ORIGIN AND KINETICS OF PULMONARY MACROPHAGES DURING AN INFLAMMATORY REACTION INDUCED BY INTRAVENOUS ADMINISTRATION OF HEAT-KILLED BACILLUS CALMETTE-GUÉRIN*

By A. Blussé van Oud Alblas, B. van der Linden-Schrever, and R. van Furth

From the Department of Infectious Diseases, University Hospital, Leiden, The Netherlands

The main defenders of the respiratory organs against microorganisms and other foreign substances are the pulmonary macrophages. The majority of these cells, which belong to the mononuclear phagocyte system (1), are normally located in the alveolar spaces, where they phagocytize surfactants (2) and various substances introduced via the airways; the remainder occur in the interstitial lung tissue (3). The origin and kinetics of the pulmonary macrophages have been controversial, mostly because of differences in the interpretation of DNA-labeling characteristics in the absence of accurate quantitative information. Recently, a method developed to study the total pulmonary macrophage population by optimal lavage of the airways followed by enzyme digestion of lavaged lung tissue after removal of circulating monocytes from the pulmonary blood vessels enabled us to demonstrate that the great majority of the pulmonary macrophages of mice in the normal steady state derive from circulating monocytes originating in the bone marrow (4), and that local proliferation of mononuclear phagocytes does not play a significant role in the maintenance of the pulmonary macrophage population.[1]

The origin and kinetics of the increased number of pulmonary macrophages during acute inflammatory reactions are, however, still a matter of debate. On the basis of studies done of inflammation induced by various stimuli ranging from inert particles to pathogenic microorganisms and noxious gases in various animal models, both an influx of circulating monocytes and interstitial multiplication of macrophages or macrophage-precursor cells have been claimed to contribute to the pulmonary macrophage population under these conditions (5–9).

The present report concerns the macrophage kinetics during an inflammatory reaction in the lungs after the intravenous injection of heat-killed bacillus Calmette-Guérin (BCG).[2] The kinetic patterns were studied by following the course and determining the DNA-labeling characteristics of both the circulating monocytes and the total macrophage population, i.e., the alveolar and interstitial macrophages.

* Supported by the Netherlands Asthma Fund.

[1] Blussé van Oud Alblas, A., H. Mattie, and R. van Furth. A quantitative evaluation of pulmonary macrophage kinetics. Manuscript submitted for publication.

[2] *Abbreviatons used in this paper:* AML, alveolar-macrophage-like; BCG, bacillus Calmette-Guérin; C, complement; HC, hydrocortisone acetate; NAML, non-alveolar-macrophage-like; t_s, DNA-synthesis time; ZN, Ziehl-Neelsen.

Materials and Methods

Animals. Specific-pathogen-free male Swiss mice (Central Institute for the Breeding of Laboratory Animals, TNO, Bilthoven, The Netherlands), weighing ~25 g, were used. All values given represent the mean of determinations in at least four animals.

Inflammatory Stimulus. Heat-killed, lyophilized BCG were derived from a supply donated by Dr. R. W. Schaedler of The Rockefeller University, New York in 1965. Each animal received 0.25 ml of a suspension of 1 mg/ml BCG in phosphate-buffered saline by injection into a tail vein.

Blood Leukocyte Counts. Two blood samples per animal were taken from a tail vein and total leukocyte counts were done in duplicate for each sample. Blood smears were made and differential counts were performed on at least 1,000 leukocytes in at least four smears. For the calculation of the numbers of blood leukocytes per mouse, the blood volume was estimated at 12.1 ml/100 g body weight (10).

Isolation of Pulmonary Macrophages. The procedure used to isolate all pulmonary alveolar macrophages and pulmonary tissue macrophages from the lungs is described in detail elsewhere (4), and, in short, is as follows: First, the thorax of the anesthetized animal is opened, and the pulmonary circulation is flushed with saline containing EDTA to remove the monocytes from the lung vasculature. Next, alveolar macrophages are collected by repeated lavage with the same saline solution, and finally, a suspension containing all remaining macrophages is obtained by pronase digestion of the lavaged lung tissue.

The macrophages among the cells harvested by lavage are exclusively pulmonary alveolar macrophages, whereas those in the suspension of enzyme-digested lung tissue are pulmonary tissue macrophages plus any alveolar macrophages not removed by lavage (4).

The cells obtained by lavage and enzyme digestion are counted, their viability is tested by trypan blue exclusion, differential cell counts are done in cytocentrifuge preparations, and the cells are cultured in Leighton tubes with flying cover slips (4). After incubation, the cover slips are washed thoroughly to remove nonadherent cells and rapidly dried in air. Numbers of pulmonary macrophages harvested are given per mouse.

Characterization. The morphological, cytochemical, and functional criteria used for the identification of monocytes and macrophages were as described elsewhere (4). Cells that had phagocytosed BCG were examined after Ziehl-Neelsen (ZN) staining for acid-fast microorganisms.

Glucocorticosteroids. Hydrocortisone acetate (kindly provided by Merck, Sharp & Dohme, Haarlem, The Netherlands) in a dose of 15 mg per mouse was injected subcutaneously.

Labeling and Radioautography. Tritiated methylthymidine ([^{3}H]thymidine) with a 6.7 Ci/mmol sp act (New England Nuclear, Boston, Mass.) was used throughout. The procedures used for in vitro and in vivo labeling and radioautography have been described in detail elsewhere (4). The preparations were stained with Giemsa stain to study cell morphology and with ZN stain for the recognition of acid-fast microorganisms. In each experiment at least 1,200 cells from each suspension were counted in several preparations. Cells with three or more grains over the nucleus were considered to be labeled.

Histology. Lung specimens for histological studies after BCG injection were fixed, and then embedded in methacrylate; 2-μm sections were cut and processed as described elsewhere, (11). The preparations were stained with Giemsa and periodic acid-Schiff's stains for light-microscope morphology and with auramine-rhodamine for the demonstration of BCG by fluorescence microscopy.

Results

Course of Circulating Leukocytes and Macrophages and Granulocytes in the Lungs after Intravenous Administration of BCG. The course of the circulating leukocytes after intravenous injection of BCG shows an increase in the number of monocytes from 1.6 $\times 10^6$ (normal) to 3.0 $\times 10^6$ per mouse within 48 h, the gain being largely maintained during the observation period, and an early granulocytosis lasting 24 h (Fig. 1A);

there was only a relatively small increase in the number of lymphocytes (data not shown).

The total number of cells isolated from the lungs by lavage and enzyme digestion rose within 12 h after BCG administration from 22×10^6 to $>32 \times 10^6$ cells and diminished gradually after 48 h to $\sim 25 \times 10^6$ at 144 h after the injection (data not shown). The increase was caused mainly by the increased number of cells isolated by enzyme digestion of lavaged lung tissue. In these suspensions, only macrophages and granulocytes could be recognized with certainty, and therefore the quantitation of cells was confined to these two cell types.

The total number of pulmonary macrophages (normally, $\sim 2 \times 10^6$ cells) showed an initial increase followed by a temporary decrease from 12 to 24 h and then a rise to maximal levels ($\sim 5 \times 10^6$ cells per mouse) at 96–120 h after BCG injection (Fig. 1 B). The total number of granulocytes in the lungs (normally, $\sim 1 \times 10^6$) peaked to $>6 \times 10^6$ at 2 h after BCG injection, remained fairly high during the next 48 h, and then dropped gradually to a near-normal level at 96 h after BCG injection. (Fig. 1 B).

In the suspension obtained by enzyme digestion, the number of macrophages rose during the first 12 h from 0.6×10^6 to $>3 \times 10^6$ cells and declined at later times to about twice the normal value at 144 h (Fig. 2 A), and this suspension contained virtually all of the granulocytes present in the lungs (data not shown). In the suspension obtained by lavage, the number of macrophages remained roughly constant for the first 48 h, and then rose to a maximum of 3.5×10^6 at 96–120 h (Fig. 2 A). Initially, there were hardly any granulocytes in this suspension, but their number increased somewhat to a maximum of 0.5×10^6 at ~ 96 h after BCG administration and thereafter decreased to near-normal numbers (data not shown).

Quantitation of Morphologically Distinct Types of Macrophages Isolated from the Lungs after Intravenous Injection of BCG. In the animals with BCG-induced pulmonary inflammation, many of the glass-adherent macrophages showed the same characteristics as pulmonary alveolar macrophages of normal mice after culture on glass for 24 h, i.e., a round or oval cell shape with an oval to slightly bean-shaped nucleus, some clearly visible nucleoli, and a cytoplasm:nucleus ratio >1; these cells do not form large pseudopods on a glass surface within 24 h, and rarely do so after longer culture. Such macrophages are called alveolar-macrophage-like (AML) cells (Fig. 3 and Table I).

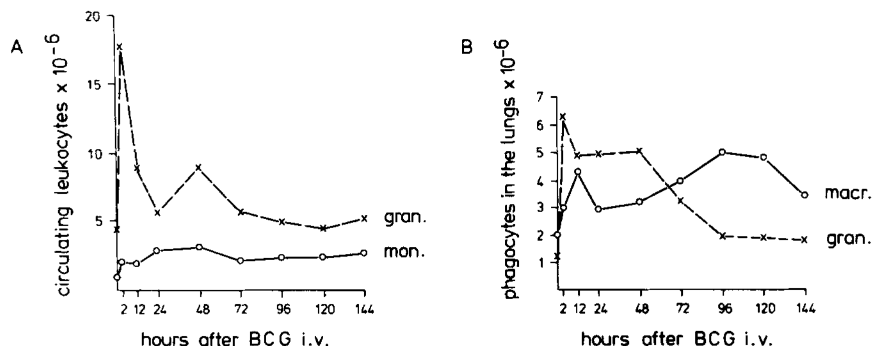

FIG. 1. Total numbers of circulating granulocytes and monocytes, and phagocytic cells in the lungs after BCG administered intravenously. (A) Circulating monocytes (mon.) (O) and granulocytes (gran.) (X). (B) Pulmonary macrophages (macr.) (O) and granulocytes (gran.) (X) isolated from the lungs by lavage and enzyme digestion.

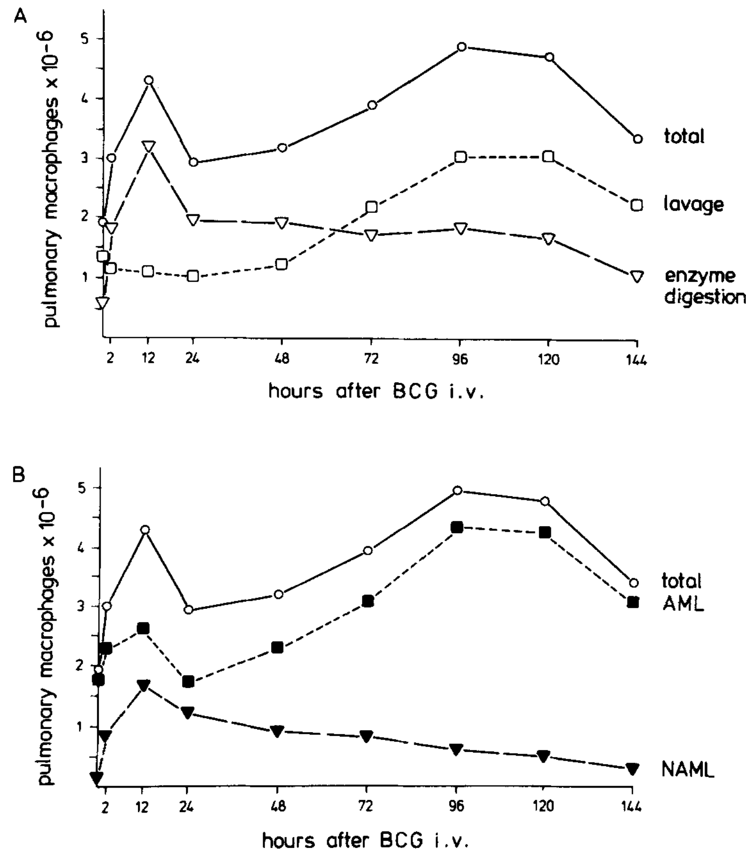

FIG. 2. Total number of pulmonary macrophages after BCG administered intravenously. (A) Subdivision on the basis of isolation method. ○, Total number of macrophages; □, macrophages isolated by lavage; ▽, macrophages isolated by enzyme digestion of lung tissue after lavage. (B) Subdivision on the basis of morphological criteria. ○, Total number of macrophages; ■, AML-type macrophages; ▼, NAML-type macrophages.

We also found cells with some divergent features, but that were certainly macrophages because they adhered to glass, were positive for nonspecific esterase staining, and often contained intracytoplasmic particles such as erythrocytes, remnants of various dead cells, and BCG bacilli—as demonstrated by ZN staining. In 24-h cultures, these cells were usually much smaller than the AML macrophages, had a lower cytoplasm: nucleus ratio, were sometimes stretched out or bore large pseudopods, and usually contained a bean-shaped or nearly horseshoe-shaped nucleus in which nucleoli could not always be discerned. These macrophages are called non-alveolar-macrophage-like macrophages (NAML) (Fig. 3 and Table I). In animals in the normal steady state, a small number of NAML macrophages are also found, but only among the cells harvested by enzyme digestion.

The characteristics of the AML and NAML macrophages are given in Table I. The great majority of both types of cell carried Fc receptors, and all phagocytosed IgG-coated erythrocytes. The presence of complement (C) receptors was much more common on NAML than on AML macrophages. However, the percentage of C-

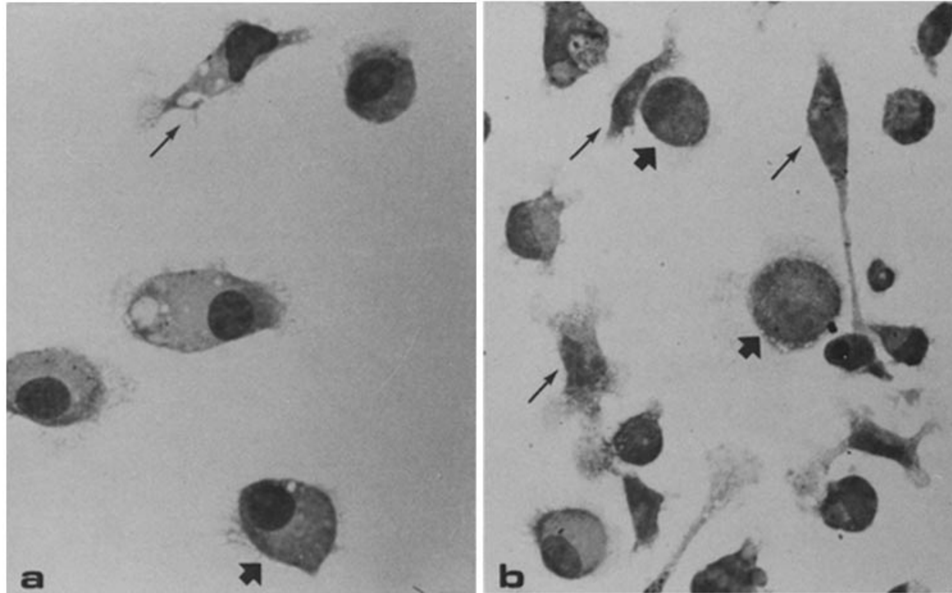

FIG. 3. Morphologically distinct types of pulmonary macrophages after 24 h of culture on a glass surface. Giemsa stain, × 400. (a) Cells harvested by lavage 48 h after BCG injection; the macrophages are predominantly AML (➚) and rarely NAML (↗). (b) Cells harvested by enzyme digestion of lung tissue after lavage 48 h after BCG injection; the numbers of AML (➚) and NAML (↗) are roughly equal.

TABLE I
Morphology, Cytochemical, and Functional Characteristics of AML and NAML

Characteristic	AML	NAML
Shape of cell*	Round to oval	Irregular
Shape of nucleus*	Oval or bean	Bean or horseshoe
Cytoplasm:nucleus ratio*	>1	<1
Large pseudopods*	Absent	Present
Minimal cell diameter*	>8 μm	<8 μm
Glass adherence*	Present	Present
Esterase‡	100%	100%
Fc receptors§	90%	93%
Fc-mediated phagocytosis§	80%	70%
C receptors‖ at 0 h after BCG	5%	81%
C receptors‖ at 12 h after BCG	17%	95%
C receptors‖ at 48 h after BCG	29%	83%
C receptors‖ at 96 h after BCG	35%	90%

* After 24 h culture on glass.
‡ With α-naphthyl butyrate as substrate.
§ Demonstrated with IgG-coated erythrocytes in 48-h cultures.
‖ Demonstrated with IgM + C-coated erythrocytes in 48-h cultures.

receptor-bearing AML increased with time after BCG administration (Table I); the presence of C receptors on AML was not correlated with the presence of intracellular BCG.

Because the morphological differences between AML and NAML macrophages were distinct and occurred in all suspensions harvested from BCG-treated animals,

we analyzed the results on the basis of not only the isolation procedures, but also on the basis of the classification according to the two types. The total number of NAML macrophages rose 10-fold in the first 12 h to 1.5×10^6, and then decreased gradually (Fig. 2B); the total number of AML macrophages initially showed a moderate rise up to 12 h after injection of BCG, followed by a decrease to normal at 24 h and then a rise to $\sim 4 \times 10^6$ at 96–120 h (Fig. 2B). Of the macrophages in the suspensions of enzyme-digested tissue isolated after BCG, >50% were of the NAML type at 12 and 24 h, and this percentage declined to ~20 at 144 h. In the lavage fluid, the percentage of NAML macrophages was much smaller, ~5% up to 72 h after injection of BCG, and slightly more at later times. Thus, the increase in the total number of macrophages is initially mainly a result of an increase of NAML macrophages, and only at later times of an increased number of AML macrophages.

In Vivo Labeling Studies with [^{3}H]Thymidine after Intravenous Administration of BCG. The mice were given an injection of 25 μCi [^{3}H]thymidine, and within 5 min, an injection of 0.25 mg BCG. The in vivo labeling index of the monocytes increased quickly to a maximum of 40% at 12 h and remained high during the next 36 h, after which it decreased (Fig. 4A). The mean labeling index of the pulmonary macrophages was a maximum of 18% at 24 h after BCG injection, and after that decreased gradually (Fig. 4A). Initially, the labeling index of the macrophages from enzyme-digested tissue was considerably higher than that of the macrophages isolated by lavage, but the former decreased after 72 h and the values were roughly the same at 120 and 144 h after BCG administration (Fig. 4B). In the morphologically defined populations, the difference in labeling indices between NAML and AML macrophages was striking (Fig. 4B). In the first 48 h after BCG administration nearly all in vivo labeled cells belonged to the NAML type, and at later times, the number of labeled NAML macrophages decreased, whereas the AML population contained an increasing number of labeled cells (Fig. 4C).

In Vitro Labeling Studies with [^{3}H]Thymidine after Intravenous Administration of BCG. After BCG injection the total number of DNA-synthesizing pulmonary macrophages per mouse, measured by in vitro [^{3}H]thymidine incorporation, showed a transient increase from 0.5×10^5 in normal mice to 3.6×10^5 maximally at 72 h after BCG (Fig. 5A). Comparison of the in vitro labeling indices for the total population after 2, 24, and 48 h of incubation shows that the number was almost doubled in the second 24 h of incubation (Table II) by cell division or an increase in the number of DNA-synthesizing cells in the cultures.

Contrary to the findings in the normal steady state (4), slightly higher in vitro labeling indices were found for the population isolated by lavage than for the population from enzyme-digested tissue (Fig. 5B, upper panel). A striking divergence was found in the morphologically determined populations: the in vitro labeling index of the AML was clearly higher than that of the NAML between 24 and 96 h after BCG administration (Fig. 5B, lower panel). The labeling indices of the NAML were constantly low (~2%), and approximately equal to the value found for pulmonary macrophages in the normal steady state (4).

Effect of Hydrocortisone on the Number of Pulmonary Macrophages after Intravenous Administration of BCG. In normal mice injected subcutaneously with 15 mg hydrocortisone acetate (HC), a severe and long-lasting monocytopenia develops (12) and the pulmonary macrophage population decreases to about two-thirds of its normal size

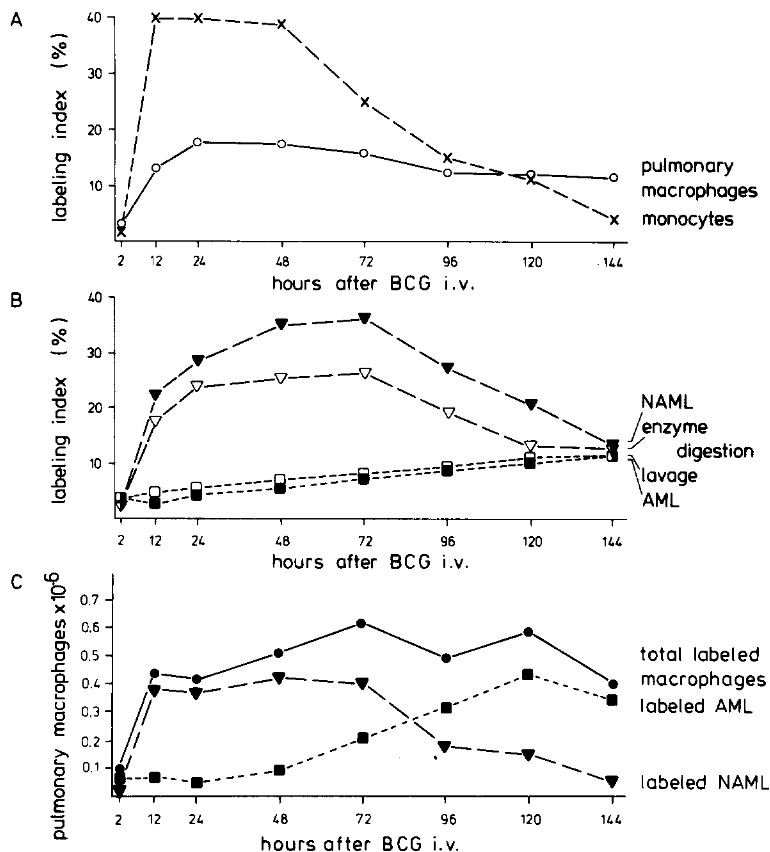

FIG. 4. In vivo labeling of mononuclear phagocytes after intravenous administration of BCG. (A) Labeling indices of circulating monocytes (X) and pulmonary macrophages (O). (B) Labeling indices of pulmonary macrophages subdivided on the basis of isolation methods and morphological criteria. □, Macrophages isolated by lavage; ▽, macrophages isolated by enzyme digestion of lung tissue after lavage; ■, AML; ▼, NAML. (C) Total numbers of pulmonary macrophages and numbers of labeled pulmonary macrophages subdivided on the basis of morphological criteria. ●, Total labeled pulmonary macrophages; ■, labeled AML; ▼, labeled NAML.

because of the diminished influx of circulating monocytes (13). In HC-treated mice given BCG intravenously 48 h after HC, the total number of pulmonary macrophages decreased during the first few days after BCG administration and remained constant after that (Fig. 6A). Analysis according to the method of cell isolation showed that after BCG injection the number of macrophages isolated by lavage decreased, whereas the number isolated by enzyme digestion remained almost constant (Fig. 6A, upper panel). Analysis according to cell morphology showed that the initial decrease was mainly a result of a moderate decrease in the number of AML, whereas a concomitant slight increase in the number of NAML was considerably smaller than normally found after intravenous administration of BCG (Fig. 6A, lower panel; cf. Fig. 2B).

Effect of HC on the in Vitro Labeling of Pulmonary Macrophages after Intravenous Administration of BCG. The in vitro labeling indices of the pulmonary macrophages isolated by lavage and enzyme digestion after BCG in HC-treated mice were initially very

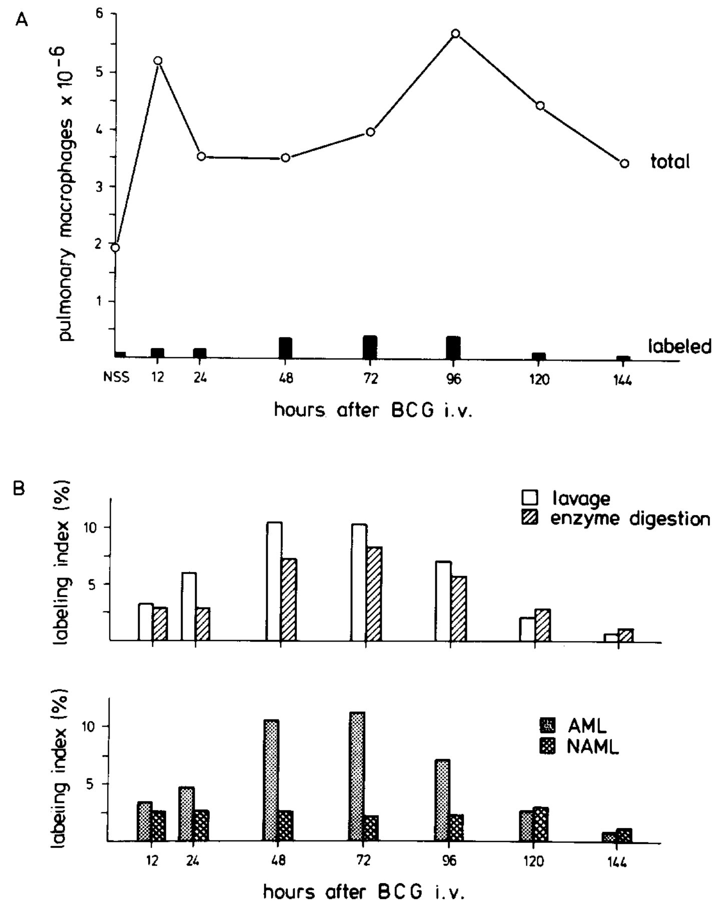

FIG. 5. In vitro labeling of pulmonary macrophages after intravenous injection of BCG determined after 24 h of incubation with [^{3}H]thymidine. (A) Total number of pulmonary macrophages (○) and the number of in vitro labeled pulmonary macrophages (bars). (B) Labeling indices of pulmonary macrophages subdivided on the basis of isolation methods (upper panel) and morphological criteria (lower panel). □, Macrophages isolated by lavage; ▨, macrophages isolated by enzyme digestion of lung tissue after lavage; ▩, AML; ■, NAML.

low, and after 48 h, rose slightly above normal levels (Fig. 6B), but remained much lower than in BCG-injected—but otherwise normal—animals (Fig. 5B, upper panel).

BCG-containing Pulmonary Macrophages in Normal and HC-treated Animals. The percentage of macrophages that had phagocytosed BCG was established at each time point after a 24-h incubation, under the assumption that the washing procedures during cell isolation and at the end of the incubation period (in a total of 12 washes) had removed bacilli adhering to the outer surface of the macrophages. In normal animals, the mean percentages of BCG-containing macrophages in the suspensions isolated by lavage and enzyme digestion show that phagocytosis of BCG is initially performed by the macrophages isolated by enzyme digestion (Table III). The number of BCG-containing macrophages in the lavage fluid increased considerably with time, whereas the number of BCG-containing macrophages in the enzyme-digested suspension decreased (Table III). ZN staining of radioautographs made it possible to

TABLE II
In Vitro Labeling Index of Pulmonary Macrophages from Animals Injected Intravenously with BCG

Time after BCG administration*	In vitro labeling index‡		
	2 h	24 h	48 h
h		%	
0	2.7	2.3	2.0
12	3.0	3.2	4.6
24	4.7	4.3	7.2
48	7.9	9.1	15.3
72	9.4	9.6	18.1
96	5.3	6.6	13.1
120	2.3	2.6	6.6
144	1.8	0.9	4.3

* 0.25 mg heat-killed BCG in 0.25 ml saline injected intravenously at 0 h.
‡ After incubation for the indicated time in culture medium containing 0.1 μCi/ml [^{3}H]thymidine.

establish the percentage of labeled and unlabeled macrophages containing BCG in the labeling experiments. After in vivo labeling, BCG was initially found more frequently in unlabeled cells than in labeled cells in both the AML and the NAML populations, but after 48 h after BCG injection, the percentages of BCG-containing labeled and unlabeled cells were roughly equal. In the in vitro labeling experiments, the percentage of BCG-containing cells was the same for labeled (i.e., DNA synthesizing) and unlabeled cells.

In the HC-treated animals, the percentage of BCG-containing macrophages was lower than in normal animals after BCG injection, but at the end of the observation period, the percentage of BCG-containing macrophages isolated by enzyme digestion equaled that found in untreated animals (Table III). In the population isolated by lavage, the percentage of BCG-containing macrophages remained very low at all times (Table III).

Histological Studies. Lungs fixed 6 h after BCG administration showed a predominantly granulocytic infiltrate in the pulmonary interstitium and engorgement of blood vessels, which were sometimes occluded by small emboli (Fig. 7); phagocytes seemed to be leaving the capillaries for the interstitial space, and no or very few signs of intra-alveolar bleeding were present. Fluorescence microscopy of auramine-rhodamine-stained preparations showed that BCG were present throughout the interstitial tissue either as clumps, ranging from aggregates of a few bacteria to emboli measuring ~10–20 μm in diameter, or as single bacteria, some of which had already been phagocytosed at 6 h after injection. At 48 h after BCG injection, the interstitial cellularity was even more pronounced, and capillaries were still moderately engorged. The interstitial infiltrate now consisted of a mixture of granulocytes, macrophages, and lymphocytes; and in the alveolar spaces, some granulocytes were present among the alveolar macrophages (Fig. 7). At 120 h after BCG injection, the cellularity of the interstitium had diminished to only slightly more than normal, but infiltrates with macrophages and lymphocytes were locally still present in the alveolar septa and perivascular areas (Fig. 7). In lungs fixed after lavage, the number of macrophages left behind in the alveoli was higher than that in normal mice. Even though cellular

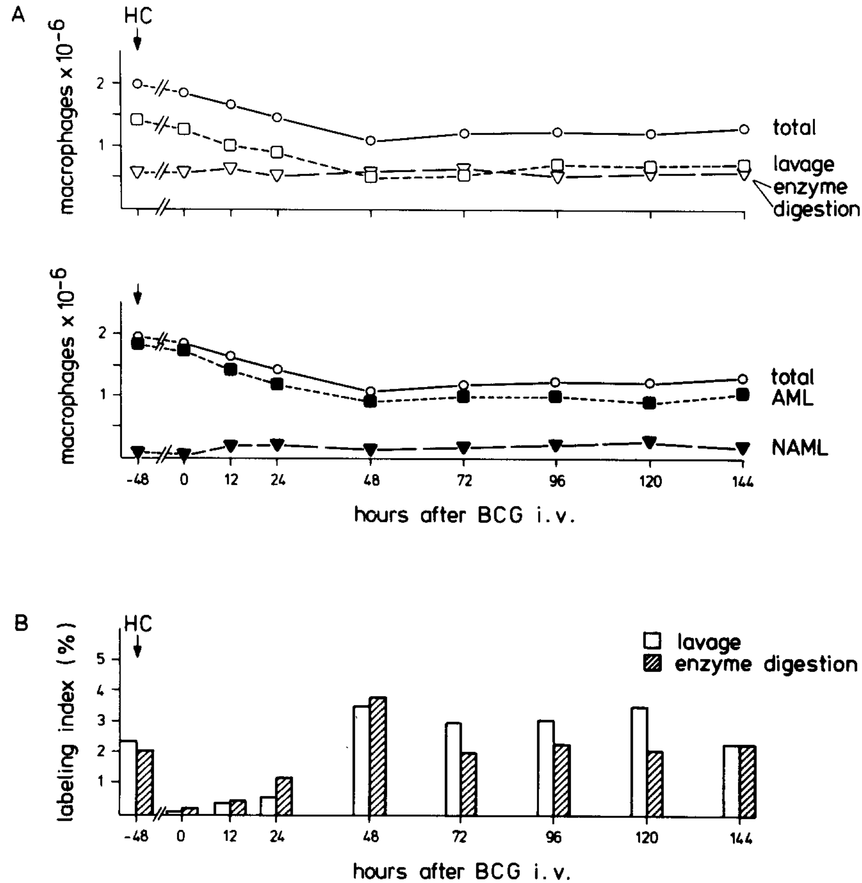

FIG. 6. The effect of HC on the number of pulmonary macrophages and their in vitro labeling indices after intravenous injection of BCG. At −48 h, 15 mg HC was injected subcutaneously. (A) Number of pulmonary macrophages subdivided on the basis of isolation method (upper panel) and morphological criteria (lower panel). HC, HC injected at −48 h, values for that time point representing the values obtained in the normal steady state; ○, total number of macrophages; □, macrophages isolated by lavage; ▽, macrophages isolated by enzyme digestion of lung tissue after lavage; ■, AML; ▼, NAML. (B) In vitro labeling indices of pulmonary macrophages subdivided on the basis of isolation methods. HC, HC injected at −48 h, values for that time point representing the values obtained in the normal steady state; □, macrophages isolated by lavage; ▨, macrophages isolated by enzyme digestion of lung tissue after lavage.

details can be very distinct in methacrylate-embedded preparations, it was impossible to identify the majority of the cells in the interstitium with certainty, and therefore quantitation of macrophages in the tissue sections was not attempted.

Calculation of Kinetic Parameters of Pulmonary Macrophages after Intravenous Injection of BCG. The total number of pulmonary macrophages is the result of the influx of blood monocytes, the production of pulmonary macrophages by local cell division, and the disappearance of pulmonary macrophages by efflux or cell death. The change in the number of pulmonary macrophages seen during an inflammatory reaction is caused by a temporary preponderance of one or a combination of these factors over the other factor(s). Because the influx rate, local production rate, and disappearance rate may change during inflammation, the overall kinetics must be approximated by

TABLE III

BCG-containing Macrophages after Intravenous Injection of BCG In Normal and HC-treated‡ Mice*

Time after BCG administration	Percentage of macrophages containing BCG§								Number of macrophages containing BCG§			
	Lavage‖				Enzyme digestion¶				Lavage‖		Enzyme digestion¶	
	Normal			HC	Normal			HC				
	AML	NAML	Mean	Mean	AML	NAML	Mean	Mean	Normal	HC	Normal	HC
h									$\times 10^4$	$\times 10^4$	$\times 10^4$	$\times 10^4$
12	2	1	2	<1	14	32	25	17	2	<1	81	11
24	6	7	6	<1	40	37	38	18	6	<1	75	10
48	17	22	18	2	43	35	39	17	22	1	78	10
72	17	28	17	4	39	26	33	26	37	2	58	17
96	17	26	18	4	31	27	30	29	54	3	56	17
120	18	34	19	4	34	29	33	37	57	3	56	21
144	20	33	21	6	40	21	35	34	49	4	39	22

* 0.25 ml heat-killed BCG in 0.25 ml saline injected at 0 h.

‡ 15 mg HC injected subcutaneously 48 h before BCG administration.

§ In ZN-stained preparations made after 24-h culture on glass.

‖ Macrophages isolated by lavage.

calculation of the mean rate constants of these processes over short intervals. During such an interval, the monocyte influx into the lungs represents a fraction of the total number of blood monocytes leaving the circulation in that period. The local production at maximum equals the quotient of the total number of DNA-synthesizing mononuclear phagocytes during the interval and the DNA-synthesis time (t_s) of these cells, under the assumption that each DNA-synthesizing mononuclear phagocyte will divide and thus contribute one additional cell to the population.[1] Although the t_s of these cells cannot be measured, it may be assumed that this time is not shorter than that of promonocytes during an inflammatory reaction (14), i.e., 7.6 h in the first 12 h and 12.8 h at later times. The number of pulmonary macrophages disappearing from the lungs during each period is a fraction of the total number of pulmonary macrophages.

The change in the total number of pulmonary macrophages over a period from t to t′ can therefore be expressed by the equation:

$$L_{t'} - L_t = pk_1 \int_t^{t'} B dt + \frac{\int_t^{t'} (I \times L) dt}{t_s} - k_2 \int_t^{t'} L dt, \qquad (1)$$

in which L_t and $L_{t'}$ are the total number of pulmonary macrophages at times t and t′; p the fraction of the circulating monocytes entering the lungs; k_1 the mean disappearance rate constant of blood monocytes over the period from time t to t′; $\int_t^{t'} B dt$ the area under the curve of the blood monocytes from time t to t′; $\frac{\int_t^{t'} (I \times L) dt}{t_s}$ the production of pulmonary macrophages by locally DNA-synthesizing

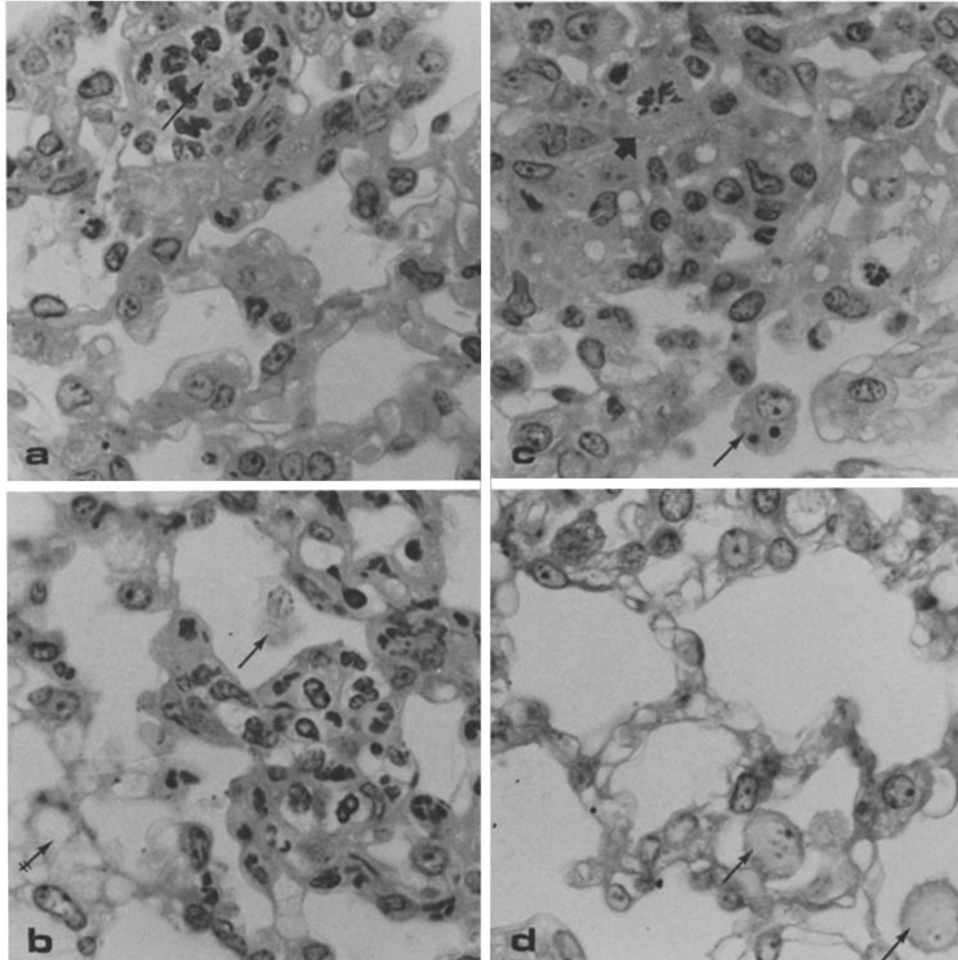

FIG. 7. Histological sections of methacrylate-embedded lung tissue fixed at various times after injection of BCG. Giemsa stain, × 400. (a) 6 h after BCG injection; lungs fixed before perfusion of the lung vessels show engorgement of the capillaries, predominantly granulocytic infiltrates, and small BCG emboli (↗). (b) 6 h after BCG injection; lungs fixed after perfusion of the lung vessels and lavage of the airways show empty capillaries (↗), predominantly granulocytic interstitial infiltrates, and an alveolar macrophage (↗) not removed by lavage. (c) 48 h after BCG injection; lungs fixed after perfusion and lavage show pronounced interstitial mononuclear infiltration. Cell type and location of the cell in mitosis (↗) is uncertain; some alveolar macrophages (↗) were not removed by lavage. (d) 120 h after BCG; lungs fixed after perfusion and lavage show decreased interstitial infiltration and some alveolar macrophages (↗) left behind after lavage.

mononuclear phagocytes in the interval t to t′, calculated with the in vitro labeling index (I), the total number of pulmonary macrophages (L), and the DNA-synthesis time (t_S); k_2 is the mean disappearance rate constant for pulmonary macrophages in the period from time t to t′; and $\int_t^{t'} L dt$ the area under the curve of all pulmonary macrophages over the period from time t to t′.

Similarly, the kinetics of labeled cells after in vivo labeling with [^{3}H]thymidine at t = 0 (the time of BCG administration) can be expressed in an equation. The difference between the numbers of in vivo labeled pulmonary macrophages present at

times t and t′ equals the difference between the numbers of labeled monocytes that have entered the lungs plus the numbers of labeled macrophages originating from cells pulse-labeled locally at time 0, minus the numbers of labeled cells that have disappeared from the lungs in the period from t to t′. It has been shown previously that under normal steady-state conditions (15) and during inflammation (14), blood monocytes leave the circulation at random. For the present calculations, it was assumed that the disappearance of pulmonary macrophages during inflammation, by cell death or efflux, also occurs at random, because the course of the total numbers of labeled and unlabeled pulmonary macrophages does not contradict a random process. The change in the numbers of labeled pulmonary macrophages over the period from t to t′ can then be expressed by the equation:

$$L_{t'}^* - L_t^* = pk_1 \int_t^{t'} B^* dt - k_2 \int_t^{t'} L^* dt + \frac{t' - t}{t_s} \times I \times L, \qquad (2)$$

in which L^* is the number of labeled pulmonary macrophages; B^* the number of labeled blood monocytes; $\frac{t' - t}{t_s} \times I \times L$ is the difference in the number of locally pulse-labeled cells plus their daughter cells at times t′ and t. After $t = t_s$, when all locally pulse-labeled cells have divided and given rise to the maximal number of labeled daughter cells ($2 \times I \times L$), this term is omitted from the equation.

With Eqs. 1 and 2, the mean values of pk_1 and k_2 for the various intervals between the determinations can be calculated by substitution of the data given in Figs. 1, 2, 4, and 5. With the values of pk_1 and k_2 and the constituents of Eq. 1, it is possible to calculate the total monocyte influx, the total macrophage efflux from the lungs, and the maximal local production for each period. The results of these calculations are given in Table IV. Because virtually no labeled monocytes enter the lungs before t = 2, the calculations are not reliable for the first 2 h after BCG administration. A minimal monocyte influx during that period can, however, be calculated by disregarding the efflux during these 2 h. In that case, the monocyte influx equals the total increase of the pulmonary macrophage population minus the calculated local production in these hours. This value for the monocyte, influx is used to calculate the mean value of pk_1 for this period.

The error in the calculation of the values of pk_1 and k_2 determined for the periods after 2 h after BCG administration is mainly dependent on the error in the values obtained for the differences between L_t and $L_{t'}$ and between L_t^* and $L_{t'}^*$. These important entities in the calculations of pk_1 and k_2 are initially large enough to permit fairly accurate estimates of the monocyte influx and the disappearance of pulmonary macrophages up to 48 h after BCG administration. At later times, these differences are too small, in view of the error in the values obtained, to permit reliable estimates, as shown by the results given in Table IV.

Thus, the calculations indicate that the total monocyte influx amounts to at least 4×10^6 cells and that the total macrophage efflux amounts to at least 3.5×10^6 cells during the total period of observation. The local production, for which the calculations provide reliable data for the total observation period, amounts to 2.1×10^6 cells in the period up to 144 h after BCG administration.

TABLE IV

*Calculated Mean Kinetic Parameters and Influx, Local Production, and Efflux for the Intervals between Observations after the Intravenous Injection of BCG into Mice**

Time after BCG administration	pk_1	k_2	Total monocyte influx	Macrophage production by local division	Total macrophage efflux
h	h^{-1}	h^{-1}	$\times 10^6$	$\times 10^6$	$\times 10^6$
0–2	0.3052		1.1	0.01	
2–12	0.1009	0.0515	2.0	0.1	1.7
12–24	0.0224	0.0498	0.6	0.1	1.7
24–48	0.0037	0.0004	0.3	0.3	0.1
48–72	0.0034	−0.0022	0.2	0.6	−0.2
72–96	−0.0029	−0.0093	−1.2	0.5	−0.9
96–120	0.0223	0.0006	0.7	0.3	0.1
120–144	−0.0224	0.0126	−0.7	0.2	1.3
NSS‡	0.0064	0.0075	0.25	0.1	0.35

* Calculated with the data from Figs. 1, 2, 4, and 5.

‡ Data concerning mice in the normal steady state (NSS) taken from Blussé van Oud Alblas et al.;[1] the total influx, local production, and efflux are given for a 24-h period.

Discussion

These results show that intravenous injection into mice of heat-killed BCG leads to an extensive inflammatory reaction in the lungs that subsides after 4–5 d. During the inflammatory reaction, the early increase and maintenance of the larger pulmonary macrophage population are brought about primarily by the influx of blood monocytes, which become exudate macrophages and later acquire the characteristics of normal alveolar macrophages. The production of macrophages by locally dividing cells is temporarily increased during a period lasting from 1–4 d after the induction of the inflammation, but this plays only a minor role in the total kinetics of the inflammatory reaction.

The total influx of monocytes during the first 48 h after BCG injection amounted to ~4×10^6 cells, or eight times the normal influx in steady-state animals.[1] The total macrophage efflux during the first 48 h after BCG administration amounted to ~3.5×10^6 or seven times the efflux in normal animals.[1] The results of the calculations concerning influx and efflux for the period after 48 h after BCG injection are less accurate, because of the relatively strong influence of the error in the experimental data. The total local production of macrophages amounted to ~0.5×10^6 cells during the first 48 h after BCG administration and to slightly $>2 \times 10^6$ cells over the period of 144 h after BCG administration, i.e., three times the normal local production during a period of the same duration.[1] The total monocyte influx is therefore at least double the total local macrophage production during the period under study and predominates particularly in the first 48 h after BCG administration. These quantitative results indicate that by 48 h after BCG administration, most of the pulmonary macrophages that were present at the time of BCG administration have been replaced by very recently arrived monocyte-derived cells.

The histological studies provide insight into the localization of the inflammatory

reaction, but the tissue sections provide no quantitative data. However, the method used to isolate all pulmonary macrophages in two phases after the removal of circulating monocytes from the pulmonary blood vessels provides quantitative information about the total population and also about the alveolar macrophages, which are more easily washed out, and the remaining alveolar macrophages plus the interstitial macrophages obtained by enzyme digestion. The initially interstitial localization of the inflammatory reaction is indicated by the early increase of the number of macrophages isolated by enzyme digestion followed by a rise in number of macrophages isolated by lavage after 48 h after BCG is administered.

The morphological characterization of the cells provides another approach for subdivision of the total number of cells. The NAML, which are virtually absent in normal lungs in the steady state, increase in number very quickly after BCG administration, accounting for most of the population increase in the first 12 h after BCG injection, and after that, their number diminishes gradually; these macrophages resemble the exudate macrophages seen in other kinds of inflammatory reactions. The number of AML increases at later times; these cells have the morphology characteristic of the alveolar macrophages found in normal animals, and these features can probably be acquired within a short time. It may therefore be assumed on morphological grounds alone that NAML represent the total population of interstitial macrophages and AML represent that of alveolar macrophages. Support for this assumption is provided by the finding that the interstitially deposited BCG are initially phagocytosed mainly by the NAML.

The intracellular BCG provide a marker that makes it possible to follow the course of the cells that are initially located in the interstitium: the gradual decrease in the number of BCG-containing NAML and the progressive increase in the number of BCG-containing AML, taken together, demonstrate that disposal of BCG takes place by migration of BCG-containing macrophages to the airways. Confirmation of this mechanism was obtained in the in vivo labeling experiments, i.e., the initially high number of labeled NAML (with a labeling index resembling that of circulating monocytes) and the gradual increase in the number of labeled AML at later times, when the number of labeled NAML was decreasing. It is therefore concluded that the NAML represent monocyte-derived exudate macrophages which, upon arrival in the alveoli, change into cells that are morphologically indistinguishable from the normal alveolar macrophages. Functionally, the AML differ slightly from the alveolar macrophages in normal animals (4) in that a larger percentage of the former phagocytose IgG-coated erythrocytes via the Fc receptor, and a larger percentage can be shown to carry C receptors. However, the presence of C receptors did not show correlation with the presence of intracellular BCG. If the presence of C receptors on AML reflects a state of activation, it is clear that in these cells activation does not depend on the intracellular presence of BCG.

In mice in the normal steady state, local production of macrophages by division of cells synthesizing DNA locally accounts for, at most, 30% of the total number needed for the maintenance of the pulmonary macrophage population.[1] The in vitro labeling of cells from animals given a BCG injection shows that local DNA synthesis by pulmonary macrophages rises from 2.7% of the population normally to a maximum of 9.6% at 72 h after BCG administration and later falls to 2.5% after 120 h after BCG administration, which indicates a temporarily enhanced production of pulmonary

macrophages by local division. The calculations show, however, that monocyte influx is the main factor in the population increase during the inflammatory reaction.

The striking difference between AML and NAML in in vitro labeling is hard to explain. The labeling experiments show that neither physical contact with BCG nor the amount of phagocytosed BCG is a decisive factor for the induction of DNA synthesis. Although a difference in the maturation stage of the cells might play a role, it must be kept in mind that both monocyte influx and macrophage efflux are initially so high that virtually all macrophages present at the time of maximal local mitotic activity have arrived very recently from the circulation. Because the cells manifesting DNA synthesis have acquired the morphological characteristics of alveolar macrophages by the time that this synthesis starts, and because NAML have only a very low in vitro labeling index, it is probable that, for the cells capable of division, there is a lag time between the arrival in the lungs and the start of DNA synthesis. It has been shown that some monocytes and exudate macrophages, when cultured in vitro in the presence of macrophage growth factors, acquire the capacity to start DNA synthesis and to divide, but only after a certain lag time (16). We may postulate that the locally dividing macrophages in the experiments reported here are similar cells, triggered in vivo to proceed to DNA synthesis during the inflammatory reaction. In reports of studies on experimental inflammation in murine livers, similar data concerning a temporarily increased number of locally DNA-synthesizing macrophages have been emphasized (17, 18), and there is evidence that the level of local mitotic activity can differ according to the inflammatory stimulus applied (17). Apparently a certain percentage of the exudate macrophages are capable of DNA synthesis and division, but the percentage that start to do so depends on the amount or characteristics of local factors generated by the inflammatory stimulus or on the type of the stimulus itself.

The observations in HC-treated mice injected with BCG show clearly that this drug prevents the initial increase in the number of NAML seen in normal animals, although a slight rise occurs at later times, probably because of some influx of monocytes despite the HC-induced monocytopenia, as has been shown to occur in mice treated with HC only (13). The increased local mitotic activity found in normal mice after BCG injection is largely absent in HC-treated mice, in which the in vitro labeling index never rises above 4%. Similar effects of HC on local mitotic activity during inflammation have been reported previously (17, 19). This response may be dependent solely on the diminished influx of cells capable of DNA synthesis, or on the diminished generation of presumed local factors inducing DNA synthesis, or on a diminished responsiveness of HC-treated macrophages to such factors, as has been found for the induction of activation in macrophages (20).

The approach used in the present study, i.e., the induction of a sterile inflammation by intravenous injection of heat-killed BCG, is relevant for the understanding of the course of other inflammatory reactions caused by microorganisms that reach the lungs via the circulation. The overall kinetic mechanisms operating in the pulmonary macrophage population as described in this paper support the general conclusion that tissue macrophages derive mainly from circulating monocytes, and also exclude the existence of an interstitial pool of dividing precursor cells and provide evidence that the limited local production of pulmonary macrophages is the result of division of

mononuclear phagocytes recently derived from the circulation and having the morphology of alveolar macrophages.

Summary

This report gives a quantitative description of the kinetics of the pulmonary macrophages and their direct precursors during the acute inflammatory reaction in the lungs induced by intravenous injection of heat-killed bacillus Calmette-Guérin (BCG) into specific-pathogen-free mice. After BCG injection, the total number of pulmonary macrophages isolated by lavage and subsequent enzyme digestion of lung tissue increased to 225% of normal within 12 h and, after a minor decrease, rose to a maximum of 250% of normal at 96 h, followed by a decrease to 150% at 144 h, the end of the observation period. The number of circulating monocytes doubled in the first 48 h and stayed close to that level. In vivo and in vitro labeling with [^{3}H]-thymidine showed that an influx of monocytes transforming into pulmonary macrophages was mainly responsible for the population increase. A temporary increase in the number of locally dividing pulmonary macrophages—manifested by an increased in vitro labeling index, reaching a maximum of 9.6% 72 h after BCG injection—made a minor contribution to the population increase.

All pulmonary macrophages were classified according to morphological criteria as alveolar-macrophage-like (AML) or non-alveolar-macrophage-like (NAML), and their respective characteristics were established. The in vivo labeling data showed NAML to represent exudate macrophages derived from circulating monocytes entering the interstitial tissue, and these cells changed morphologically into AML upon entering the alveolar hypophase. This mechanism was confirmed by the finding that the interstitially deposited BCG were found first inside NAML and later in AML. The in vivo labeling data showed that local production was mainly a result of division of macrophages that were morphologically identical with normal alveolar macrophages. The former cells, however, derived most probably recently from the circulation, because the turnover of the total population was very high before local macrophage production became maximal. In mice treated with HC before the injection of BCG, this population increase was absent, because of virtual abolition of the initial monocyte influx and absence of the increased local production of macrophages.

Calculations showed that the monocyte influx in the first 48 h amounted to $\sim 4 \times 10^{6}$ cells, i.e., eight times that found in the normal steady state, and that the efflux of pulmonary macrophages in that period amounted to $\sim 3.5 \times 10^{6}$ cells, i.e., seven times the normal efflux. The local production over the total period of 144 h was only three times that found normally. The results of these quantitative studies show that the increase of the pulmonary macrophage population during an acute inflammation is brought about mainly by monocyte influx and to a minor extent by a temporary increased local production of macrophages. Disposal of interstitially deposited BCG occurred by phagocytosis by local macrophages and the subsequent efflux of the latter.

We thank Dr. H. Mattie (Department of Infectious Diseases, Leiden University Hospital) for critical discussion of the calculations and Dr. J. te Velde (Department of Pathology, Leiden University Hospital) for help in performing the histological studies.

Received for publication 11 March 1981 and in revised form 13 April 1981.

References

1. Van Furth, R., Z. A. Cohn, J. G. Hirsch, J. G. Humphrey, W. G. Spector, and H. L. Langevoort. 1972. The mononuclear phagocyte system. A new classification of macrophages, monocytes, and their precursor cells. *Bull. W. H. O.* **46:**845.
2. Nichols, B. A. 1980. The vacuolar apparatus of alveolar macrophages and the turnover of surfactant. *In* Mononuclear Phagocytes—Functional Aspects. Part I. R. van Furth, editor. Martinus Nijhoff, The Hague. 119.
3. Weibel, E. R., P. Gehr, D. Haies, J. Gil, and M. Bachofen. 1976. The cell population of the normal lung. *In* Lung Cells in Disease. Proceedings of a Brooklodge Conference. A. Bouhuys, editor. North Holland Publishing Company, Amsterdam. 3.
4. Blussé van Oud Alblas, A., and R. van Furth. 1979. Origin, kinetics, and characteristics of pulmonary macrophages in the normal steady state. *J. Exp. Med.* **149:**1504.
5. Adamson, I. Y. R., and D. H. Bowden. 1980. Role of monocytes and interstitial cells in the generation of alveolar macrophages. II. Kinetic studies after carbon loading. *Lab. Invest.* **42:** 518.
6. Velo, G. P., and W. G. Spector. 1973. The origin and turnover of alveolar macrophages in experimental pneumonia. *J. Pathol.* **109:**7.
7. Fritsch, P., R. Masse, G. Stanislau, and J. Chrétien. 1975. Etude dynamique comparative de la population cellulaire intra-alvéolaire après injection intratracheale d'hydroxide de beryllium et d'hydroxide d'aluminium chez le rat. *Biomedicine (Paris).* **23:**98.
8. Onoé, K., and K. Morikawa. 1972. A study on the origin of alveolar macrophages. *The Japanese Society of Researchers.* **12:**100.
9. Thomas, E. D., R. E. Ramberg, and G. E. Sale. 1976. Direct evidence for a bone marrow origin of the alveolar macrophage in man. *Science (Wash. D. C.).* **192:**1016.
10. Kaliss, N., and D. Pressman. 1950. Plasma and blood volumes of mouse organs as determined with radioactive iodoproteins. *Proc. Soc. Exp. Biol. Med.* **75:**16.
11. te Velde, J., R. Burkhardt, K. Kleiverda, L. Leenheers-Binnendijk, and W. Sommerfeld. 1977. Methyl-methacrylate as an embedding medium in histopathology. *Histopathology. (Oxf.).* **1:**319.
12. Thompson, J., and R. van Furth. 1970. The effect of glucocorticosteroids on the kinetics of mononuclear phagocytes. *J. Exp. Med.* **131:**429.
13. Blussé van Oud Alblas, A., B. van der Linden-Schrever, H. Mattie, and R. van Furth. The effect of glucocorticosteroids on the kinetics of pulmonary macrophages. *J. Reticuloendothel. Soc.* In press.
14. Van Furth, R., M. M. C. Diesselhoff-den Dulk, and H. Mattie. 1973. Quantitative study on the production and kinetics of mononuclear phagocytes during an acute inflammatory reaction. *J. Exp. Med.* **138:**1314.
15. Van Furth, R., and Z. A. Cohn. 1968. The origin and kinetics of mononuclear phagocytes. *J. Exp. Med.* **128:**415.
16. Stewart, C. C., and H. Lin. 1975. Macrophage growth factor and its relationship to colony stimulating factor. *J. Reticuloendothel. Soc.* **23:**269.
17. Diesselhoff-den Dulk, M. M. C., R. W. Crofton, and R. van Furth. 1979. Origin and kinetics of Kupffer cells during an acute inflammatory response. *Immunology.* **37:**7.
18. North, R. J. 1970. The relative importance of blood monocytes and fixed macrophages to the expression of cell-mediated immunity to infection. *J. Exp. Med.* **132:**521.
19. North, R. J. 1971. The action of cortisone acetate on cell mediated immunity to infection: suppression of host cell proliferation and alteration of cellular composition of infective foci. *J. Exp. Med.* **134:**1485.
20. Rosenthal, A. S., and J. E. Balow. 1975. Mechanisms of glucocorticosteroid suppression of cell-mediated immunity. *In* Mononuclear Phagocytes in Immunity, Infection and Pathology. R. van Furth, editor. Blackwell Scientific Publications Ltd., Oxford. 702.