MACROPHAGES AS ORIGIN OF FACTOR INCREASING MONOCYTOPOIESIS

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During an acute inflammation the number of circulating monocytes increases, and a large proportion of these cells migrate to the site of inflammation and differentiate into macrophages (1). In previous reports (2-5) we described a factor stimulating the monocyte production, called factor increasing monocytopoiesis (FIM), occurring in the circulation of mice and rabbits during the onset of an inflammation. FIM has been fairly well characterized (5, 6), and others have reported (7) a similar factor during an inflammatory response. This factor is a protein of ~20 × 10^3 mol wt that accelerates the rate of division of promonocytes and monoblasts in vivo and is not CSF-1 (3, 5).

FIM is not detectable in the circulation of normal mice and rabbits with the two available bioassay methods, which determine the increase of the number of blood monocytes after intravenous injection of the factor into untreated animals (2, 5), or establish the increased rate of the proliferation of a macrophage cell line in vitro (4). FIM is detectable in the circulation during the initial phase of an inflammatory reaction and indications have been obtained (8) that this factor is produced at the site of inflammation. This paper reports a study performed to establish the cellular origin of FIM and to determine the kinetics of its production and release.

Materials and Methods

Animals. Specific pathogen-free, 6-12-wk-old male Swiss (CPB:SE) mice obtained from The Central Institute for the Breeding of Laboratory Animals (Zeist, The Netherlands) were used in all but a few experiments were done on male C57BL/LiARij (C57BL) and female CBA/BrARij (CBA) mice obtained from REPGO-TNO (Rijswijk, The Netherlands). All animals were maintained on a 12-h light/dark cycle, given sterilized food (type 1410; Hope Farms, Woerden, The Netherlands) and tap water ad libitum, and were not used before the 7th day after arrival to allow time for acclimatization to the new environment.

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1 Abbreviations used in this paper: FIM, factor increasing monocytopoiesis; ppFIM, partially purified FIM.

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Peritoneal Macrophages. Peritoneal macrophages were collected from chloroform-
anesthetized animals by lavage with 2 ml PBS containing 50 U/ml heparin. The cells were
counted in duplicate with a Coulter counter model ZF (Hoek-Loos, Schiedam, The
Netherlands), checked for viability by trypan-blue exclusion, and identified in cytocen-
trifuge preparations stained with Giemsa stain (2). If the viability was <95% the suspensions
were discarded. Suspensions of peritoneal cells from normal mice contained 2.3 (± 0.6
SD) x 10^6 cells/ml of which 76.9% (± 4.2 SD) were macrophages, 21.6% (± 4.6 SD)
lymphocytes, and 1.5% (± 2.5 SD) granulocytes.

Alveolar Macrophages. Alveolar macrophages were harvested from animals anesthe-
tized with an intravenous injection of 45 mg pentobarbitone sodium per kilogram body
wt (Sanofi Santé Animale, Paris, France). After perfusion of the lungs with 5 ml 0.6 mM
EDTA in PBS (PBS-EDTA) to remove monocytes from the blood vessels, the alveolar
cells were collected by bronchial lavage with 15 ml PBS-EDTA at 37°C (9). If the viability
was <95%, the suspensions were discarded. The cell yield of lavage amounted to 0.95 (±
0.24 SD) x 10^6 cells per mouse, 99.2% (± 2.3 SD) of which were macrophages and 0.8%
(± 2.5 SD) lymphocytes, as identified in cytocentrifuge preparations; there were no
granulocytes (± 1.3 SD).

Cell Lines. Cells of two continuous macrophage cell lines, PU5-1.8 (PU5) (10, 11) and
J774.1 (J774) (11, 12), were obtained from end-log-phase cultures in RPMI 1640 medium
supplemented with 10% FCS, 1,000 U/ml sodium penicillin G (Gist-Brocades NV, Delft,
The Netherlands), 50 µg/ml streptomycin (Gist-Brocades NV), and 2.0 mM L-glutamine.
Murine lymphoblastic cells (L1210; kindly donated by Dr. E. R. Rijke, Department of
Clinical Immunology, Utrecht University Hospital, The Netherlands) were cultured in
suspension in RPMI 1640 medium supplemented with 10% FCS, 1,000 U/ml sodium
penicillin G (Gist-Brocades), 50 µg/ml streptomycin (Gist-Brocades), and 2.0 mM L-
glutamine.

Blood Lymphocytes and Granulocytes. Blood lymphocytes and granulocytes were ob-
tained from heparinized blood drawn by cardiac puncture from chloroform-anesthetized
animals. After differential centrifugation of the blood (diluted fivefold with PBS) on a
Ficoll-Hypaque gradient, the lymphocyte-rich interface layer was collected and washed
four times with PBS containing 0.5 U/ml heparin (PBS-heparin). In this suspension, in
which lymphocytes predominate (87%), the proportion of granulocytes was 7% (± 5 SD)
and monocytes accounted for 6% (± 5 SD). The viability of the cells, as checked by trypan-
blue exclusion, was at least 95%. A granulocyte-rich cell suspension was prepared from the
pellet of the Ficoll-Hypaque gradient by sedimentation of the erythrocytes with a 5%
solution of dextran (mol wt 200,000) in PBS, followed by two washes with PBS-heparin,
removal of the remaining erythrocytes by hypotonic lysis, and two additional washes with
PBS-heparin. In this suspension enriched in granulocytes (76%), monocytes accounted for
1% (± 1 SD) and lymphocytes for 23% (± 10 SD). If the viability of the cells was <95%,
the suspensions were discarded.

Cell Lysates. Lysates of various types of cell were prepared as described elsewhere (3).
In short, 10^7 cells were suspended in 1 ml of pyrogen-free distilled water, quick-frozen to
-70°C, and then lyophilized at -20°C. After addition of 1 ml sterile pyrogen-free saline,
the suspension was stirred, allowed to stand for 5 min at 4°C, and then passed through a
0.45-µm Millex filter (Millipore, Molsheim, France) to be assayed for FIM activity.

Cell Culture. The cell suspensions were centrifuged at 120 g for 10 min at 4°C, after
which the cells were resuspended in RPMI at a concentration of 10^7 viable cells/ml and
incubated on Teflon film dishes (13) or microtiter plates (Nunc Products, Roskilde,
Denmark) in a humidified atmosphere of 7.5% CO_2 in air at 37°C. Preopsonized latex
was used as phagocytic stimulus (latex-to-cell ratio ~100:1). Polystyrene latex (0.81 µm
diameter; Difco Laboratories Inc., Detroit, MI) was opsonized by incubation of 10^9
particles per milliliter of RPMI with 20% FCS at 37°C for 30 min under slow rotation (4
rpm). The incubation medium was discarded after passage through a 0.22-µm Millipore
filter; the opsonized particles were then collected from the filter with serum-free RPMI
and suspended to a concentration of 10^9 particles per milliliter.

At various time points the culture supernatant was sampled to be assayed for FIM
activity. After the dishes had been cooled to 4°C, the remaining cells were recovered from the Teflon film by careful rinsing and aspiration with pyrogen-free saline, and were then checked for phagocytosis of latex in cytocentrifuge preparations (14) and for viability by trypan-blue exclusion. After only 3 min, 55% of the cultured macrophages had ingested latex beads. This percentage gradually increased to 90 at 6 h, and after 18 h of incubation all of the macrophages had phagocytosed latex. During the 48-h incubation period in serum-free medium, the viability of the cells only decreased slightly, i.e., from 95 to 80%.

**In Vitro Bioassay of FIM Activity.** The FIM activity of cell lysates and culture supernatants was assessed by determining the stimulatory effect of the factor on the proliferation rate of the macrophage cell line PU5-1.8 (4). For this purpose, PU5 cells obtained from end-log-phase cultures were suspended at a concentration of 2 × 10⁶ cells/ml in RPMI together with 2% (vol/vol) of the cell lysate or supernatant under study and 10% FCS, unless otherwise stated. The suspended cells were cultured for 24 h in Teflon culture bags, after which the bags were cooled to 4°C and the cells were recovered by rinsing and aspiration of the suspension, stained with 0.2% trypan blue in PBS, and living and dead cells were then counted in triplicate with a Bürker hemocytometer. As reference for the proliferation of the macrophage cell line, PU5 cells were cultured in RPMI with 10% FCS. Evaluation of the statistical significance of the stimulatory effect of the sample under study was performed on the raw data. To facilitate comparison, the effect on the proliferation of the cell line was expressed as the index of FIM activity (I), which was calculated as the percentage increase of the number of viable cells in cultures containing the sample under study (Nₛ) in relation to the increase in the number of viable cells in the reference culture (Nᵥ), according to the formula: $I = 100 \times (Nₛ/Nᵥ - 1)$.

The effect of FIM on the proliferation rate of PU5 cells was also studied by determining the change in the cell-cycle time of PU5 cells on the basis of the course of the stathmokinetic (blocked divisions) index (15). In short, 2 × 10⁶ cells/ml were incubated in medium containing 2% supernatant with or without FIM activity in the presence of 0.25 μg/ml vincristine sulphate (Eli Lilly France, St. Cloud, France) in Teflon culture bags. Cells were harvested at 2-h intervals during a 6-h incubation period, living and dead cells were counted, cytocentrifuge preparations were made and stained with Giemsa stain, and the percentage of cells in metaphase was determined. The cell-cycle time was assessed from the regression line calculated by the least-squares method.

**In Vivo Bioassay of FIM Activity.** FIM activity of samples was assessed in normal Swiss mice by determination of the number of monocytes ($\times 10^{-3}$/ml) in blood collected from the tail vein (6) 24 h after intravenous injection of 0.2 ml of a serial twofold dilution of the sample under investigation, and was expressed as the difference ($\Delta$) between that value and the number of monocytes of the untreated control. The total numbers of blood monocytes, granulocytes, and lymphocytes were calculated from the total numbers of leukocytes counted in triplicate with a Coulter Counter (Hoek-Loos), and the differential counts of at least 400 leukocytes in four Giemsa-stained blood smears, and they were expressed as number per milliliter blood.

**In Vivo $[3H]$Thymidine Labeling of Monocytes.** To find out whether FIM stimulates the production of monocytes, we followed the course of $[3H]$thymidine-labeled blood monocytes after intravenous injection of 1 μCi $[3H]$thymidine (sp act, 6.7 Ci/mmol; New England Nuclear, Boston, MA) per gram body weight together with supernatant-containing FIM. The labeling index of blood monocytes was determined in blood smears, prepared for autoradiography as described (1), by counting 100 monocytes per mouse at given time points; cells with three or more grains over the nucleus were considered positive. The number of circulating labeled blood monocytes per milliliter at each time point was calculated with the labeling-index values and the number of monocytes per milliliter.

**Ultrafiltration.** Fractionation of supernatants was performed at 4°C by centrifugation for 5–10 min at 300 × g in Centriflo ultrafiltration cones (Amicon Corp., Lexington, MA) with molecular weight exclusion limits of 25 × 10⁴ (CF25) and 50 × 10⁴ (CF50A). For ultrafiltration through a Diaflo membrane UM10 (exclusion limit 10⁴ mol wt) we used a micro-ultrafiltration system (Model 8MC; Amicon Corp.), which was operated at
4°C under an air pressure of 1.5–2.5 atm until the volume of the filtrate reached 60–75% of the original sample volume.

**CSF-1.** The concentration of CSF-1 in culture supernatants of peritoneal cells was determined with an RIA (16) and expressed in units per milliliter (17), mouse L cell CSF being used as the standard (determinations kindly performed by Dr. E. R. Stanley, Albert Einstein College of Medicine, Bronx, NY).

**IL-1.** The cloned murine helper T cell line D.10.G41 was used to monitor the presence of IL-1 in culture supernatants (18). In short, T cells from a 10–14-d culture were suspended at a concentration of 10^5 cells/ml in RPMI containing 5% heat-inactivated FCS, 1 μg PHA/ml, and dilutions of the supernatant under study, and were added to flat-bottomed microtiter wells in triplicate. The proliferative response of these cells was measured after 72 h as the uptake of [³H]thymidine present during the last 16 h of culture, which was determined with a Rackbeta liquid scintillation counter (LKB Produkter, Bromma, Sweden).

**Lysozyme and β-glucuronidase.** The synthesis and secretion of lysozyme and β-glucuronidase by resident peritoneal macrophages in the presence of preopsonized latex were assessed on the basis of the levels in the supernatant. The amount of lysozyme was determined with the lysoplate method (19), and β-glucuronidase was determined with methylumbelliferyl glucuronide as substrate (20) (both assays were kindly performed by Dr. J. Schnyder, Wander Research Institute, Berne, Switzerland).

**Chemotactic Activity.** The chemotactic activity towards resident peritoneal macrophages of the culture supernatants in 5 and 10% dilutions in Gey's solution was determined with the leading-front method (21). The assay was performed in a Millpore chamber with nitrocellulose nitrate filters (pore size, 12 μm) (Sartorius, Gottingen, Federal Republic of Germany), as described elsewhere (11).

**Endotoxin Determination.** Supernatants, reagents, and glassware were checked with the Limulus amebo cytolytic gelation test (22) (kindly performed by Dr. M. van der Veer, National Institute for Public Health and Environmental Hygiene, Bilthoven, The Netherlands), and they all were shown to contain <0.03 ng/ml endotoxin. Furthermore, in rabbits no significant rise in rectal temperature (≤0.33°C) was found over a 3-h observation period after intravenous injection of culture supernatant of up to 3 × 10^7 normal peritoneal macrophages that were incubated for 18 h in the presence of latex beads.

**Analysis of Data.** The data given here represent the mean and one standard error. A significant increase occurring in the in vivo or in vitro bioassay was taken as indicating the presence of FIM in a supernatant or cell lysate under study (4, 6). In all in vivo experiments, at least 4 but usually 8–12 mice were used per time point. The in vitro experiments were performed in triplicate. Differences between groups were evaluated on raw data only for significance at the 5% significance level on the basis of Student's two-tailed test or, where appropriate, by analysis of variance according to Bonferroni (23, 24).

The strength of the linear association between the numbers of macrophages, granulocytes, and lymphocytes in suspensions and FIM activity in supernatants was evaluated by analysis of correlation performed with the Pearson correlation formula (23). Unless stated otherwise, all reported p values refer to differences relative to the untreated controls.

**Results**

**Presence of FIM in Lysates of Various Types of Cells**

For identification of the types of cells containing FIM, various cell lysates were assayed for FIM activity. The results showed that intravenous injection of lysed resident peritoneal cells (76.9% macrophages) evoked early monocytosis in mice and stimulated proliferation of the continuous macrophage cell-line PU5–1.8 (Table I). Lysates of normal alveolar cells (99.2% macrophages) and PU5–1.8 cells gave similar results. Lysates of blood granulocytes and blood lymphocytes did not evoke a response (Table I). Lysates of a lymphoblastic cell line (L1210)
TABLE I

Presence of FIM in Lysates of Various Cells

| Cell type          | FIM index | In vivo* | FIM index | In vitro§ |
|--------------------|-----------|----------|-----------|-----------|
|                    | × 10³/ml  | in %     | × 10³/ml  | in %      |
| Resident peritoneal cells | 346 ± 60  | <0.001   | 25.2 ± 3.2 | <0.001   |
| Resident alveolar cells   | ND        | ND       | 28.2 ± 4.3 | <0.001   |
| PU5 cells             | 319 ± 56  | <0.001   | ND        | ND       |
| Blood granulocytes    | −65 ± 26  | NS       | 1.8 ± 1.9 | NS       |
| Blood lymphocytes     | 3 ± 8     | NS       | 5.6 ± 3.7 | NS       |
| L1210                | 53 ± 36   | NS       | −7.2 ± 1.9 | <0.02    |

* FIM activity determined in vivo and expressed as numerical increase of blood monocytes 24 h after injection of 0.2 ml of a suspension of 10⁷ lysed cells/ml, relative to the number of blood monocytes of untreated mice; mean ± SE.
§ Significance level of the difference relative to untreated controls; NS, p > 0.05.
$ FIM activity determined by in vitro bioassay and expressed as percent increase of the number of PU5 macrophages in cultures containing 2% (vol/vol) of a suspension of 10⁷/ml lysed cells under investigation in relation to the value of the reference culture; mean ± SE.
§ Negative values in vivo indicate a decrease in the number of blood monocytes relative to untreated mice, and in vitro, a decrease in cell proliferation relative to control.

FIGURE 1. Course of FIM activity in the supernatant of resident peritoneal cells incubated at a concentration of 10⁷/ml in the absence (a) and presence (b) of preopsonized latex particles (latex-to-cell ratio, ~100:1) from between 0.05 and 48 h. The FIM activity was assayed in vivo and expressed as the difference in number of blood monocytes 24 h after injection of 0.2 ml culture supernatant relative to the control. Data are means plus one standard error for triplicate experiments. 8–12 mice were used per time point. Negative values, which were obtained at 2 h (a) and 12 h (b), are put at zero here, because analysis of variances did not show any significant difference relative to the control.

did not induce monocytosis in vivo either, and in vitro even retarded the proliferation of the macrophage cell line slightly (Table I).

In Vitro Release of FIM

In a preliminary study (8) we found that peritoneal cells release FIM in vitro. To study this in more detail, these cells were incubated in Teflon film dishes in the absence and presence of a phagocytosable material. After various intervals up to 48 h the culture supernatants were sampled for determination of FIM activity by the in vivo assay. During incubation in the absence of latex, none of the supernatant samples injected intravenously into recipient mice induced a statistically significant monocytosis (p = 0.33) (Fig. 1a). The supernatant of
peritoneal cells incubated with preopsonized latex was, however, able to induce monocytosis; supernatant collected after 3 min of incubation caused the number of blood monocytes in the recipients to increase by $329 \times 10^3$ monocytes/ml at 24 h after intravenous injection ($p < 0.0001$ for the difference relative to the control) (Fig. 1b). The FIM activity in supernatants collected after that time point was much lower, and became minimal at 30 min ($\Delta, 24 \times 10^3$ monocytes/ml; $p = 0.72$) before rising to a maximum ($\Delta, 235 \times 10^3$ monocytes/ml; $p < 0.009$) after 2–6 h of incubation. The supernatant collected after 18 h of incubation showed another peak of activity ($p < 0.0001$) that subsided slowly (Fig. 1b). This course of FIM activity in the supernatant of peritoneal cells among which macrophages predominate (76.9%) is statistically significant ($p < 0.0001$ for time-dependent effect).

To find out whether this response is specific for Swiss mice and whether FIM in supernatant can be detected with the in vitro assay as well, we incubated resident peritoneal cells of Swiss mice and two other mouse strains in the presence of latex. In this experiment the course of FIM activity released by peritoneal cells of Swiss mice, as determined on the basis of its stimulatory effect on the proliferation rate of the macrophage cell line PU5, was almost the same as had been found with the in-vivo assay (Fig. 2a). Peritoneal cells from CBA and C57BL mice released FIM into the supernatant almost instantaneously upon exposure to latex and again in a later phase ($p < 0.001$ for time-dependent effect), but the courses were not exactly the same as that found for peritoneal cells from Swiss mice (Fig. 2, b and c).

**Effect of Inhibition of Protein Synthesis on FIM Release in Vitro**

To find out whether FIM released into the supernatant of a macrophage suspension was newly synthesized or already present in the cells, as found in cell
TABLE II
Effect of Cycloheximide on FIM Release by Resident Peritoneal Macrophages

| Addition to cultures* | FIM activity in supernatant after: |  | |
|-----------------------|----------------------------------|--|--|
|                       | 3 min   | 6 h    | 18 h   |
|                       | $\times 10^3/ml$ | $\times 10^3/ml$ | $\times 10^3/ml$ |
| None                  | 369 ± 64 | 282 ± 74 | 335 ± 32 |
| Cycloheximide         | 273 ± 10 | 188 ± 49 | 5 ± 45  |

FIM activity expressed as the increase in the number of blood monocytes 24 h after injection of 0.2 ml culture supernatant into mice relative to the number of blood monocytes in untreated mice; mean ± SE.

* Preopsonized latex particles were added to $10^7$ resident peritoneal cells per ml (latex-to-cell ratio about 100:1) in the presence or absence of cycloheximide in a final concentration of 0.5 μg/ml.

...lysates (see above), the effect of an inhibitor of protein synthesis was studied. The culture supernatant of resident peritoneal cells incubated for 3 min or 6 h in the presence of preopsonized latex and 0.5 μg/ml cycloheximide contained a significant amount of FIM activity ($p < 0.01$ and $p < 0.02$, respectively, relative to the controls) (Table II). After 18 h of incubation no FIM activity was shown by the in vivo bioassay ($p > 0.9$). At that time point, the number of macrophages containing latex and their viability did not differ from the control cultures.

**Association between the Number of Phagocytes in Various Cell Suspensions and FIM Activity in the Supernatant**

Because we wanted to know which cell type synthesizes and secretes FIM, we incubated cell suspensions differing in composition in the presence of latex for 18 h and then determined in vivo the FIM activity in the supernatant. FIM activity was found in the supernatant of resident peritoneal cells (Table III). Serial twofold dilutions of the resident peritoneal cell suspension incubated for 18 h in the presence of latex showed a dose relationship between the number of cells and FIM activity in the supernatant (Table III). This relationship can be described by the equation $n = 341 - 173 \times 2^{\log \text{(dose)}}$ ($r^2 = 0.803; p < 0.001$), where $n$ is the increase (Δ) in the number of blood monocytes ($\times 10^{-3}$/ml) at 24 h after injection and dose is the dilution factor.

When adjusted to the same number (about $2 \times 10^6$) of macrophages, supernatants of resident peritoneal and alveolar cells, J774.1 macrophages, and exudate peritoneal cells harvested 6 h after an intraperitoneal injection of latex showed about the same FIM activity (Table III). Since the numbers of granulocytes and lymphocytes in these cell suspensions varied, it is improbable that the release of FIM is attributable to these two types of cell. This point was studied in more detail by diluting a suspension of peritoneal exudate cells 100-fold to adjust the number of granulocytes to $4 \times 10^4$, i.e., the same number as in the resident peritoneal cell suspension. After incubation with preopsonized latex for 18 h, the supernatant showed no FIM activity ($p > 0.05$).

An analysis of correlation performed with the present data showed a significant linear association between the number of macrophages in the cell suspension and the FIM activity in the supernatant ($r^2 = 0.95; p < 0.001$); a relationship between
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TABLE III

Correlation Between the Number of Phagocytes in Various Cell Suspensions and FIM Activity in the Supernatant

| Cell type            | Cellular composition* | FIM activity in supernatant<sup>±</sup> |
|----------------------|-----------------------|-----------------------------------------|
|                      | Macrophages  | Granulocytes  | Lymphocytes  |                      |
|                      | × 10<sup>4</sup>  | × 10<sup>4</sup>  | × 10<sup>4</sup>  | × 10<sup>3</sup>/ml |
| Resident peritoneal cells | 180  | 90.0  | 2.0  | 16  | 8.0  | 331 ± 64 |
| Resident peritoneal cells (diluted 1:2) | 90  | 90.0  | 2.0  | 8  | 8.0  | 199 ± 44 |
| Resident peritoneal cells (diluted 1:4) | 45  | 90.0  | 1  | 2.0  | 4  | 36 ± 39 |
| Resident alveolar cells | 198  | 99.2  | 0.4  | 0.2  | 1.2  | 0.6  | 335 ± 156 |
| J774.1 macrophages | 200  | 100  |                      |                      |                      | 458 ± 82 |
| Exudate peritoneal cells<sup>§</sup> | 172  | 22.4  | 590  | 76.7  | 7.2  | 0.9  | 285 ± 67 |
| Exudate peritoneal cells<sup>§</sup> (diluted 1:100) | 1.3  | 24.3  | 4  | 74.6  | 0.06  | 1.1  | -25 ± 34 |

* Cells of various origin were suspended in RPMI 1640 and incubated for 18 h in the presence of preopsonized latex particles. Next, 0.2 ml of supernatant conditioned by the indicated number of cells, was assayed in vivo for FIM activity. The numbers of macrophages, granulocytes, and lymphocytes in each suspension are given, as well as the cellular composition, expressed as a percentage of total.

<sup>±</sup> FIM activity assayed in vivo and expressed as the increase in the number of blood monocytes 24 h after injection of 0.2 ml culture supernatant, relative to the number of blood monocytes in untreated mice; mean ± SE.

<sup>§</sup> Exudate peritoneal cells were harvested 6 h after intraperitoneal injection of ~5 × 10<sup>8</sup> latex particles and adjusted to obtain the same numbers of macrophages or granulocytes as in the resident peritoneal-cell suspension, the objective being to establish the relationship between cell type and the amount of FIM activity.

FIM activity and the number of granulocytes ($r^2 = 0.05; p = 0.62$) or lymphocytes ($r^2 = 0.14; p = 0.40$) was not observed. Together, these results indicate that FIM is secreted by macrophages and not by granulocytes or lymphocytes.

Characteristics of FIM Derived from Peritoneal Macrophages

Molecular Weight. The molecular weight of FIM present in supernatant of peritoneal macrophages was determined by filtration of the supernatant on a series of ultrafiltration membranes with different exclusion limits, after which the FIM activity of the various fractions was assessed in the in vivo assay. The results showed that macrophage-derived FIM passes through membranes that retain molecules of >50 × 10<sup>3</sup> mol wt and must be much smaller, since membranes with an exclusion limit of 25 × 10<sup>3</sup> mol wt did not retain FIM either (Table IV). Ultrafiltration of FIM-containing macrophage supernatant through a membrane with an exclusion limit of 10 × 10<sup>3</sup> yielded a residue that showed significantly more FIM activity than the filtrate ($p < 0.0001$) and the original supernatant did ($p = 0.008$) (Table IV). From these experiments it may be concluded that the molecular weight of FIM in macrophage supernatant lies between 10 and 25 × 10<sup>3</sup>.

Half-life in Cell-free Supernatant. To establish the half-life of FIM activity at 37°C, we used the filtrate obtained from a CF25 filter (exclusion limit, 25 × 10<sup>3</sup>), which will be referred to here as partially purified FIM (ppFIM). Incubation
Table IV

**Estimation of the Molecular Weight of FIM in Peritoneal Macrophage Supernatant by Ultrafiltration**

| Exclusion limit of membrane | Residue $\times 10^3$/ml | Filtrate $\times 10^3$/ml | Unfractionated supernatant $\times 10^3$/ml |
|-----------------------------|--------------------------|--------------------------|------------------------------------------|
| $50 \times 10^3$ (CF50)     | 119 ± 55                 | 234 ± 61                 | 218 ± 51                                 |
| $25 \times 10^3$ (CF25)     | 26 ± 38                  | 527 ± 74                 | 240 ± 88                                 |
| $10 \times 10^3$ (UM10)     | 510 ± 82                 | -20* ± 48                | 198 ± 86                                 |

FIM activity of each fraction assayed in vivo and expressed as the increase in the number of blood monocytes 24 h after injection of 0.2 ml, relative to the number of blood monocytes in untreated mice, mean ± SE.

* Negative values indicate a decrease in the number of monocytes relative to the control mice.

FIGURE 3. Course of the number of blood monocytes (O), lymphocytes (□), and granulocytes (△) per milliliter after intravenous injection of 0.2 ml of ppFIM. Data are means plus one standard error. 8-12 mice were used per time point.

of ppFIM for various intervals at 37°C before in vivo assay of residual FIM activity showed rapid inactivation with a half-time of about 2.5 min. During incubation of ppFIM at 37°C, addition of 0.5 mg/ml soybean trypsin inhibitor, which did not itself induce monocytosis ($p > 0.5$), inhibited the degradation of the activity of FIM for at least 45 min.

**Cell-lineage Specificity.** The cell-lineage specificity of ppFIM was studied by intravenous injection of 0.2 ml into mice. The number of blood monocytes increased from 338 (± 32 SE) $\times 10^3$ monocytes/ml to a maximum of 775 (± 70 SE) $\times 10^3$ monocytes/ml 24 h after the injection ($p < 0.0001$), and then gradually decreased, returning to control values at 72 h ($p < 0.0001$ for time-dependent effect) (Fig. 3). The number of blood granulocytes showed only a small and insignificant ($p = 0.28$) increase from 835 (± 117 SE) $\times 10^3$ cells/ml to 1225 (± 237 SE) $\times 10^3$ cells/ml at 12 h and then remained at that level for the rest of the observation period ($p = 0.48$ for time-dependent effect) (Fig. 3). The number of blood lymphocytes decreased slightly from 11.0 (± 1.2 SE) $\times 10^6$/ml to 8.3 (± 1.2 SE) $\times 10^6$/ml at 12 h ($p = 0.16$), but returned to the control level at 24 h ($p = 0.15$ for time-dependent effect).

**Stimulation of the Production of Monocytes in the Bone Marrow.** The course of the numbers of labeled monocytes after administration of $[3^H]$thymidine reflects
the effects of stimulation of monocyte production in the bone marrow. After intravenous injection of ppFIM together with $[^3]H$thymidine into Swiss mice, the number of labeled monocytes in the blood increased rapidly to a maximum of 2.78 ($\pm$ 0.41 SE) × 10$^5$ cells/ml at 24 h, which is twice the increase in the number of labeled monocytes in normal mice given only an injection of $[^3]H$thymidine ($p = 0.0002$) (Fig. 4). During the next 24 h the number of labeled monocytes in mice given ppFIM gradually decreased. At 48 h the difference with respect to normal mice amounted to only 25%, and after that time point the numerical course of the labeled monocytes was similar under both conditions (Fig. 4).

**Effect on the Cell-cycle Time of the Macrophage Cell Line PU5.** During incubation of PU5 cells in the presence of ppFIM with an in vitro FIM index of 18.3% and vincristine, the percentage of cells arrested in metaphase increased from 4.1% ($\pm$ 0.5 SE) at 0 h to 41.1% ($\pm$ 1.2 SE) at 6 h. Calculated with these values, the cell-cycle time of PU5 cells amounted to 16.3 h, that of the controls to 23.8 h. Thus, ppFIM decreased the cell-cycle time by 31.3%, which is greater than the effect on the population doubling time, i.e., a decrease of 18.3% relative to the control. This indicates that the FIM index underestimates the effect of FIM on the rate of proliferation of PU5 cells, in all probability because a proportion of the PU5 cells die during incubation and therefore the increase in the number of the cells is smaller than would be expected from the cell-cycle time.

**Presence of Other Biologically Active Factors in Peritoneal Cell Supernatant**

The presence of other factors relevant for the regulation of proliferation of monocyte precursors was studied in peritoneal macrophage supernatants with an FIM index of 22.7%. The supernatant contained only 10 U CSF-1 per milliliter, had no IL-1 activity, and showed no chemotactic activity. This supernatant contained 0.217 µg lysozyme and 2.5 mU $\beta$-glucuronidase per milliliter, which indicates that other biologically active substances were secreted normally under the culture conditions applied.

**Discussion**

The main conclusion to be drawn from the present study is that macrophages contain and are able to synthesize and secrete FIM. Granulocytes and lymphocytes neither contain nor secrete FIM.
FIM was found in lysates and supernatants of cultures of resident and exudate peritoneal and alveolar macrophages and a macrophage cell line. After exposure of phagocytosable particles, the macrophages released FIM into the culture supernatant, the course of the release showing a biphasic pattern, which is in agreement with preliminary findings (8). This pattern of FIM release is significant, because the standard deviation is much smaller at each time point than the fluctuation of the level of FIM activity in time, and is in all likelihood determined by the rates of synthesis, secretion, and in vitro inactivation, since FIM is readily inactivated by proteolytic enzymes in the culture supernatant. Thus, after the secretion of preformed FIM upon phagocytosis of particles, FIM synthesis recommences and this newly synthesized FIM is secreted too.

The characteristics of macrophage-derived FIM are in many respects similar to those of FIM in serum. The estimated molecular weight of macrophage-derived FIM lies between 10 and $25 \times 10^3$ and that of FIM in serum between 18 and $25 \times 10^3$. In all likelihood, macrophage-derived FIM, like FIM in serum, is a protein, its synthesis being inhibited by cycloheximide. Both FIMs stimulate the production of monocytes in the bone marrow. The courses of the labeled blood monocytes are not entirely identical; in the present study an injection of macrophage-derived FIM led to a maximum of $2.8 \times 10^5$ labeled monocytes per milliliter at 24 h followed by a gradual decrease to $0.8 \times 10^5$ per milliliter at 96 h, whereas an injection of serum containing FIM led to a further increase to a maximum of $5.1 \times 10^5$ cells per milliliter at 48 h (9). This difference could be due to either a smaller amount of FIM in FIM-containing macrophage supernatant (protein concentration, 0.82 mg/ml) than that in serum or to the difference in the half-life of FIM at 37°C, which was 2.5 min in macrophage supernatant and about 25 min in serum (7). In addition, the kinetic studies showed that macrophage-derived FIM is cell-lineage specific since it does not have any effect on the production of granulocytes or lymphocytes.

During inflammation induced by an intraperitoneal injection of latex, FIM activity in the serum, unlike that in supernatants of resident peritoneal macrophages incubated with preopsonized latex in vitro, increased steadily to a maximum at 18 h and then decreased gradually (3). No explanation can be offered for the more gradual increase of FIM activity in the circulation, but it seems possible that a loss of synchronization of FIM secretion after the injection of latex might be responsible, since, in contrast to the in vitro situation where the number of macrophages is constant, the number of FIM-secreting macrophages in the inflammatory exudate gradually increases.

For the present study, we used an in vivo and an in vitro bioassay with equal sensitivity for the detection of FIM activity in culture supernatants. The in vivo assay is based on the ability of monocyte precursors in the bone marrow to respond to FIM by increased monocyte production, which is measured as the increase in the number of monocytes in the circulation. The reliability of this assay for assessment of the stimulation of monocyte production is evident from the finding that the increase in the number of $[^3H]$thymidine-labeled monocytes was greater after injection of supernatant with FIM activity than for the controls. The validity of the in vitro assay, which is based on the ability of the continuous macrophage cell line PU5 to respond with an increased rate of proliferation, is
shown by the decrease of the cell-cycle time of PU5 cells cultured in the presence of FIM. Assay of FIM in vitro based on its effect on the cell-cycle time instead of on the population-doubling time is more sensitive but more laborious and therefore is not suitable for routine use.

What is the relevance of the present findings? Tissue macrophages are among the first cells to come into contact with deleterious material that must be eliminated. This requires a rather large number of phagocytes at the site where such material has become localized. The necessary increase in macrophages is effectuated by an influx of monocytes. The present results show that the macrophages themselves regulate the supply of circulating monocytes by stimulating the increase in the production of monocytes in the bone marrow via the secretion of FIM during phagocytosis.

Summary

Earlier investigations (3, 5, 8) had indicated that the factor increasing monocytopoiesis (FIM), present in the serum of mice and rabbits during the onset of an inflammatory response, is released by cells of the inflammatory exudate. The present study was performed to determine which cells produce and secrete this factor and to establish the kinetics of its production and secretion. FIM was assayed in vivo by intravenous injection of samples into untreated mice and monitoring the course of the number of blood monocytes in the recipients. FIM was assayed in vitro by adding samples to cultures of the macrophage cell line PU5 and determining the rate of proliferation of the cells.

The results show that only macrophages contain and synthesize FIM. This factor is secreted upon exposure to a phagocytic stimulus, and after the release of preformed FIM, macrophages secrete newly synthesized FIM. Granulocytes and lymphocytes neither contain nor secrete FIM.

The characteristics of FIM derived from macrophages are in all aspects similar to those of FIM in serum. Macrophage-derived FIM is a protein with a molecular weight between 10 and $25 \times 10^3$, its activity is cell-lineage specific and dose dependent, and it stimulates monocyte production in the bone marrow. Macrophage-derived FIM is not identical to either CSF-1 or IL-1, and has no chemotactic activity.

Taken together, the present results show that FIM occurring in serum during an inflammatory response originates from macrophages at the site of the inflammation. In this way the macrophages themselves regulate the supply of circulating blood monocytes that can migrate to the site of injury when needed.

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