PRODUCTION OF MACROPHAGE MIGRATION INHIBITION FACTOR BY CONTINUOUS CELL LINES*

By DAVID G. TUBERGEN,‡ JOSEPH D. FELDMAN, E. M. POLLOCK, AND RICHARD A. LERNER§

(From the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037)

Since the description of the capillary tube technique by George and Vaughan (1) the macrophage migration inhibition test has been widely used as an in vitro correlate of delayed hypersensitivity and cellular immunity (1–20). Migration inhibition factor (MIF),[1] which is responsible for this inhibition, was found to be released by sensitized lymphocytes of several species when sensitized cells were incubated in vitro with antigen. It has been repeatedly emphasized that the production of MIF was the result of a specific immunologic reaction (1–5, 8, 10, 15, 16, 19, 20) and its detection in vitro reflected a state of cellular immunity in vivo (2, 10, 14). Rarely, a report suggested that MIF might be released by mitogen that was not a specific antigen (21) or that correlation of migration inhibition in vitro and skin test reactions in vivo was poor (22). Recently, MIF has been detected in the medium of continuously growing cell lines, under conditions in which there was presumably no antigenic stimulation (23, 24).

In this paper we present evidence that MIF is released by both lymphoid and fibroblast cell lines in continuous growth, that its release is dependent upon activation of cells to enter the mitotic cycle, and that MIF is associated with delayed hypersensitivity and cellular immunity because specific antigen induces a small number of sensitized cells to divide.

Materials and Methods

Cell Lines.—Five cell lines were used. Lines WIL2 and 8866 are human diploid lymphocyte lines and their biology in culture has been described (25, 26). YCAB is a mouse lymphocyte

[1] *Abbreviations used in this paper:* FCS, fetal calf serum; MEM, Eagle's minimal essential medium; MIF, migration inhibition factor; PBS, phosphate-buffered saline.

line originating from A strain mice (27). The mouse fibroblast line, 3T3, derived from Swiss mice, was provided by Dr. M. Vogt of the Salk Institute and has been maintained by us. WI38 is a diploid fibroblast line that originated from human embryonic lung and was a gift from Dr. Fred Jensen of the Wistar Institute. Repeated efforts to identify mycoplasma by culture, by chemical analysis of microbial DNA and RNA, and by electron microscopical surveillance have been negative. Control lymphocytes were enriched to a 98% plus purity from normal human peripheral blood by Dr. John Mendelsohn, as described elsewhere (28).

Culture Media and Conditions.—Cells were routinely grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and antibiotics as previously described (26). The lymphoid lines were seeded at a concentration of 2×10^5 cells/ml in a final volume of 500 ml and were incubated at 37°C in stoppered 2-liter flasks on rotating platforms. In approximately 5 days they reached a concentration of $2-3 \times 10^6$ cells/ml. At this point cells were arrested in the G_1 "stationary phase" of the cell cycle (29). At appropriate time intervals after replacement of media, supernatants were removed and tested for MIF. Fibroblasts were grown at 37°C in 1-liter flat bottles containing 50 ml of medium and 5% CO_2 in air atmosphere. Media were changed by decantation and replacement with fresh media. In some experiments, as will be specified, cells were grown in the absence of FCS or with 10% A strain mouse serum substituted for FCS.

Supernatants.—All media and supernatants to be tested for MIF activity were centrifuged for 20 min at 1500 rpm, 4°C, and frozen at −20°C until assayed. To eliminate nutritional depletion as a cause of migration inhibition, samples were dialyzed against fresh MEM for 6–18 hours before testing. Material used for column chromatography was concentrated approximately 20 times by lyophilization and resolution in distilled water. Approximately 6 ml of the concentrated material was applied to a 90×2.5 cm G-100 Sephadex column (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) and eluted with 0.14 phosphate-buffered saline (PBS). Trace quantities of HSA-^{131}I were added to the redissolved material as a molecular weight marker. The eluted fractions were pooled as shown in Fig. 1, lyophilized, redissolved in 5 ml of distilled water, and dialyzed against PBS. 0.2–0.4-ml aliquots of these fractions were then added to 2.2 ml of media for MIF testing. For control migrations (100%), unused fresh complete media (MEM plus FCS) were similarly concentrated and fractionated; to this was added 0.2–0.4 ml of PBS.

MIF Tests.—MIF was assayed, as described by Bloom et al. (12), with peritoneal exudate cells obtained from normal Hartley guinea pigs 3–4 days after the intraperitoneal injection of 20 ml of mineral oil. The cells were collected by lavage of the peritoneal cavity with cold Hanks' balanced salt solution, washed two times, and resuspended at a concentration of 5×10^7 cells/ml in MEM containing 15% normal heat-inactivated guinea pig serum. The cell suspensions, containing 60–70% large mononuclear cells, were put into capillary tubes sealed at one end and were centrifuged at 600 rpm for 2 min. Capillary tubes were cut at the cell-medium interface and the sealed ends were fixed with silicone grease to the bottom of a Mackaness chamber. The chambers were filled with test or control media, sealed, and incubated at 37°C for 20 hr. After incubation, chambers were photographed, the negatives projected, the outlines of the migration patterns were traced on bond paper, cut out, and weighed. The weight of the cut out pattern was directly proportional to the area of migration. Each sample was tested in at least two chambers so that a minimum of four migration patterns was obtained for one sample. The results are expressed as percentage of inhibition:

$$\text{Per cent inhibition} = 1 - \frac{\text{mean area of migration of experimental (mg)}}{\text{mean area of migration of control (mg)}} \times 100.$$

Inhibition of 20% or less was not regarded as significant.

To control and test media, heat-inactivated guinea pig serum was added at a concentration of 5% just before use. Controls comprised: complete medium in which cells had not been

grown; complete medium fractionated and handled in the same manner that supernatants were handled; supernatants from fresh human peripheral blood lymphocytes that were incubated for periods of time equal to or greater than the periods during which supernatants of continuous cell lines were collected; and supernatants of two equal time intervals removed from the same flask of cells. In the experiment in which YCAB cells were grown in mouse A strain serum and FCS for comparison, the supernatants were assayed with mouse peritoneal exudate cells collected 3 days after intraperitoneal injection of proteose peptone.

RESULTS

MIF Production by Continuous Cell Lines.—As indicated in Table I, in 16 of 17 trials with the supernatants of four cell lines there was significant inhibition. Controls consisted of either complete fresh medium and/or medium in which unstimulated human peripheral lymphocytes had been incubated for 24–96 hr. No significant differences were seen between the controls.

TABLE I

Production of MIF Activity by Continuous Cell Lines*

Supernatant of cell strain	Per cent inhibition		
8866 (Human lymphocyte)	66	51	77
	0	66	69
WIL2 (Human lymphocyte)	67	68	68
YCAB (Mouse lymphocyte)	61	69	74
	72		
WI38 (Human fibroblast)	74	25	
Normal human lymphocytes	0	0	0
Media not incubated with cells	0	0	0

* $P < 0.05$ for inhibition of 20% or greater.

Recovery of MIF Activity from Sephadex G-100 Chromatography.—Concentrated supernatants from human (8866) and mouse (YCAB) lymphoid lines were eluted from Sephadex G-100. Fig. 1 demonstrates the MIF activity in each pooled fraction in relation to the recovery of HSA-^{131}I for the human lymphoid line. Significant inhibition was seen in those fractions eluting with and behind albumin, comparable to the elution behavior of MIF described by Remold et al. (30). Fig. 2 presents similar data for mouse lymphoid MIF, namely that MIF begins to elute in the fraction containing albumin although the greatest amount of activity occurs in the fraction eluting behind albumin. In terms of estimated molecular size, 30,000–55,000, MIF produced by continuous human and mouse cell lines corresponded to that described for MIF produced by sensitized cells incubated with antigen (30–32).

MIF Release Related to Phase of the Cell Cycle.—A synchronized culture of 8866 cells was initiated with greater than 95% of the cells in the G_1 phase of the cell cycle. DNA synthesis, as measured by the uptake of thymidine-^{14}C, began at about 8 hr after synchronization, peaked at 20 hr, and then rapidly declined.

Fig. 3 demonstrates the amount of inhibition found with supernatants from G_1 (0–8 hr), early S (8–12 hr), and late S (12–24 hr) phases in such cultures. Significant inhibition was present in the late S phase of the cell cycle. No MIF was detected in supernatants of G_1 and early S phases. This experiment was repeated three times and yielded similar results each time.

Supernatants of the different phases were concentrated and tested at varying dilutions to measure semiquantitatively the amount of MIF released during each phase of the cell cycle. Fig. 4 illustrates that the 12–24-hr S phase supernatant, spanning a 50% greater time interval than the 0–8-hr G_1 supernatant,
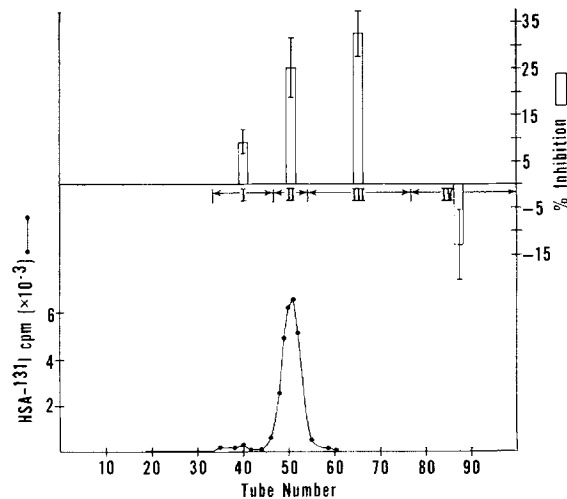

FIG. 1.   Recovery of MIF produced by human lymphoid cells (8866) from Sephadex G-100. The column eluates were pooled into fractions as indicated by the roman numerals. Vertical bars represent per cent inhibition; vertical lines indicate ±SE. Continuous solid line constructed from counts per minute of radioactivity.

contained four times more MIF activity than was detectable in G_1 material. There was, however, some inhibition with the eight times concentrated samples of both 0–8- and 8–12-hr periods. This was most likely related to incomplete synchrony and did not detract from the observed relationship between DNA synthesis and MIF release.

MIF Production by Growing and Contact Inhibited Fibroblasts.—Previous reports have documented the loss of mitotic activity by and synchrony in G_1 of 3T3 fibroblasts during contact inhibition (33–35). Therefore we compared the amount of inhibition produced by supernatants of 3T3 fibroblasts during the growth phase of cultures with those contact-inhibited cells collected over a similar length of time, as shown in Table II. The greatest inhibition occurred with supernatants of growing fibroblasts. There was also MIF activity in supernatants of contact-inhibited cells, but it was substantially less than that

in supernatants of growing cells. The presence of MIF in supernatants of contact-inhibited fibroblasts may be related to the observation that merely adding fresh serum, as was done in this experiment, triggers a small proportion of the G_1 cells to enter the mitotic cycle, divide, and again stop in G_1 (35, 36).
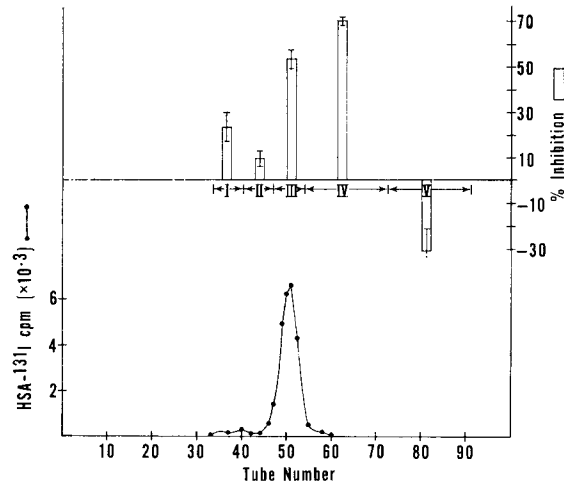

FIG. 2. Recovery of MIF produced by mouse lymphoid cells (YCAB) from Sephadex G-100. The column eluates were pooled into fractions as indicated by the roman numerals. Vertical bars represent per cent inhibition; vertical lines indicate ±SE. Continuous solid line constructed from counts per minute of radioactivity.
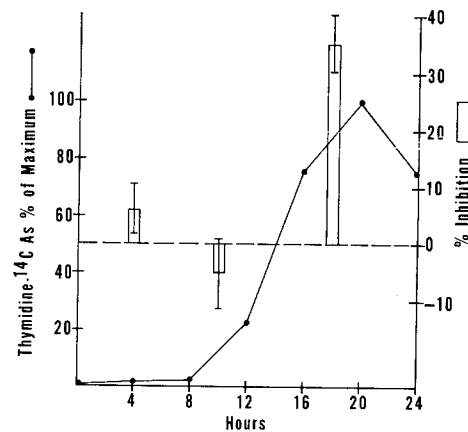

FIG. 3. Production of MIF by synchronized lymphocytes (8866) in relationship to the thymidine-^{14}C uptake. Three time periods 0-8 (G_1), 8-12 (early S), and 12-24 hr (late S) are shown. Per cent inhibition is indicated by the vertical bars; vertical lines represent ±SE.

MIF Production by Mouse Lymphocytes in Homologous Serum.—The possibility existed that lymphocytes in media containing FCS released MIF in response to an antigenic stimulation provided by FCS. This possibility was examined by growing thoroughly washed YCAB cells in parallel, in 10% FCS and 10% A strain mouse serum. After 4 days the cells were sedimented and the supernatants tested for MIF activity against mouse peritoneal exudate cells.
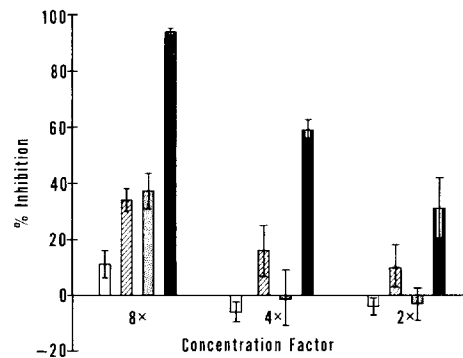

Fig. 4.   MIF activity in concentrated supernatants from synchronized lymphocytes (8866). Vertical bars depict per cent inhibition; vertical lines represent ±SE. Open bars, control normal medium; hatched bars, 0–8-hr collection (G₁); dotted bars, 8–12-hr collection (early S phase); solid bars, 12–24-hr collection (late S phase).

TABLE II

Production of MIF by Growing and Contact-Inhibited Fibroblasts

Supernatant	Per cent inhibition (± SE)		
	Exp. 1	Exp. 2	Exp. 3
Control media	0 (± 5.1)	0 (± 5.2)	0 (± 4.8)
Contact inhibited	6.6 (± 4.7)	59.2 (± 3.8)	13.5 (± 8.0)
Growth phase	56.4 (± 6.9)	81.4 (± 4.2)	74.4 (± 8.2)

As shown in Table III both supernatants contained significant amounts of MIF activity.

MIF Production by Human Lymphoid Cells in Serum-Free Media.—If purification of MIF is to be accomplished by the use of continuous cell lines, it is desirable to have as little extraneous protein as possible in the starting material. Also, a protein-free medium would eliminate any possibility of antigenic stimulation. Lymphoid cells (line WIL2) were planted at 2×10^5 cells/ml in media containing FCS. When the cell concentration reached 9.0×10^5 cells/ml, the cells were washed with MEM and resuspended at the same concentration in either MEM with 10% FCS or in MEM without added serum. After 24 hr the cell concentrations were 19.3×10^5/ml in the culture containing

serum and 14.1 × 10^5/ml in the culture free of serum. The supernatants were tested for MIF activity after adding FCS to the protein-free supernatant and as shown in Table IV the production of MIF was essentially the same in both supernatants. The above experiment was repeated with 8866 cells and yielded similar results.

TABLE III

Production of MIF by 24-Hr Cultures of Mouse Lymphoid Cells in Isologous Mouse Serum and Fetal Calf Serum

Supernatant	Per cent inhibition (± SE)
Fresh media with 10% mouse serum	0 (± 9.0)
Supernatant with 10% fetal calf serum	85 (± 3.9)
Supernatant with 10% isologous serum	93 (± 1.6)

Each figure represents the mean of four measurements.

TABLE IV

Production of MIF by 24-Hr Cultures of Human Lymphoid Cells in Serum-Free Media and Media Containing 10% Fetal Calf Serum

Supernatant	Per cent inhibition (± SE)
Fresh media with 10% fetal calf serum	0 (± 13.0)
Supernatant with 10% fetal calf serum	68 (± 7.4)
Supernatant with 10% fetal calf serum added after cell incubation in protein-free media	75 (± 3.3)

Each figure represents the mean of four measurements.

DISCUSSION

The evidence presented here indicated that MIF release was associated with activation of cells from a resting state into the mitotic cycle, particularly with S-phase cells. Resting lymphocytes, almost 100% purified from peripheral blood of normal human donors and almost all in G_0, did not release MIF into media throughout cultivation periods of 24–96 hr. By contrast, MIF was readily and reproducibly assayed in media of continuously proliferating lymphocyte lines, both human and murine. Furthermore, when synchrony was established in one human cell line, i.e. about 95% of the cells in culture were in G_1 and then permitted to grow, MIF was detected only in S phase. These results were consistent with the observation of Bennett and Bloom (37) that after antigen was added to sensitized lymphocytes a period of 6 hr elapsed before MIF could be assayed, and also with the observations that certain metabolic inhibitors, notably actinomycin D and puromycin, prevented release of MIF (38). These reagents may have stopped cells from entering S phase of

the cell cycle. It should be emphasized that actual cell division was not required to release MIF.

The results of the experiments with 3T3 fibroblasts corroborated those obtained with the lymphocyte lines. Actively growing fibroblasts in culture released significantly greater quantities of MIF than did the same cells during periods of contact inhibition. These results appeared even more striking when we calculated that during growth phase of 4 days needed to reach a state of contact inhibition the average number of cells in culture was about 25 % of the number of cells present during contact inhibition. Thus a smaller number of proliferating cells produced a greater quantity of MIF than did four times as many cells in a state of contact inhibition (G_1), a state that is somewhat analogous to G_0 (26, 29). Some MIF was assayed in supernatants of contact-inhibited cells and this was attributed to the fact that addition of fresh medium to an inhibited culture activated a small proportion of G_1 cells to enter S (35, 36).

The association of MIF release with cycling cells provoked a number of important questions. First, was the MIF of continuously growing cell lines the same as or similar to MIF generated by the interaction of sensitized lymphocytes and specific antigen? Since MIF has not been chemically nor immunologically identified, one can only compare the few biophysical features of MIF from different sources. MIF released by continuously proliferating cell lines was eluted from Sephadex G-100 in the same manner and under the same conditions as MIF generated by the interaction of antigen with sensitized lymphocytes (30–32). This finding was suggestive of similarity between MIF from human, murine, and guinea pig cells, produced under different circumstances, but was in no way proof of identity nor did it certify the singularity of MIF, i.e., there may be several such factors. Indeed the assay for MIF is a nonspecific test that measures inhibition of macrophage migration and there are certainly many reagents, without immunologic specificity, that might accomplish this (39–41). Further, Amos and Lachmann described an MIF that was antigen dependent; it inhibited macrophage migration only in the presence of antigen (15).

A second question provoked by the results of this report was what is the relationship of MIF to other lymphokines that are generated by activated cells (23, 32, 39, 40, 42) e.g., lymphotoxin, chemotactic factors, agglutinating agents, etc. Nonspecific stimulants of cell proliferation such as concanavalin A and phytohemagglutinin have been reported to release skin reactive factor (40) and lymphotoxin (39), respectively. Furthermore, continuously growing cell lines, that replicate in the absence of known stimulants, produce lymphotoxin and MIF (23) and our line 8866 makes interferon (S. H. Baron, personal communication). Until one or more of these factors has been isolated and identified it will not be possible to establish a relationship, if any, among them.

A third important question was what is the relationship of MIF to delayed

hypersensitivity and cellular immunity. Originally, MIF was found in medium after incubation of sensitized lymphocytes with specific antigen and was presumed to be an in vitro correlate of delayed hypersensitivity. Our studies indicated that MIF was a product of mitotic activity or of cells preparing to divide, and many stimuli, immunologic or nonimmunologic, that would activate cells to enter S phase would suffice to cause release of MIF. Several of our experiments were designed to rule out the possibility that proliferation of continuously growing cell lines was a response to antigens by cells cloned for these antigens. First, it would be highly unlikely that fibroblasts divided in response to antigens and thus produced MIF as an immunologic product. More telling arguments rested on the fact that MIF was released by cells grown in homologous serum in the absence of FCS, a medium that did not contain antigenic substances, and also on the fact that MIF was released by cells in medium lacking any protein. That mycoplasma or other bacterial contaminants played no role was confirmed by our inability to identify them on numerous occasions by culture and electron microscopical surveillance.

MIF released by the interaction of sensitized cells and specific antigen would then reflect a restricted situation in which a small number of the cells were stimulated by antigen to divide. In circumstances in which most of the proliferating cells were activated by antigen, the release of MIF might be regarded as a product of an immunologic reaction, but when cells stimulated to divide by antigen represented only a fraction of all dividing cells, then MIF could not be considered an immunologic reagent.

SUMMARY

Macrophage migration inhibitory factor (MIF) was found in media of human and mouse lymphocyte and fibroblast cell lines that were continuously growing. Its release was dependent on activation of the cells to enter the mitotic cycle, particularly on cells in S phase. The greatest quantity of MIF was detected in supernatants of lymphocytes collected during S phase after the cells were synchronized in G_1 and in supernatants of growing fibroblasts. When the latter were contact inhibited little or no MIF was found in media. MIF was also released into media of cells proliferating in homologous serum in the absence of fetal calf serum and into media lacking any protein. The MIF produced by lymphocyte lines eluted from Sephadex G-100 in the same fashion as MIF produced by the interaction of sensitized guinea pig cells and antigen. The results indicated that MIF is not a specific mediator of delayed hypersensitivity and cellular immunity and that MIF released by sensitized lymphocytes incubated with antigen merely reflects that fraction of cells activated by antigen to enter the mitotic cycle.

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