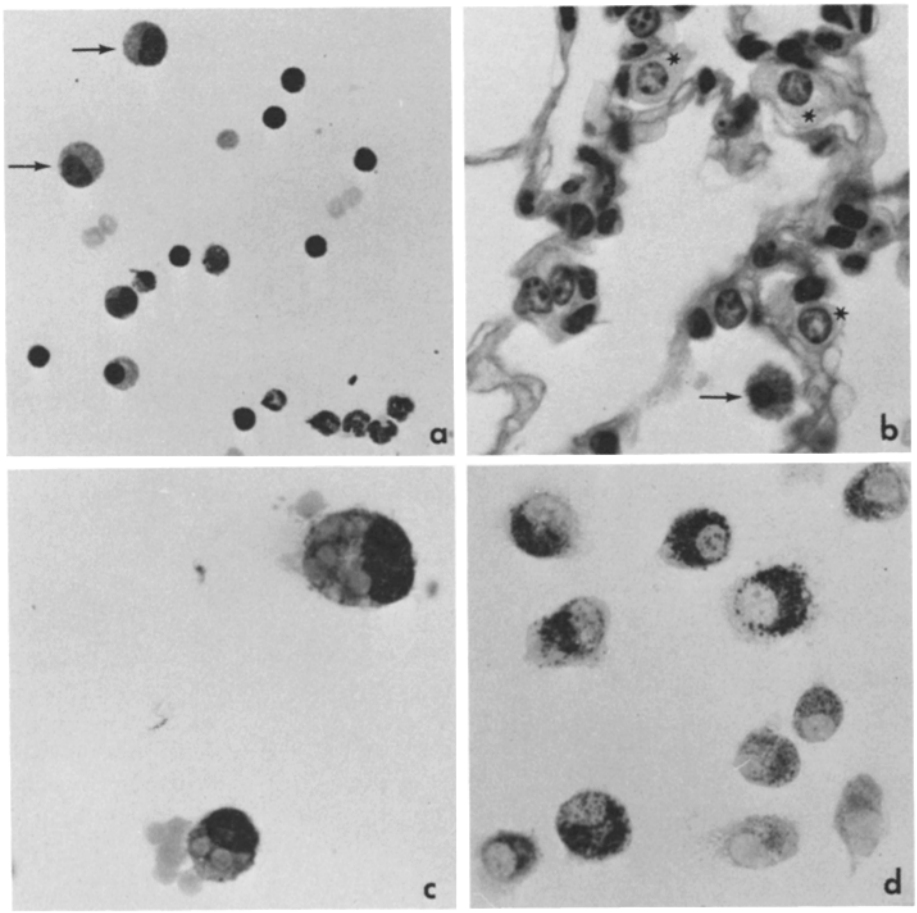

FIG. 1. Mouse pulmonary macrophages. (a) Giemsa-stained cytocentrifuge preparation of a cell suspension obtained by enzyme digestion of lung tissue. Arrows indicate pulmonary macrophages (×630). (b) PAS-stained section of methacrylate-embedded lung tissue after lavage. Arrow indicates a pulmonary macrophage still present in the alveoli; asterisk indicates type II alveolar epithelial cells (×800). (c) Giemsa-stained 24-h culture of PAM isolated by lavage; rosette formation and ingestion of IgG-coated SRBC (×1,000). (d) Toluidine blue-stained 24-h culture of PAM and PTM isolated by enzyme digestion; pinocytosis of dextran sulphate (×800).

epithelial cells on the basis of morphology and esterase staining (see below) was only partially possible.

Cytochemistry. In cytocentrifuge preparations of lavage-fluid the macrophages were all clearly positive for esterase (Table II) and weakly positive for glucose-6-phosphate dehydrogenase. In such preparations of enzyme-digested cell suspensions, type II alveolar epithelial cells and bronchial epithelial cells were present and stained very intensely for esterase and strongly for glucose-6-phosphate dehydrogenase. These epithelial cells could thus be differentiated from macrophages on the basis of both morphology and staining. Peroxidase staining of cells obtained by lavage and enzyme digestion showed that virtually none of the macrophages contained peroxidase-positive granules (Table II).

Immune Receptors. Fc receptors were present on most of the PAM isolated by lavage

TABLE II

Cytochemical and Functional Characteristics of Pulmonary Macrophages

Characteristics	PAM* cultured		PAM and PTM‡ cultured	
	24 h	48 h	24 h	48 h
	%		%	
Esterase activity§	100	100	100	100
Peroxidase‖	0.9	0.2	1.1	0.2
Fc receptor¶	72.4	87.1	55.9	83.2
C receptor¶	2.2	6.6	16.0	28.9
Phagocytosis				
Opsonized bacteria**	94.2	98.0	92.3	95.6
IgG-coated SRBC	61.5	76.3	41.4	65.8
IgMC-coated SRBC	1.8	3.6	3.9	13.0
Pinocytosis‡‡	89.7	92.9	91.7	93.6

* Macrophages isolated by lavage.
‡ Macrophages isolated by enzyme digestion.
§ Diffuse cytoplasmic staining; α-naphthyl butyrate as substrate.
‖ Located in granules; determined by the Kaplow method.
¶ Cells with SRBC rosettes (and[or] ingested SRBC).
** Ingestion of opsonized *S. epidermidis*.
‡‡ Dextran sulphate (mol wt 500,000).

(Fig. 1c) and most of the PAM and PTM isolated by enzyme digestion (Table II). Very few of the PAM isolated by lavage carried C3 receptors. The PAM and PTM isolated by enzyme digestion showed a higher percentage of C3-receptor-carrying cells (Table II).

To determine whether the slightly different percentages of Fc-receptor-carrying macrophages in the population of PAM and that of PAM and PTM were due to the pronase treatment or to a difference in the membrane receptors of PAM and PTM, a pure PAM population isolated by lavage was incubated for 1 h with pronase and DNA-ase under the conditions for enzyme digestion. When these cells were cultured on glass for 24 and 48 h, the percentage carrying Fc receptors was 55 and 75%, respectively, which is about equal to that of the population of PAM and PTM isolated by enzyme digestion. This indicates that the Fc receptors are slightly affected by the enzyme treatment, and in all probability, the percentage of macrophages carrying Fc receptors is similar for the cells isolated by lavage and those isolated by enzyme treatment.

The influence of pronase treatment on C3 receptors was similarly investigated: the percentage of PAM with C3 receptors after lavage and pronase treatment was 7 and 6% after 24 and 48 h, respectively; i.e., similar to that of nonenzyme-treated PAM, which indicates a real difference in the detection of C3-receptor-carrying cells in the two populations.

Phagocytosis and Pinocytosis. Almost all of the macrophages ingested opsonized bacteria, whether isolated by lavage or by enzyme digestion (Table II). Ingestion of IgG-coated SRBC (Fig. 1 c) was lower, but increased when the macrophages were cultured longer before phagocytosis (Table II). Ingestion of IgMC-coated SRBC was low in both the PAM population and the population containing PAM and PTM (Table II). Most of the PAM isolated by lavage and the PAM and PTM isolated by enzyme digestion (Fig. 1 d) pinocytised dextran sulphate (Table II).

TABLE III

In Vitro Labeling of Pulmonary Macrophages in the Steady State and after Hydrocortisone*

Time after hydrocortisone‡	PAM§ incubation time			PAM and PTM‖ incubation time		
	2 h	24 h	48 h	2 h	24 h	48 h
h		%			%	
0¶	2.7	2.3	2.0	2.9	2.1	1.9
6		2.8			2.2	
12		1.8			1.9	
24		0.4			0.4	
48		0.1			0.7	

* All fluids used after the first phase of isolation and the culture medium contained 0.1 μCi/ml ^{3}H-thymidine.
‡ s.c. injection of 15 mg hydrocortisone.
§ Macrophages isolated by lavage.
‖ Macrophages isolated by enzyme digestion.
¶ Normal steady state.

In Vitro Labeling Studies in the Steady State. In mice in the steady state, the in vitro labeling index of pulmonary macrophages was <3% after 2 h of incubation and did not increase with longer incubation. The indices were equal in macrophages isolated by lavage and by enzyme digestion (Table III). This means that the mitotic activity of freshly isolated pulmonary macrophages in the normal steady state is very low.

In Vitro Labeling Studies after the Administration of Hydrocortisone Acetate. Hydrocortisone acetate in high doses causes severe monocytopenia (20) and arrests the efflux of monocytes from the bone marrow, but does not affect the mitotic activity of promonocytes (21). When pulmonary macrophages isolated at various times after s.c. injection of 15 mg hydrocortisone acetate were labeled in vitro, the labeling index fell to very low values (Table III). This implies that the small number of DNA-synthesizing pulmonary macrophages found under normal conditions diminishes considerably when the efflux of monocytes from the bone marrow is arrested.

In Vivo Labeling Studies in the Steady State. The labeling index of pulmonary macrophages in mice given one i.v. injection of ^{3}H-thymidine was established at various times after the injection (Fig. 2). After 1 h, the labeling index was 2.9%, which is comparable to the in vitro value. The labeling index increased to a maximum of 10.5% at 48 h and decreased at later time points. The highest values coincide with the highest value of the labeling index of blood monocytes in these mice (Fig. 2). Labeling indices after four i.m. injections of ^{3}H-thymidine at 4-h intervals, showed a maximum of 20% at 60 h, followed by a gradual decline (Fig. 3). No differences were found between the labeling indices of macrophages isolated by lavage or enzyme digestion.

In Vivo Labeling Indices after the Administration of Hydrocortisone Acetate. When the mice were given hydrocortisone acetate 3 h after the last of four i.m. injections of ^{3}H-thymidine, the labeling index was about equal to that in untreated mice at 12 h, but decreased at later time points (Fig. 3). Here again, the same labeling indices were seen in the macrophages isolated by lavage and those isolated by enzyme digestion.

The Effect of Total Body X-Irradiation with Partial Shielding of Bone Marrow on the Labeling Index of Pulmonary Macrophages In Vivo. Between 24 and 36 h after x-irradiation the mice received four i.m. injections of ^{3}H-thymidine. The total number of circulating monocytes decreased to about 25% of the normal value at 48 h after irradiation and

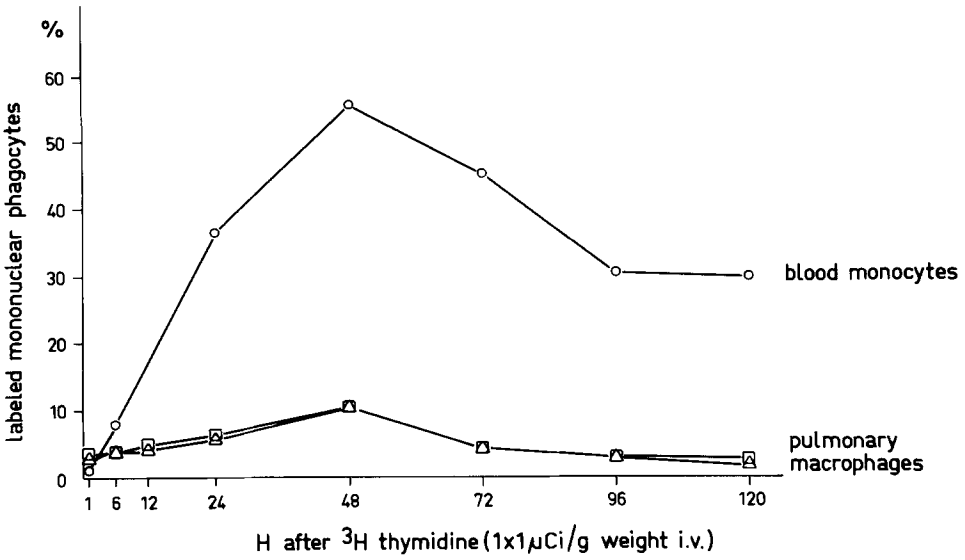

FIG. 2. Labeling indices of blood monocytes and pulmonary macrophages after a single i.v. injection of ^{3}H-thymidine. ○, blood monocytes; □, macrophages isolated by lavage (PAM); △, macrophages isolated from enzyme-digested lung tissue (PAM and PTM).

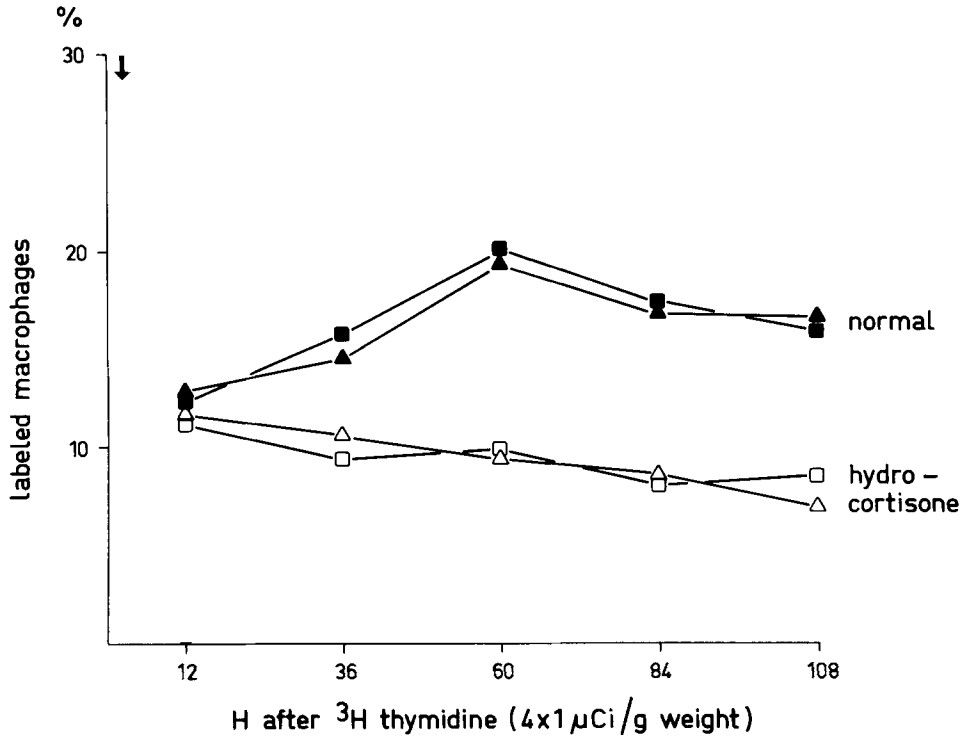

FIG. 3. Labeling indices of pulmonary macrophages after four i.m. injections of ^{3}H-thymidine. □, macrophages isolated by lavage (PAM); △, macrophages isolated from enzyme-digested lung tissue (PAM and PTM). "normal": mice in normal steady state; "hydrocortisone": 3 h after the last i.m. injection of ^{3}H-thymidine, 15 mg hydrocortisone acetate was given s.c. (arrow).

TABLE IV

*Numbers and Labeling Indices of Blood Monocytes and Pulmonary Macrophages After X-Irradiation with Partial Bone Marrow Shielding**

Time after ^{3}H-thymidine‡	Number of circulating monocytes	Labeling index of monocytes	Total number per mouse		Labeling index of macrophages	
			PAM§	PAM and PTM‖	PAM§	PAM and PTM‖
h	*per mm*^{3}	%	× 10^6		%	
Steady state	350		1.37	0.58		
12	95	60	1.39	0.96	0.4	0.5
36	156	75	1.13	0.80	2.1	2.3
60	82	76	1.27	0.64	2.7	2.9
84	96	80	0.99	0.55	2.4	3.3
108	100	60	0.96	0.60	1.8	2.4
132	90	58	0.89	0.66	1.6	1.6

* X-irradiation (650 rad) with shielding of hind limbs, 24 h before the first i.m. injection of ^{3}H-thymidine.
‡ Four i.m. injections of 25 μCi ^{3}H-thymidine given within 12 h.
§ Macrophages isolated by lavage.
‖ Macrophages isolated by enzyme digestion.

remained at that level for the next 4 d (Table IV). The labeling indices of the monocytes at the various time points (Table IV) were similar to those found in the steady state (15, 18); i.e., the number of labeled monocytes in the circulation was very low. The total number of pulmonary macrophages was slightly higher than normal on the 2nd d after x-irradiation, but decreased gradually to subnormal levels at later time points (Table IV). The labeling indices of the pulmonary macrophages were considerably lower than normal (Table IV, compare with Fig. 3). Since the lungs were irradiated at a dose which inhibits proliferation of mononuclear phagocytes, the labeled pulmonary macrophages must represent newly formed monocytes produced in the shielded part of the bone marrow. The labeling indices of the PAM population and the population containing PAM and PTM were similar for each time point, thus reflecting a random distribution of the newly arrived cells over the lungs.

Discussion

The present study shows that both PAM and PTM have the characteristics of mononuclear phagocytes similar to those of other macrophages studied in mice in the steady state (15, 22). The data from the in vitro and in vivo labeling studies with ^{3}H-thymidine provide conclusive evidence that in the steady state neither PAM nor PTM proliferate in a compartment in the lung but are derived from circulating monocytes, as has been established for murine peritoneal macrophages (18) and Kupffer cells (15).

The isolation method described here provides a means to study all macrophages in the lung and not just some of the alveolar macrophages. A critical component of this method is the vascular perfusion carried out initially, since the numerous monocytes present in the lung circulation (13) may contaminate the harvested pulmonary macrophages. The vascular perfusion with PBS containing 0.6 mM EDTA (to prevent clotting and diminish adhesion to vessel walls) is, however, very effective in washing out the monocytes, as shown by histological sections of perfused lungs. Furthermore,

hardly any mononuclear phagocytes with peroxidase-positive granules were seen in the cytocentrifuge or 24-h culture preparations of enzyme-digested tissue, whereas under similar conditions most blood monocytes still have peroxidase-positive granules (22).

The lavage procedure provides a cell suspension containing only PAM. For optimal recognition of possible differences in characteristics between PAM and PTM, it is necessary to have a minimal number of PAM in the suspension of PTM and PAM obtained by enzyme digestion. This is sought by ensuring a maximal cell yield in the lavage procedure before enzyme digestion of the lung tissue, which contains all PTM and those PAM that were not washed out during the lavage procedure. To obtain macrophages from lung tissue, pronase treatment is used, as in the isolation of Kupffer cells from liver tissue (15). Pilot studies showed that alveolar macrophages were resistant to pronase in 0.2% solution and that survival amounted to more than 95%. A large number of other lung cells also survive pronase treatment; satisfactory differentiation of macrophages from other cells can be performed on cytocentrifuge preparations by morphology and cytochemistry.

The total number of pulmonary macrophages isolated from SPF Swiss mice in the steady state is about 2×10^6 per mouse or about 8×10^4/g body wt. Masse et al. (7) found a total of about 1×10^5 pulmonary macrophages/g body wt in SPF rats, estimated on the basis of lavage and histology, and Godleski and Brain (4), using only lavage, isolated 3.3×10^4 PAM/g body wt in C57B mice. To our knowledge, no estimation of the total number of pulmonary macrophages in mice has been made.

Almost all of the macrophages isolated by lavage and enzyme digestion are viable; morphological, cytochemical, and functional studies can be performed as usual for comparison to other mononuclear phagocytes. The pulmonary macrophages are positive for esterase and negative for peroxidase, carry Fc receptors, and show avid phagocytosis and pinocytosis. The study of immune receptors is slightly hampered by pronase treatment (15), but no difference in Fc receptors is found between the macrophages in the suspension containing PAM only and the suspension with both PAM and PTM, if the results of the control experiments on the influence of pronase treatment are taken into account.

Unlike other mature macrophages, those in the lavage fluid rarely carried C3 receptors, and when they did, very few coated SRBC adhered. Failure to demonstrate C3 receptors on murine (22) and guinea pig (23) alveolar macrophages has been reported earlier, but in rabbits (24) and humans (24–26) the presence of these receptors has been shown with similar techniques. It is probable that failure to demonstrate C3 receptors on alveolar macrophages is caused by their exposure to the micro-environment in the alveoli. It may then be expected that the pulmonary macrophages in the interstitium (PTM), that have not been in contact with the alveolar environment, carry C3 receptors in their cell membrane, like the other tissue macrophages of the mouse (22). The percentage of macrophages with C3 receptors in the population isolated by enzyme digestion was only 28.9% (Table II) instead of 100%, so the number of PAM left behind after lavage and lacking C3 receptors, is apparently rather large. Since C3 receptors are restored by incubation for 48 h after pronase treatment (15), we may assume that at that time 100% of the PTM and 6.6% of the PAM (see Table II) carry C3 receptors. Calculated with these values, the population isolated by enzyme digestion comprises 23% PTM and 77% PAM. This would mean

that $0.23 \times 0.58 \times 10^6 = 0.13 \times 10^6$ macrophages, or 7% of the total pulmonary macrophage population (see Table I), is located in the interstitial lung tissue. This value is in agreement with the results of morphometric studies of mouse lung tissue (27) and quantitative electron-microscopical studies of rat lungs (7) in which 2.5% of the cells counted were PTM.

The results of the in vitro labeling experiments confirm the findings of other studies in mice (5, 6) and humans (28) indicating that the labeling index in unactivated alveolar macrophages is very low. The low percentage of cells synthesizing DNA in both the suspension containing PAM and the suspension containing PAM plus PTM argues against multiplication of macrophages within the lung as a mode of population renewal and against local proliferation in the interstitial tissue specifically. Further evidence supporting this conclusion is provided by the decrease in the in vitro labeling index after an injection of hydrocortisone acetate. The cells that incorporate ^{3}H-thymidine apparently belong to the group newly recruited from the bone marrow, because hydrocortisone acetate causes a rapid and deep monocytopenia (20, 29) (thus preventing the delivery of monocytes to the tissues), but does not arrest the mitotic activity of promonocytes (21).

The in vivo labeling studies have shown an increase in the number of labeled cells that can only be attributed to labeled monocytes migrating from the circulation into the lungs, since the labeling index found 1 h after i.v. injection of ^{3}H-thymidine (representing the maximal number of macrophages able to divide at any one time) is too low to account for these numbers. Support for the monocytic origin of pulmonary macrophages was obtained from the experiments in which hydrocortisone acetate was given shortly after labeling: as soon as the influx of monocytes into the tissue stopped, there was a steady decrease of the labeling indices in the PAM population and the population containing PAM and PTM. Additional evidence for the monocytic origin of pulmonary macrophages was found in the animals x-irradiated with partial shielding of bone marrow: labeled PAM and PTM were seen (the indices reflecting the lower output of labeled bone marrow monocytes) in a situation where local proliferation in the exposed lung is impossible.

Comparison of these data with other investigators' results (considered by some authors to support the presence of an interstitial division and maturation compartment [5, 6, 30]) clarifies the issue further. For instance, Pinkett et al. (8) found a mean of only 2.4 mitoses per mouse in the pulmonary macrophages when no adjuvant or irritant was given and only 14 mitoses per mouse after administration of both stimuli, thus demonstrating the paucity of dividing macrophages in the lung in the steady state and after stimulation. Virolainen (9), Soderland and Naum (31), and Naum (32) induced proliferation of unstimulated alveolar macrophages in vitro after a lag of several days by adding different conditioned media to the cultures, but found no signs of proliferation in the absence of these stimuli. In comparable experiments, Lin et al. (33) found that, at most, 8% of the alveolar macrophages could be stimulated to proliferate. In cultures of lung-tissue explants, Bowden et al. (5, 6) found proliferating pulmonary macrophages concurrently with decomposition of the lung tissue and rapid multiplication by fibroblasts. When the explants were taken out of the cultures, division of macrophages ceased. Other investigators (2, 34, 35) found few macrophages in the interstitium of the lung and no mitotic figures. Taken together, these results indicate that pulmonary macrophages can be induced to proliferate in vitro under

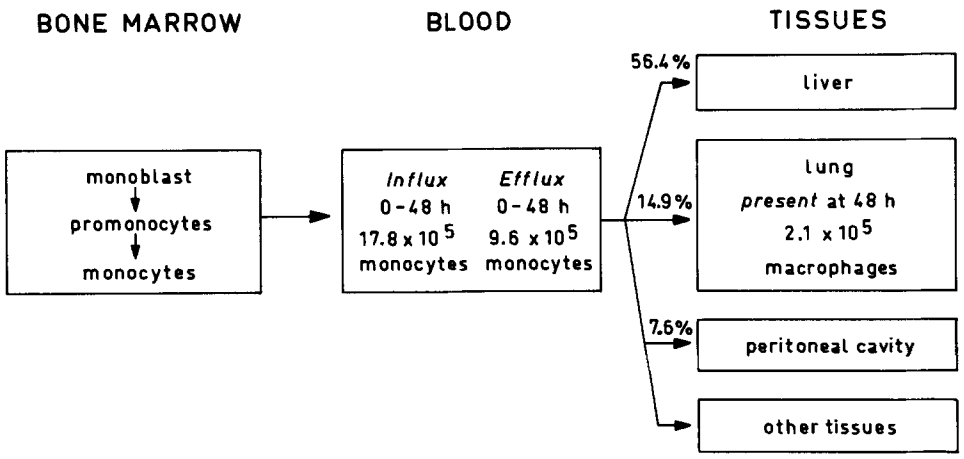

FIG. 4. Kinetics of labeled monocytes in the blood compartment, and migration of these cells into the lungs during the normal steady state. For comparison, the influx into the liver and the peritoneal cavity are given (from references 15 and 36).

certain conditions, but provide no evidence for the presence in the normal steady state of dividing precursors of macrophages in the pulmonary interstitial tissue.

Masse et al. (7) and Fritsch et al. (13) suggested, on the basis of experiments in rats, that monocytes divide in the pulmonary capillaries or the alveoli to give rise to alveolar macrophages. Since the generation time of promonocytes in SPF Swiss mice is ≅16 h (36), and the generation time of a dividing monocyte would presumably be of the same order, the peak labeling index for pulmonary macrophages, should, if their hypothesis were correct, be reached later than that for peripheral blood monocytes. The similarity found in the present study between the time courses for labeled monocytes and pulmonary macrophages, and the very low in vitro labeling indices of both cell types (22), exclude the existence of a compartment where monocytes from the alveolar capillaries undergo division in mice in the steady state.

Most of the macrophages in the lung leave the alveoli by way of the airways and are expelled via the mucociliary pathway (19, 37), but there are indications that PTM may leave the interstitium by way of the lymphatic channels (38, 39) or perhaps the blood vessels (19), or travel locally to the bronchus-associated lymphoid tissue, where they might enter the airways at the level of the terminal bronchioli (40). Although our results do not provide any additional evidence for the proposed pathways of clearance, they do support the conclusion that there is no significant subpopulation of pulmonary macrophages having different kinetics, because such a subpopulation would have caused differences in labeling indices between the two populations in vivo.

Since the total number of macrophages in the lung is known from the present study, the input of monocytes needed to maintain a constant population in the steady state can be calculated (15, 36, 41) by using the total calculated efflux of labeled monocytes

from the circulation in the first 48 h after a single ^{3}H-thymidine injection, the number of labeled macrophages present in the lung at 48 h, corrected for the 1-h labeling index, and a half time of 17.4 h for blood monocytes (36). The data in Fig. 4 show that about 15% of the monocytes that leave the circulation become pulmonary macrophages. If we assume that the total population of pulmonary macrophages is kinetically homogeneous, the calculated influx of 1.43×10^5 cells in a 48-h period gives a mean turnover time of 27 d for pulmonary macrophages. These data fit well with the 21–28 d found by Godleski and Brain (4) and are comparable to the mean turnover time of 27 d established in SPF rats (42).

Summary

Pulmonary macrophages of mice in the steady state were isolated by lavage with PBS containing EDTA and subsequent enzymatic digestion of tissue with pronase and DNA-ase. By this method, the total pulmonary macrophage population was obtained in two cell suspensions, one with a pure population of pulmonary alveolar macrophages (PAM) and the other with a mixed population of pulmonary alveolar and pulmonary tissue macrophages (PTM). The morphological, cytochemical, and functional characteristics of both PAM and PTM were like those of mature tissue macrophages except for the presence of C3 receptors. These receptors were almost absent on PAM and present on a larger number of the cells in the mixed population of PAM and PTM. The total pulmonary macrophage population of mice in the steady state is $\cong 2 \times 10^6$, of which about 93% are PAM and about 7% are PTM.

In labeling experiments with ^{3}H-thymidine, the low in vitro labeling indices (<3%) for both PAM and the mixture of PAM and PTM, showed that both are essentially nondividing cells. In vivo labeling studies showed an increase in the number of labeled macrophages that can only be attributed to labeled monocytes migrating into the lungs. Additional evidence was provided by a decrease in the labeling indices of pulmonary macrophages when mice were treated with hydrocortisone acetate, which causes a severe monocytopenia, thus preventing monocyte influx into the lungs. Confirmation of the bone marrow origin was obtained in mice labeled after x-irradiation with partial bone marrow shielding: labeled pulmonary macrophages were found in the exposed lungs. In all experiments, the labeling indices were identical in the two macrophage populations isolated.

These results show that the influx of monocytes is the source of cell renewal for the pulmonary macrophages. No indications for an interstitial division or maturation compartment in the lung were found. Quantitation of the efflux of labeled monocytes from the blood, and the number of labeled pulmonary macrophages, showed that in the steady state about 15% of the monocytes leaving the circulation become pulmonary macrophages and that the turnover time of pulmonary macrophages is $\cong$27 d.

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