

Lymphocyte-Derived Factors Affecting Cell Migration *In Vitro*

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1 INTRODUCTION

The phenomenon of cell migration inhibition *in vitro*, in the present context, was initially described in 1932 by Rich & Lewis. They showed that the migration of cells from spleen fragments taken from guinea pigs sensitized to tubercle bacilli was reduced in the presence of tuberculin. In 1962, George & Vaughan improved the technique; they demonstrated the antigen-induced inhibition of the migration from small glass capillary tubes of oil-induced peritoneal exudate cells (PEC) from sensitized guinea pigs. Soon the migration inhibition phenomenon in this system was examined in more detail, especially by David and co-workers and by Bloom and Bennett. The test proved to be a reliable *in vitro* correlate of delayed type hypersensitivity; with few exceptions, antibody and/or complement components were found to be uninvolved in the reaction. The participating cells were identified as lymphocytes and macrophages, the former responsible for the immunologic specificity, which was carrier dependent. A positive reaction could be obtained when lymphocytes constituted no more than 1% of the cell population, but the optimum figure was found to be 20-40%. The macrophages were found to serve as indicator cells. No migration inhibition was observed in the presence of protein synthesis inhibitors, indicating that a newly synthesized protein played an essential role in the reaction. Conforming to this, it was shown that, when sensitized lymphoid cells were stimulated by antigen, they produced a soluble factor

which inhibited the migration of normal macrophages out of the glass capillaries. The lymphocyte-produced factor responsible for this effect was called Migration Inhibitory Factor (MIF). This "factor" is the earliest and today the most extensively described member of the group of lymphocyte-produced mediators of cellular immunity, i.e. the lymphokines (recently reviewed by Bendtzen in this journal). MIF-like activities have now been studied in a number of experimental systems with the aid of several different techniques. The present article will briefly review the properties of these factors and their mutual relation. It will become clear that a factor which is effective in one specific system does not necessarily show activity in other systems. Thus, one can differentiate MIF-activity according to cell type used as indicator. Furthermore, the possibility that under some circumstances the total MIF-activity in a single system could be due to more than one specific factor is considered, and the relation between factors produced by various cell types (e.g. B-cells contra T-cells) at stimulation by different inducers (e.g. specific antigen contra mitogen) is discussed. The existing situation has posed problems with respect to the terminology. Thus it seems that the term MIF should be understood as a general description of an activity shown by a variety of factors in various systems and not as a descriptive term for a single moiety in a single system, unless conditions for its production (producing cell type and inducing

agent) and assay (indicator cell and technical details) are also clearly indicated. The inadequacy of the present terminology from this and from other aspects has been previously stressed (56).

The present review is in no way exhaustive. Circumstances made us choose to cite recent articles in which extensive lists of references to original reports can be found. Therefore some important original reports and some statements in the text are not adequately covered by explicit reference. The pertinent references can be obtained in previous reviews that treat the migration inhibition phenomenon in detail (e.g. refs. 9, 10, 16, 56, 57, 60).

II MIF ACTING ON GUINEA PIG MACROPHAGES

1. *Methods of assay*

The technique originally described by Rich and Lewis, where the migration of cells from

lymphoid tissue fragments was examined, is seldom used today. David & David (16) have reviewed the work performed with this method. The procedure developed by George and Vaughan and by David et al. (see 16) is presently in general use and a number of modifications have been described. Standardized procedures are given by both Bloom and Bennett and by David & David in ref. 10, by Hughes (38), and by Morley et al. (57).

Fig. 1 shows schematically the principles of the direct and indirect forms of the test. The following comments seem justified with respect to the technique.

(i) Leu et al. (48) have shown that the PEC respond best to MIF when taken 2–3 days after the oil injection. Normal PEC and alveolar macrophages and PEC obtained more than 3–4 days after oil injection respond less well to MIF unless the migration is performed in the presence of

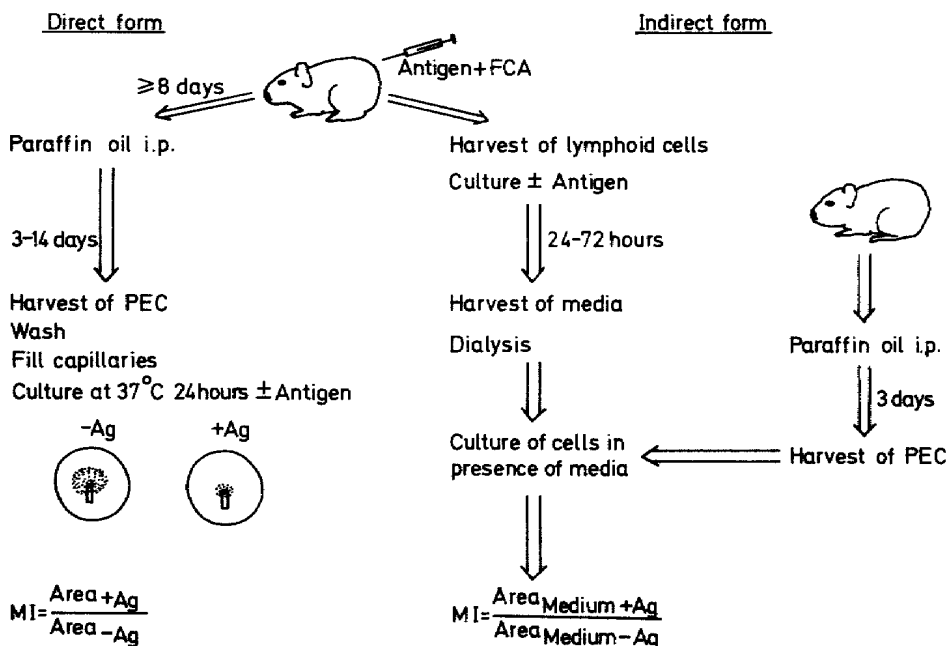


Fig. 1. Principles of the direct and indirect forms of the macrophage migration inhibition test in guinea pigs.

certain MIF-promoting sera and also show reduced capacity to adsorb MIF from active supernatants. In the direct test, Hughes (38) found that the cell yield is higher and the cell reactivity is apparently not decreased 7 to 14 days (compared with 3 days) after oil injection.

(ii) Some authors have reported that cells escape from migration inhibition if cultures are analysed at 48–72 h instead of at 18–24 h after initiation (direct test) (16, 38, 85). The escape phenomenon is probably due to a progressively decreasing pH of the culture medium to below pH 7.2, where migration inhibition is decreased, and/or that cells which initially show inhibition of migration in later phases of the culture migrate faster than cells in control cultures. Watanuki & Haga (85) suggest that the escape phenomenon is more expressed at 24 h at the indirect than at the direct test. Thus adequate control of pH and of culture time is essential.

(iii) It has been shown that serum is required for migration of macrophages (38, 63), that the migration area of individual macrophage batches varies with individual sera used for supplementing culture medium (6), and that LPS induces direct migration inhibition in the presence of fresh C4 deficient serum, but not in the presence of heat-inactivated or zymosan-treated serum, indicating that activation of complement through the alternative pathway could have an effect mimicking that of MIF (32). It has also been shown that high concentrations of serum (30%) in the cultures block the expression of MIF-activity (63), that the activity of ConA-induced MIF of human blood lymphocyte origin is governed by a heat sensitive (56°C for 2 h) inhibitor (CFI), which is a normal constituent of serum (84), and that fresh serum enhances part of the migration inhibitory activity of lymph from sensitized and PPD-challenged sheep (55). Thus the nature and concentration of serum used in the culture influence the outcome of the test.

(iv) The influence on the test results of a number of other important variables, such as storage of cells, dimension of capillary tubes, storage time of filled capillary tubes, force used to sediment cells in capillaries, use of anticoagulants, and optimum antigen concentration, is discussed in refs. 10, 16, 38, 57.

(v) The area of cell migration is usually quantified by tracing a magnification of it on paper, followed by planimetric measurement, or by cutting out and weighing the tracing (see 38). Variability between identically treated capillaries could be large; therefore a sufficient number of capillaries should be used for each reagent (6, 38, 56). The effect is often expressed as the migration index (MI), which is the ratio of mean areas of migration in the presence of antigen to those in the absence of antigen. If MI is less than 0.8, many authors conclude that the effect is significant. Experience shows that a more stringent statistical treatment of the data than the above-mentioned is highly desirable (6, 54, 56, 58) and also that measurement of migration distance instead of migration area is justified (85). At measurements of areas, square root transformation of data normalizes the distribution and reduces the scatter of the values (58).

(vi) Mixed success has attended attempts to apply the cell migration under agarose gel technique (see section IV. 1.B.) to guinea pig peritoneal cells. Carpenter et al. initially developed the technique for use in the guinea pig and demonstrated inhibition of migration of peritoneal cells by specific antigen. Braendstrup et al. (11) using comparably low numbers of cells per hole, demonstrated migration inhibitory activity for peritoneal exudate cells from non-immunized animals with supernatants of tuberculin-stimulated guinea pig lymph node cells. However, some groups have reported failure to obtain migration of the peritoneal exudate cells. Harrington and Stastny have introduced a technique where the macrophages migrate from an agarose

droplet. This method seems to be simple and economical with reagent; it has also been applied to tests in mice and human systems (for ref. see 1, 47, 65).

2. MIF. Production and properties

Fig. 2 gives a schematic representation of various factors shown to influence the production and biological properties of antigen- and mitogen-induced guinea pig MIF. Fig. 3 shows a comparison between the properties of this and a number of MIF-activities derived from other sources. These Figures merit the following comments.

A. General comments

The only way of assaying MIF is still the original test where the activity of a supernatant from an appropriately stimulated culture is compared with that of a reconstituted one from a saline-treated

culture. This type of biological assay has many pitfalls (56, 57, 82). The necessity of quantifying MIF with the aid of dose response curves and standard preparations has repeatedly been stressed (54, 56, 57). It should be noted that many reports regarding production and properties of MIF fail to show even simple dose response curves for the claimed effect; where such curves are given, it is striking that, already after a 10-fold to 20-fold dilution, the effect is almost lost (see, e.g., 57). McDaniel et al. (54) were unable to obtain correlation between concentration of MIF-sample and degree of inhibition. Furthermore, whereas the antigen bovine gamma globulin (BGG) can inhibit the migration of BGG sensitive PEC at concentrations between 0.1 $\mu\text{g/ml}$ and 0.01 $\mu\text{g/ml}$ in the direct test (16), the same antigen has to be used at concentrations between 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ to induce substantial MIF production in supernatants

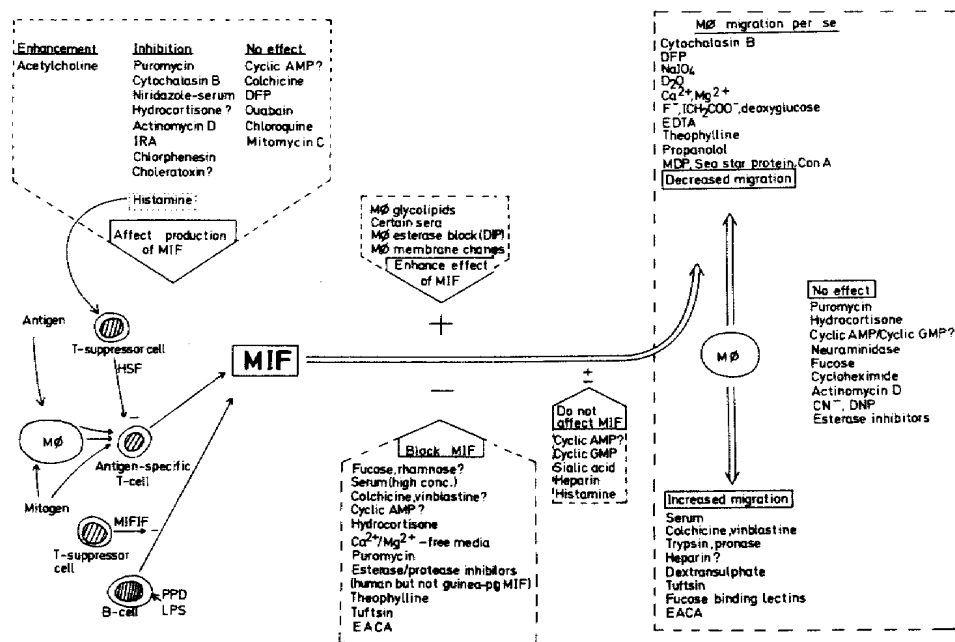


Fig. 2. A schematic representation of various factors that influence the production and biological effect of guinea pig MIF.

from sensitized lymph node cell cultures (45). In the last-mentioned experiment, even a strong MIF-activity was lost after a 4-fold dilution. Possibly, the sensitivity of the assay for MIF can be increased—at the expense of cell economy—by incubating the PEC with the supernatants before filling the capillaries (48, see also 54). Nonetheless, only the postulation of a very high local concentration of MIF in the vicinity of the macrophage in the direct form of the test seems sufficient to explain these data within the framework of a soluble mediator concept.

Taylor et al. (82) reported in detail on difficulties in producing MIF at stimulation of human peripheral leukocytes with ConA in serum-free cultures. On the other hand, they showed that inadequately removed ConA in low concentrations (0.1–1 $\mu\text{g/ml}$)

can mimic MIF-activity (see also 26). Leukocytes prepared from heparinized blood by sedimentation in dextran are often contaminated with erythrocytes. The red cells are agglutinated and lysed by the mitogen during culture, which leads to higher concentrations of protein in supernatants from stimulated cultures than in reconstituted control culture supernatants. Removal of ConA by adsorption to Sephadex gel is then more efficient for the former than for the latter supernatant. The authors also found that MIF-like activity induced by stimulation with PHA could largely be reduced by dialysis or by the addition of glucose. Remaining MIF-activity with macromolecular properties constituted only a small part of the total initial activity. The authors point out that care should be observed

Source of MIF-activity

	Gp Ag ^{MIF}	Gp Ag(low) ^{MIF}	Gp ConA ^{MIF}	Homo Ag,ConA ^{MIF}	FCS ^{MIF}
Cellular source	LNC	LNC	LNC	PBL	
App. molecular weight	~ 50,000	~ 25,000	<del>~ 65,000</del> ~~25–43,000~~	(~ 25,000)	~ 50,000
pI			3–4.5 5–5.5	3.5–4	
Nature	Glyco-protein		Glyco-protein	(Protein)	
Inactivation by neuraminidase	Controversial		Yes (Partial?)	(No)	
Inactivation by 56°C 30 min	No		No	No	(Yes)
Inactivation by DFP	No			(Yes)	
Antigen-dependence	No	(Yes)			

Fig. 3. A schematic comparison of MIF-activities derived from various sources but all affecting guinea pig macrophages. Encircled figures indicate difference from GP Ag^{MIF}.

before a migration inhibitory effect is attributed to the activity of a macro-molecular lymphocyte-derived product. It is worth mentioning that, according to published reports, there are many workers who do not dialyze their supernatants before testing.

B. MIF produced by guinea pig lymphoid cells

1. MIF produced by T-cells stimulated by specific antigen or mitogen

a. Culture conditions

Supernatants exhibiting MIF-activity are produced at culture of more or less purified lymphoid cells in the presence of specific antigen or mitogen. Corresponding control supernatants receive the stimulant after separation of the cells. As a further control, fresh, appropriately reconstituted medium can serve, but it cannot replace the reconstituted control supernatant (57, 60).

Cells: Most authors use draining lymph node cells (LNC) for MIF-production (when mitogen is used as stimulant, animals are immunized with FCA to increase cell yield). LNC seem to be better MIF-producers than spleen cells (16, 57). Doroszczak et al. (18) found that the LNC taken 8 days after immunization were more efficient MIF-producers than 3-week cells. According to Morley et al. (57), strong sensitivity is maintained for a longer period, but with lymph nodes taken later than 14 days post-immunization, there is a reduced yield of viable cells. Purified peritoneal T-cells are also highly efficient as MIF-producers (for ref. see 26, 57, 60). The presence of viable macrophages seems necessary for antigen-induced production of MIF to occur; the presence of 2-mercaptoethanol or "particular" PPD cannot replace the macrophages (for ref. see 46, 60). The MIF-producing cells of the lymph node are the large, most rapidly sedimenting cells that do not proliferate on exposure to antigen or mitogen (18, 60). These data confirm

previous reports that MIF-production does not depend on cell proliferation (for ref. see 33, 60). MIF-producing cells are usually T-cells, although B-cells can also give rise to migration inhibitory activities. Erythrocyte contamination in the culture should be kept low, because: (i) terminal fucose residues of the blood-group substances have been claimed to block or even adsorb the MIF-activity (for ref. see 2, 17, 24, 60); (ii) agglutination and lysis of red cells could lead to differing protein concentrations in active and control supernatants (82).

Conditions for culture. Cell concentrations used at culture range from less than 10^6 to 50×10^6 /ml. Cultures are performed with or without the addition of 5–15% fresh frozen or heat-inactivated, homologous or heterologous serum to the medium at 37°C for 24 h or up to 4 days. Morley et al. (57) recommend flat-bottomed glass culture vessels and a lymphocyte concentration of 5×10^6 /ml at a cell density of about 5×10^6 /cm². Most authors prefer to use serum-free medium in order to avoid large amounts of interfering protein at concomitant purification of MIF. Specific antigen is added at a final concentration of 10–200 µg/ml, sometimes even at 1 mg/ml. ConA is preferably used at 10 µg/ml (see, e.g., 71, 72); the mitogen is then removed from active and reconstituted supernatants by adsorption to Sephadex (cf. 82). Migration inhibitory activity, attributable to remaining ConA in active or control supernatants, can be blocked with α -methyl-D-mannoside; this is not the case with MIF-activity (26).

Insoluble ConA (covalently bound to Sepharose 4B) also yields sizeable amounts of MIF activity at culture with guinea pig lymphoid cells. This MIF-activity is not due to free ConA released during culture (26). MIF-activity can be demonstrated 6 h after antigen addition, and production continues for 3–4 days (16, 21). Remold et al. (71) found that LNC stimulated by ConA produce more MIF-activity than sensitized

cells at antigen stimulation and also that simultaneous antigen stimulation reduces the production of MIF in ConA-treated cultures. However, Ford et al. (21), at a cell concentration of $50 \times 10^6/\text{ml}$, find equal degrees of MIF-production at optimum antigen and ConA concentrations. At this high concentration of cells in the culture, even control culture supernatants are slightly inhibitory, which is not normally the case (e.g., 95, but see also 60).

b. Agents influencing production of MIF

The production of MIF by the lymphoid cells demands protein synthesis (9, 16, 33, 60). There is no influence on MIF-production by Mitomycin C (9, 60), whereas Actinomycin D is inhibitory (16, 60). Moreover, an intact microfilament system seems necessary because cytochalasin B ($5\text{--}10 \mu\text{g}/\text{ml}$) completely blocks production of MIF, but only when added initially; if added 2 h after antigen, no effect is seen (60).

Niridazole is an antischistosomal drug that after oral administration to guinea pigs, gives rise to a metabolite which has the property of reversibly inhibiting the production of MIF. This effect is interesting because, in order to demonstrate its activity, the metabolite-containing serum must be added to the cells together with MIF (for ref. see 14). Despite these observations, the action of neither cytochalasin B nor the niridazole metabolite is believed to be at the phase of macrophage antigen-processing (14, 60).

Hydrocortisone (approximately $2 \times 10^{-5}\text{M}$) suppresses the production of PPD-induced MIF in serum-free, but possibly not in serum-containing, cultures, apparently by interfering with lymphocyte function (for ref. see 60). Histamine (acting via the H_2 receptor) in concentrations from 10^{-5}M suppresses the production of antigen-induced MIF. If the MIF-producing lymph node cells are filtered through a column material bearing histamine as an insoluble ligand, part of the cells (probably T-cells)

stick to the column. The cell population passing through the column now produces MIF even in the presence of histamine. Histamine acts by inducing the production (by suppressor cells) of a suppressor factor (HSF) with an estimated molecular weight of about 23–40000. Production of HSF is most efficient with cells from immunized animals but it is demonstrable also with normal cells. The action of HSF is concentration-dependent and reversible (see 76). Recently, it was also reported that preculture of lymphoid cells for 24 h before antigenic challenge, increased their capacity to produce MIF, presumably by elimination of suppressor cells (25).

Exogenous cyclic AMP (10^{-4}M), theophylline (10^{-4}M), and isoproterenol ($10^{-7}\text{--}10^{-5}\text{M}$) show, at most, a borderline effect on MIF-production by antigen-stimulated guinea pig lymphocytes, whereas the effect of cholera toxin is controversial (see 33, 60). Thus the opinion that the intracellular level of cyclic AMP governs MIF production is clearly not free from objections. On the other hand, there is unanimity that colchicine ($10^{-7}\text{--}10^{-4}\text{M}$) and vinblastine do not affect MIF-production, indicating that the microtubuli system is not involved in the process (see 60). Ouabain and chloroquine and the esterase inhibitor diisopropyl fluorophosphate (DFP) are also without effect, whereas immunoregulatory α -globulin (IRA) and chlorphenesin suppress and acetylcholine enhances MIF-production (for ref. see 60). The possibility that MIF-production is under feedback regulation through the action of macrophage-produced prostaglandins has been suggested by Morley (for ref. see 60).

c. Properties of MIF

(i) *Size.* True MIF-activity is linked to macromolecular component(s). The activity is not reduced after dialysis (e.g., 9, 16, 60). There are a number of reports describing the characteristics of MIF at gel filtration. The

yield of MIF after such a separation could be low (3, 72), possibly due to absorption of MIF to Sephadex gels, to degradation of it during the procedure by proteolytic enzymes, or to the presence of low molecular weight false "migration inhibitory activity" in the original supernatant (see 82), but apparently it can be increased if chromatography is done at room temperature (3). It is seldom pointed out that the estimated molecular weights attributed to MIF on the basis of data from columns calibrated with globular proteins are reliable only if MIF has globular dimensions and is not retarded on the column. None of these prerequisites have yet been shown to be valid. Moreover, practical reasons permit tests of only a limited number of fractions which decrease the resolution of the test.

A general opinion (see 9, 16, 60, 71) has long been that antigen-induced MIF is recovered in Sephadex column eluates at a position where albumin is found to one where chymotrypsin appears. This suggests a molecular weight for MIF between the limits 80,000 and 20,000. In some situations, two components with MIF-activity have been demonstrated. The elution pattern of MIF does not change in the presence of 0.1 M 2-mercaptoethanol. Postlethwaite & Kang (65) reported the hitherto most-detailed elution profile of antigen-induced MIF; the molecular weight was estimated at 53,000. These authors take advantage of the enhanced reactivity of DFP-treated indicator cells to increase the sensitivity of the test. MIF induced by ConA shows a more heterogeneous elution pattern than antigen-induced MIF with a substantial part of the activity appearing at the position of chymotrypsin (16, 71). Remold & Mednis (72) recently electrophoretically separated MIF into two components: at gel filtration, the separated components eluted in positions corresponding to molecular weights of 65,000 and 43,000–25,000. By centrifugation in a sucrose gradient, the molecular weight of MIF was determined at about 40,000 (16).

(ii) *Charge.* At DEAE cellulose chromatography, MIF elutes at a position corresponding to that of either α -globulins or albumin and electrophoretic separations have shown MIF to migrate either slightly faster or slightly slower than albumin, depending on conditions (16, 60, 71, 72). The two components of MIF_{ConA} separated by Remold & Mednis (72) were attributed isoelectric points of 3–4.5 and 5.0–5.5.

(iii) *Influence of enzyme treatment.* MIF-activity is sensitive to treatment with chymotrypsin and with trypsin (9, 16, 60, 65, 71), but it is resistant to DNase and RNase (16). The effect of neuraminidase treatment is controversial (3, 16, 71) but the glycoprotein nature of antigen- and also ConA-induced MIF is supported by its properties at centrifugation in a cesium chloride gradient (16, 71).

(iv) *Thermal sensitivity.* It is generally agreed that MIF activity resists treatment at 56°C for 30–60 min (16, 60, 65). However, Ashworth et al. (3) claim that at 60°C diluted MIF loses activity after 20 min, although concentrated MIF can resist 90°C for a similar length of time. MIF-activity can be stored at –20°C or even at 4°, and it resists lyophilization from volatile buffers (57).

(v) *Influence of chemicals.* MIF-activity is not precipitated by 50% saturated solutions of ammonium sulphate (56, 57). It is soluble in 1 M HClO₄ and is not affected by reversible treatment with DFP, 1 M 2-mercaptoethanol, 6 M urea, 6 M guanidinium-HCl, or 1% sodiumdodecylsulphate, but is destroyed at treatment with 0.02 M NaIO₄ (ref. 9, 16, and Kotkes and Pick in ref. 20).

(vi) *Affinity for macrophages and ConA.* MIF-activity can be adsorbed by treatment with macrophages for 1 h at 37°C in a process which obeys saturation kinetics (48, 60). No serum is needed for this reaction. It has also been reported that ConA adsorbs MIF; dissociation can be performed with 0.5 M

methyl- α -D-glucopuranoside (4, Kotkes and Pick in ref. 20).

d. *Antigen dependence of MIF*

The question whether MIF requires specific antigen in order to express full activity is controversial. David and co-workers (16) could find no effect on MIF activity by adding antigen, whereas Bennett & Bloom (see 9) showed that the presence of PPD enhanced the effect of PPD-induced MIF. Similar results were reported by a few other groups (see 9, 16, 57, 60). It has been suggested that two different MIF-activities exist; at low cell- and/or antigen concentrations, antigen-dependent MIF is preferably produced, whereas at higher cell- and antigen concentrations, both antigen-dependent and antigen-independent MIF activities are synthesized.

e. *Antisera to MIF*

The production of antisera with the property of blocking guinea pig MIF-activity has been described by a few groups (45, 60, 80, 95). Yoshida et al. immunize rabbits with control culture supernatants, and the resulting antiserum is covalently attached to agarose which is packed into a column. A MIF-rich supernatant obtained by antigen-stimulation (PPD, BGG, or OA) of sensitized cells is passed through the column. The eluate, containing MIF, is concentrated and used for the immunization of another rabbit. The resulting MIF-antibodies suppress the activity of MIF and some but not all other examined lymphokine activities, and it could reduce the degree of a delayed type skin test *in vivo*. Gerzy et al. have produced rabbit antibodies against ConA-induced MIF which has been purified by gel filtration and electrophoresis. This antibody adsorbs both ConA-induced MIF and antigen-induced MIF, but not skin reactive factor. Material eluted from a rabbit anti-guinea pig lymphokine immunoabsorbent at pH 3 was used to raise anti-lymphokine antibody in a goat. Given intradermally, this "second generation" antibody totally suppressed

delayed skin reactivity to PPD. The anti-lymphokine antibody reacted primarily with three T- and B-cell-derived lymphocyte activation products of molecular weight 60,000, 45,000, and 30,000 (all with an *pI* of 5.2) (see ref. 80).

2. *MIF produced by B-cells*

It was initially thought that only T-cells could produce MIF. However, Yoshida, Cohen et al. demonstrated MIF activity in supernatants from cultures of sensitized or non-sensitized lymph node or spleen B-cells stimulated by specific antigen (PPD) or by a B-cell activator (LPS). Other specific antigens could not stimulate purified B-cells to produce MIF; thus it was suggested that PPD and LPS act because of their properties as polyclonal B-cell activators and that the B-cells produce MIF only at such stimulation. Non-separated lymph node and spleen cells do not produce detectable amounts of MIF under these circumstances. This is probably due to the production by suppressor T-cells of a soluble, heat sensitive (56° , 30 min), non-dialysable inhibitor (MIFIF) which inhibits the production by B-cells of MIF. The MIF-activity produced by B-cells was not sensitive to treatment at 56°C for 30 min. It was eluted from a Sephadex G-100 column mainly at a position corresponding to a molecular weight of 45,000. Bloom and co-workers and Pick et al. confirmed that PPD induces B-cells to produce MIF; however, the question whether non-sensitized cells can produce MIF at PPD-stimulation is debatable (for ref. see 13, 18, 60).

C. *MIF produced by human lymphoid cells*

Rocklin and co-workers first described the production by antigen-stimulated human peripheral leukocytes in culture of a MIF-like activity. The streptokinase-streptodornase (SK-SD) induced MIF-activity eluted from Sephadex G-100 columns at the same position as did chymotrypsin. At polyacrylamide gel electrophoresis, the MIF-activity migrated as albumin; at centrifugation in cesium chloride gradients, it behaved like a

protein. Neuraminidase treatment did not impair its activity. Rocklin found no difference in the effect of human MIF when human monocytes, instead of guinea pig macrophages, are used as indicator cells (see 60, 74). The MIF-like activity was produced after antigenic stimulation of B-cells and T-cells; B-cells seemed even more efficient than T-cells as producers of the activity. In contrast to T-cell MIF production, B-cell production was not impaired by arresting proliferation. The B-cell and T-cell produced MIF-activity could not be differentiated. MIF-production by human lymphocytes induced by soluble antigen in general required the participation of macrophages, but cultures severely depleted of macrophages did not show decreased MIF production at stimulation by ConA (for ref. see 60, 83). At suboptimum concentrations of ConA, the drug levamisole dose dependently enhanced MIF-production. Levamisole did not induce MIF production in the absence of ConA (90). α -Fetoprotein had no effect on antigen-induced MIF production (49). Block et al. (8) recently reported extensive purification of ConA-induced MIF by Sephadex gel filtration (yield about 20%) and isotachopheresis. Total yield of MIF is not remarkably high; 80 l of blood results in 250 μ g final product. The increase in specific activity obtained by purification is estimated at 300–400 $\times$ at 30–40% migration inhibition. The purified product is heterogeneous at polyacrylamide gel electrophoresis; the molecular weight determined in SDS gels is 23,000 for the major component and its isoelectric point 3.5–4.0.

Production of MIF-activity is also induced by pulse treatment of cells with PHA (5–10 μ g/ml) for 2–4 h at 37°C followed by washing and culture (e.g., 64).

D. MIF produced by continuously growing or transformed cell lines

Granger and co-workers initially showed that seven out of eight continuously growing human lymphoid cell lines secreted into the culture medium products with migration in-

hibitory activity. These findings were further confirmed by Papageorgiou et al. and Tubergen et al. who found that not only lymphoid cell lines but also some non-lymphoid cell lines produced migration inhibitory activity. The MIF-activity produced by two lymphoid cell lines (SWD-46A and PGLC-33H), and in some respects also that produced by HeLa cells, were compared with PHA- and PPD-induced MIF produced by human peripheral leukocytes. No significant difference between these activities was observed with respect to gel filtration behaviour (apparent molecular weight 10,000–25,000), heat stability, and chymotrypsin sensitivity. Yoshida and co-workers examined eight continuously growing cell lines all of which produced MIF-activity independently if they were of B- or T-cell origin at culture of 5×10^6 cells/ml (but not of 10^6 cells/ml). B-cell lines seem to produce MIF activity faster than T-cell lines (peak times for production were 18 and 45 h, respectively; supernatants that were harvested at $\frac{1}{2}$ –2 hours after initiation of culture actually stimulated the migration of the indicator macrophages). The MIF-activity produced by these cell lines is *not* inactivated by anti-guinea pig MIF-antiserum which does react with a MIF-activity produced by monkey kidney cells injected with SV 40 virus (for ref. see 45, 60, 95). MIF is produced by continuously growing human and murine lymphoid and murine fibroblast cell lines during the G1 and S phases of the cell cycle (see 60); this was not found with MIF produced by 3T3 cells transformed by SV 40 (64). The MIF-activity produced by 3T3 cells transformed by SV 40 has been compared to PHA-induced MIF from human lymphocytes. Both activities have a similar apparent molecular weight of about 25,000 (Sephadex G-75) and both are inhibited by α -fucose but neither is sensitive to heat treatment (56°C for 30 min) or to dialysis (64).

E. MIF in body fluids

Foetal calf serum expresses MIF-like activity. Similar activity is not demonstrable in

normal guinea pig or horse serum. This MIF-activity did not resist dialysis, repeated freezing and thawing, heat treatment (56°C for 30 min), or exposure to pH lower than 6.0 or higher than 7.7. The activity that was blocked in the presence of 0.1 M L- α -fucose could be purified by affinity chromatography on an agarose column bearing fucosamine as a ligand. From 10 ml foetal calf serum, 10 μ g protein showing MIF-activity was obtained at elution with 1 M fucose. The apparent molecular weight of this MIF-activity, which easily aggregates at 4°, was 50,000 (for ref. see 24). Purification was also claimed of PPD-induced MIF produced by guinea pig lymph node cells by this procedure; others have failed to reproduce this result (17).

There are a number of reports describing the detection of MIF-like activity in body fluids from sensitized animals after appropriate challenge with antigen (for ref. see 60). Within 6 h after intrapleural challenge of sensitized guinea pigs with PPD, cell-free pleural cavity exudates and sera show migration inhibitory activity. This activity is antigen-dependent and can be separated into one high molecular component associated with immunoglobulin and one low molecular weight compound. 12–24 h after challenge, the exudate, but not serum, contained two components that show antigen-independent migration inhibitory activity, one of them seemed to be identical with classic MIF (92).

Peritoneal fluids from guinea pigs, which 3 weeks after sensitization to HRPO received an i.p. injection of antigen, showed migration inhibitory activity which was maximum 24–48 h after challenge. The properties of this MIF activity were virtually identical to HRPO-induced MIF from lymph node cells with respect to apparent molecular weight, heat stability, and sensitivity to treatment with trypsin (66).

Lachmann and co-workers have characterized the migration inhibitory activities found in efferent lymph 6–24 h after injections of PPD into sensitized pigs and sheep. This activity can be separated into

two forms. The first is heat-sensitive (56°C, 30 min) and is associated with complement; it constitutes the major part of the migration inhibitory activity of pig lymph. It is inhibited by heparin (1 U/ml) and by streptokinase. Two components are required to express this kind of non-specific migration inhibitory activity; one is fibrinogen; the other, a component precipitated by 40% saturated $(\text{NH}_4)_2\text{SO}_4$. Soybean trypsin inhibitor (SBTI) (100 μ g/ml) but not DFP, is inhibitory for this activity; the use of fresh serum in the assay enhances its effect (55). The second migration inhibitory activity in efferent lymph is antigen-dependent (PPD). It is particularly abundant in sheep lymph from where it has been purified. It is precipitated by $(\text{NH}_4)_2\text{SO}_4$ between 40 and 90% saturation, is not sensitive to heat treatment, shows an apparent molecular weight and electrophoretic mobility at gel filtration and polyacrylamide gel electrophoresis similar to that of albumin, and binds to ConA-Sepharose, indicating that it is a glycoprotein. It could also be purified by affinity chromatography on PPD-Sepharose (for ref. see 79).

F. Thymus MIF

Using rabbit peritoneal macrophages as indicator cells and a procedure where the migration of cells into a newly formed scratch in the cell layer is examined, Houck and co-workers characterized a migration inhibitory activity, derived from extracts of acetone defatted guinea pig or bovine thymic tissue. This MIF-activity is precipitated by 70% but not by 50% ethanol. The molecular weight of the purified material is approximately 35,000 (SDS gel electrophoresis, gel chromatography). It has no detectable proteolytic activity, but the molecule which contains sialic acid residues is inactivated by treatment with neuraminidase (37).

G. Migration inhibition due to antigen-antibody complexes and cytophilic antibody

There is no doubt that under certain

circumstances antigen-antibody complexes (formed in excess of antigen) can produce migration inhibition. Apparently, the complexes act directly on the macrophage and not by inducing lymphocytes to produce MIF, and they have to be present during the entire culture period to promote the effect (9, 16, 40). Cytophilic antibodies can also mediate antigen-induced migration inhibition (9, 16).

3. *Influence by MIF on the migration of guinea pig macrophages*

A. *The macrophage migration per se*

Before we discuss how the macrophage responds to MIF and which factors regulate this response, it is important to know the factors governing the macrophage migration *per se*. This topic was recently reviewed in detail by Pick (60).

1. Macrophage motility is dependent on the presence of serum; the migration area increases with increasing serum concentration up to 30–40% (38, 63). Both the factor "medium (within animal)" and the factor "animal (within medium)" are significant sources of variation when the effect of individual sera is examined on migration of cells from individual animals (see 6). Macrophages elicited by i.p. injection of mineral oil migrate faster than cells from non-injected animals (see 60).

2. The macrophage migration is probably dependent on an intact microfilament system; cytochalasin B in concentrations down to 1 $\mu\text{g/ml}$ blocks the movement and the microtubuli-disruptive drugs colchicine and vinblastine markedly enhance migration (60). According to Pick et al. (in ref. 20), D_2O , which stabilizes microtubuli, reduces migration.

3. Pick (60) maintains that cyclic nucleotides have a central role in the regulation of macrophage migration. However, the combined evidence for this is not wholly convincing. Although dibutyryl cyclic AMP

at 10^{-4} M reduces migration, isoproterenol, prostaglandins E_1 , E_2 , and F_{2a} , cholera toxin, cholinergic agents, and cyclic GMP were ineffective or showed only a borderline effect (60, 63). Theophylline at concentrations down to 10^{-4} M inhibits migration (63); this is apparently also the case with the β -adrenergic antagonist propranolol (39).

4. Inhibitors of protein synthesis or RNA synthesis, such as puromycin, cycloheximide, actinomycin D, chloramphenicol, and hydrocortisone, do not influence macrophage migration (for ref. see 16, 60, 63).

5. In Ca^{2+} - and Mg^{2+} -free medium migration of macrophages is enhanced (63). In media added either 10 mM EDTA or 10 mM MgCl_2 together with 10 mM EGTA (but not 10 mM EGTA alone), migration is completely blocked (32).

6. Fluoride, iodoacetic acid, and L-deoxyglycose (inhibitors of glycolysis) prevent macrophage migration, but low concentrations of cyanide and dinitrophenol do not (16, 60).

7. A number of plasma esterase inhibitors, including α_2 -macroglobulin and α_1 -antitrypsin, do not affect the migration of macrophages but pulse treatment of macrophages at 37°C for 1 h with 10^{-5} M DFP produced a low degree of inhibition ($\text{MI} : 78.8 \pm 2.0 \text{ SEM}$; $n = 34$) (for ref. see 73). Epsilon amino caproic acid at 10 mg/ml markedly enhances migration (30, 60).

8. Treatment of macrophages with proteolytic enzymes tends to increase their migration (16, 60, 63) but neuraminidase does not influence it (63).

9. Oxidative treatment of cells with NaIO_4 (10^{-5} – 10^{-3} M at 4°C for 10 min) leads to a strong reduction of migration capacity, which can be reversed by reduction with NaBH_4 (22).

10. According to Pick and Manheimer (63), treatment with heparin and dextran sulfate results in markedly enhanced migration. The

effect of heparin has recently been reevaluated (94).

11. ConA (0.16–10 $\mu\text{g/ml}$) inhibits the migration of normal purified macrophages (44, 82), whereas low concentrations of fucose-binding lectins and the phagocytes-stimulating tetrapeptide tuftsin (Thr-Lys-Pro-Arg), at 1 mg/ml enhance migration (Newman et al. in ref. 20).

12. *N*-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP) the minimum effective component for adjuvant activity of cell wall peptidoglycan, even in low concentrations (0.1 $\mu\text{g/ml}$), directly inhibits macrophage migration (93).

13. A neutrophil-derived factor and a 32,000 molecular weight, heat sensitive (56°C 30 min) sea star protein have been shown to possess migration inhibitory activity (for ref. see 9).

B. Inhibition and enhancement of the effect of MIF

It is sufficient to expose macrophages to MIF for only 30 min to 2 h to obtain migration inhibition (60). As mentioned above, the macrophages respond most effectively when harvested 2–3 days after oil injection, but some sera promote efficient migration inhibition at MIF exposure even with "older" macrophages or alveolar macrophages (48). Incubation at 4° of macrophages together with MIF, followed by washing of the cells and preculture for 2 h, also results in inhibition of migration at a subsequent test, indicating that macrophages express receptors for MIF. Activated peritoneal but not alveolar macrophages or trypsin-treated peritoneal macrophages can adsorb MIF; in this respect, even non-viable cells are effective. However, subsequent elution of MIF activity has not been accomplished (48, 60).

1. Inhibition

The postulated macrophage MIF receptor is thought to incorporate L- α -fucose as an

important component, because Remold found that 0.1 M fucose specifically blocks the activity of MIF (see 17, 60). L-fucose also blocked the effect of foetal calf serum MIF (see 24), MIF produced by transformed cell lines, and PHA-induced MIF from human lymphoid cells (64). Poste (64) confirmed that the effect of fucose was specific; none of several of other examined monosaccharides were inhibitory. Recently, Amsden et al. (2) re-examined the issue. They found that the MIF-blocking effect of fucose is dependent on the strength of the MIF preparation. Reproducible inhibition of a high activity MIF preparation obtained by stimulation of lymph node cells with PPD could only be achieved at a limited dose interval of fucose. It is confusing that, when the MIF activity in the test is increased, the fucose concentration producing 50% inhibition of the MIF effect decreases. In this work, where the authors apparently used nondialysed MIF-preparations, L-rhamnose was found to mimic the effect of fucose. Remold reported that treatment of macrophages with L- α -fucosidase blocks the response of the cells to MIF. MIF-activity as such is not affected by the enzyme. Furthermore, in the presence of 0.1 M fucose, macrophages cannot be sensitized to migration inhibition by pulse treatment with MIF for 1 h at 37° (17). As mentioned above, the claim by Fox & MacSween (24) that MIF binds to fucose-Sepharose was not confirmed by David & Remold (17). Fucose-binding lectins do not block the action of MIF (Newman et al. in ref. 20). Rocklin showed that ConA-induced MIF produced by human lymphocytes (assayed with human monocytes) was blocked by 0.1 M fucose, but in this situation, other 5-methylpentoses (e.g., rhamnose and 6-deoxyglucose) were also effective (75). Thus a few paradoxical findings, not yet convincingly explained, contradict the opinion that fucose is a constituent of the macrophage receptor for MIF. Treatment of macrophages with trypsin suppresses their response to MIF (see 48, 60). The effect of colchicine and vinblastine (which strongly

enhance macrophage migration *per se*) on MIF-induced migration inhibition seems to be controversial. Henney et al. (33) found no effect of these agents examining PPD-induced MIF, whereas Pick and Abrahamer (for ref. see 60) in a closely similar system observed that both agents exerted a dose-dependent blockade of the effect of MIF.

In the presence of theophylline (10^{-4} – 10^{-3} M), prostaglandins E_1 or E_2 (0.05–5 μ g/ml), dibutyryl cyclic AMP (10^{-3} M), isoproterenol (3×10^{-4} – 10^{-3} M), cholera toxin, or hydrocortisone (approximately 2×10^{-5} M), or in media free from Ca^{2+} and Mg^{2+} (for ref. see 33, 39, 60, 63), the macrophage response to MIF is reduced or abolished. The effects obtained with the putative adenylate cyclase stimulators and phosphodiesterase inhibitor are, even at high concentrations, sometimes contradictory; the evidence that the cyclic AMP/cyclic GMP level of the macrophage controls its response to MIF is not compelling (see also 11.3.B.3 below). In the presence of puromycin (1–2 μ g/ml) and cycloheximide (0.1 μ g/ml), MIF-activity is partially blocked (63). The tetrapeptide tuftsin at 1 mg/ml abolishes the MIF-activity (Newman et al. in ref. 20). The activity of human ConA- and PPD-induced MIF is blocked by DFP and pancreas trypsin inhibitor, whereas SBTI is only partially inhibiting (for ref. see 60). Furthermore, human MIF is inactivated by a serum component (84). On the other hand, DFP does not affect the activity of guinea pig MIF but EACA (10 mg/ml) reduces its activity (30, 60). Incubation of supernatants from virus transformed cells with DFP (5×10^{-4} M) and SBTI (500 μ g/ml) partially blocked the migration inhibitory activity, whereas TPCK (10^{-6} M) and TLCK (5×10^{-5} M) had no effect (64).

2. Enhancement

Remold and co-workers (for ref. see 17) have shown that inhibition of esterase activity associated with the macrophage membrane enhances the response of the cells to ConA-induced MIF. Thus when macrophages are

pretreated with a suitable esterase/protease inhibitor at 37°C for 1 h and then washed, the migration inhibitory effect of a weak MIF-activity is more clearly observed. The responsible esterase activity has an enzymatic specificity different from those of trypsin and thrombin.

Recently, Higgins et al. (34) reported that fucose containing guinea pig macrophage glycolipids specifically enhanced the effect of MIF. The possible role of these glycolipids as receptors for MIF was discussed.

Leu and co-workers (48) have shown that some selected serum batches have the property of enhancing the macrophage response to MIF; moreover they showed that preincubation of macrophages, in such high response heat-inactivated serum before culture, also increased the macrophage response to MIF.

Chemical modification of the indicator macrophages in various ways by treatment with the diazonium salt of sulfanilic acid, with acetic anhydride, with $NaIO_4$, or with hydroxylamine enhances their response to ConA-induced MIF (70). The treatments did not affect the migration of the macrophages as such.

3. No effect

The following compounds or events have been shown not to influence the macrophage response to MIF: (i) Sialic acid or treatment with neuraminidase (16, 33), (ii) Actinomycin D (0.1 μ g/ml; 63), (iii) Heparin (63, 94), (iv) Histamine (see ref. 76), (v) Exogenously added cyclic AMP or cyclic GMP or an intracellular increase in the level of cyclic AMP; thus the prevailing opinion today is that cyclic AMP is not a second messenger for MIF (60, 61).

C. Migration enhancing factors

Despite a prevailing opinion that migration-enhancing factors are conceivably released from antigen- or mitogen-stimulated lymphocytes, there are few reports that characterize such activity in an indirect

system. Fox et al. (23) showed that, after DEAE-cellulose fractionation of fetal calf serum, one of the separated protein fractions had migration stimulating properties. No such activity was observed after separation of normal guinea pig serum, but could be demonstrated in supernatants from cultures of antigen-stimulated lymphocytes. It was found that *N*-acetyl-D-galactosamine, but not fucose, blocked the effect of this protein fraction.

Continuously growing B- and T-cell lines of human origin, cultured for only $\frac{1}{2}$ –2 hours in the absence of serum, give rise to supernatants showing macrophage migration stimulating activity (95); supernatants harvested 24–48 h later show migration inhibitory activity.

D. The use of alveolar macrophage as indicator cells

Antigen-induced guinea pig MIF produced by lymph node cells does not inhibit the migration of guinea pig alveolar macrophages unless a specified batch of MIF-promoting serum is used at cultures (48). However, apparently the migration of alveolar macrophages is inhibited by cytophilic antibodies (see 16). Bartfeld and Atoyntan early used the alveolar macrophages to show that human MIF produced by peripheral lymphocytes at stimulation with PPD can be adsorbed by normal, but not by trypsinized, alveolar macrophages suggesting that this MIF showed cytophilic properties. Moreover, these authors showed that tryptic treatment of alveolar macrophages blocks the capacity of the cells to respond to MIF (for ref. see 60).

4. General effects of MIF-containing supernatants on macrophages in vitro

Macrophages incubated and cultured in MIF-rich supernatants undergo a number of morphologic, metabolic and functional changes. There are many reports that describe in detail these changes (for reviews see 9, 15–17, 60). On the other hand, the

factor responsible for macrophage aggregation has been separated physically from MIF by Sephadex gel filtration (65). At treatment with MIF-rich supernatants, the aggregation and the adherence of the macrophages increase, the phagocytic capacity increases, and after the initial migration inhibition, the capacity to migrate increases. The electrophoretic mobility of the macrophages also changes. These effects can usually be registered 48–72 h after treatment with MIF-rich supernatants. At the biochemical level, among others, the following effects of treating macrophages with MIF have been observed: Increased protein synthesis (doubling after 72 h), increased glucose-carbon-1-oxidation (4–6 times at 72 h), and early changes in capacity to utilize hydrogen liberated from the hexose monophosphate shunt, early changes of cellular permeability, loss of glycoprotein from the surface of the cell, and an enhanced uptake of glucosamine (for ref. see 15, 16, 60). According to a recent workshop report (Pick et al. in 20), MIF shifts the tubulin dimer polymer equilibrium of macrophages towards the polymerized state in a similar way to that of D_2O . MIF does not induce a change in the macrophage cyclic AMP content (35, 61), but still induces in the cells a state of reduced responsiveness to other agents that stimulate the adenylate cyclase enzyme (62).

5. Effects of MIF-containing supernatants in vivo

MIF-containing supernatants have been shown to produce a number of biological effects when administered to living animals (review 60). Thus intradermally injected MIF-containing supernatants produce local inflammatory reactions; intraperitoneally administered, they cause a macrophage disappearance reaction; i.v. injected, they cause a reduction in the number of monocytes in peripheral blood and suppression in the degree of a delayed type skin reaction to another antigen. Furthermore, biological effects exerted by antisera against MIF *in vivo* have been described. Locally administered

goat anti-lymphokine serum suppressed delayed-type skin reactivity to PPD; intravenously injected antibody was less effective (see 45, 80).

III MIF-ACTIVITY ON INDICATOR CELLS FROM MICE

In recent years, a number of reports have appeared that describe and characterize the macrophage migration inhibition phenomenon in some detail also in mice. MIF-activity is usually assayed on peritoneal exudate cells either by the capillary tube technique (43) or by the agarose droplet assay (31, 46). Attempts to inhibit migration of mouse peritoneal cells with guinea pig MIF were unsuccessful (9) but Lohmann-Matthews et al. (50) showed that bone marrow cells cultured 7–9 days *in vitro* can be used as indicator cells to assay ConA-induced MIF-activity both of mice and human origin. This technique has not yet been extensively examined, but it seems to have several advantages; the cells are not pre-activated but yet respond well to MIF; inhibition of their migration by MIF is highly reproducible, and the cell yield is high. The cells can be stored frozen until used. McDaniels et al. (54) recently introduced a short-time technique employing ³H-labelled lymphoid cells as indicator cells.

Kühner & David (43) examined ConA-induced MIF produced by spleen cells. At gel filtration, the activity showed a comparably broad elution profile (apparent molecular weight 67,000–48,000). It was sensitive to treatment with chymotrypsin, but resistant to heat treatment (56°, 30 min). At centrifugation in a cesium chloride gradient, the activity showed characteristics of a glycoprotein, but it was not inactivated by treatment with neuraminidase. This MIF-activity is not stable at storage for 2–3 months at –20°, but is enhanced after storage at 4° for 7 days in the presence of heat inactivated guinea pig serum. This MIF-activity has not yet been separated from the nonspecific *soluble immune*

response suppressor factor (SIRS) which can also be demonstrated in active supernatants.

Non-H-2 dependent strain variation exists with respect to capacity to produce MIF at stimulation of spleen cells with ConA or with PPD (1). Cells from the C57BL/6 strain did not produce MIF at serum-free cultures; but when 2% FCS was added to the cultures at termination of the incubation, MIF-activity could be found in stimulated, but not in control, supernatants. Thus MIF-activity was generated by interaction with FCS.

MIF-activity is also produced by spleen cells from animals rejecting a tumour allograft. This activity is resistant to heat treatment; it is inactivated by trypsin, exhibits an apparent molecular weight of about 45,000, and an isoelectric point 5.0–5.5, but it does not inhibit the migration of guinea pig macrophages (31).

Recently, immune spleen cells were shown to produce MIF activity to tumour-associated antigens by two different mechanisms. The response against soluble antigens required viable and metabolically active macrophages for antigen processing and contact between T-lymphocytes and macrophages sharing genes of the I-A portion of the H-2 complex for antigen presentation. The response against intact tumour cells did not require macrophages, and the interaction between immune lymphocytes and tumour cells was not under genetic control of genes mapping in the H-2 complex (46). Supernatants from spleen cells activated in mixed leukocyte culture also exhibit MIF-activity (59). This MIF-activity is eluted from a BioGel P 100 column at a position corresponding to a molecular weight of 25,000–60,000. The production of the activity requires T-cells either of the Ly 1 or the Ly 23 phenotype. Simultaneously with the MIF-activity, the cells produce a principle, IPA, which is able to *induce* activated macrophages to *plasminogen activator* production. The IPA- and MIF-activities have not yet been separated from each other. An antigen-dependent MIF-activity, which is not identical with classic MIF or due to antibody

components, is produced by spleen and lymph node cells (42). Its production requires T-cells, its molecular weight is lower than 100,000, its isoelectric point is 6.4–6.5, and it shows affinity for Sepharose bound ConA.

IV FACTORS EXERTING INHIBITION/ STIMULATION OF THE MIGRATION OF HUMAN POLYMORPHONUCLEAR (PMN)/MONONUCLEAR CELLS *IN VITRO*

Much work has been devoted to adapt the cell migration inhibition phenomenon to a human system with the aim of obtaining an *in vitro* correlate to cell-mediated immunity. As a result of this work, antigen- and mitogen-induced lymphocyte produced soluble principles mediating the cell migration inhibition of polymorphs/mononuclear cells have been characterized.

1. *Methods of assay*

A. *Migration from capillary tubes*

Søborg and Bendixen introduced a capillary tube technique in which buffy coat cells were allowed to migrate out of the tubes; if cell donors were skin test positive to *Brucella* antigen, the migration of their cells out of the tubes was inhibited in the presence of particular *Brucella* antigen. This method has been examined by many authors; some have experienced difficulties. Standardized procedures have been described by, among others, Federlin et al., Maine et al., and Bendixen et al. (see 4). The following are some of the factors found important in the test:

(i) *The serum used for culture.* Different batches vary with respect to both capacity to mount migration as such and capacity to promote migration inhibition. The way that heat inactivation (56°C for 30 min) of serum influences the outcome of the test is not fully clarified. The presence of autologous serum is claimed to enhance migration inhibition in the direct test.

(ii) *The time for culturing the cells.* Escape from migration inhibition has been observed at prolonged culture time.

(iii) *Inhibition of clotting.* Defibrinated blood, but not heparinized blood, is recommended for use because heparin probably forms a complex with thrombin and the heparin-antithrombin cofactor (AT III), the esterase-inhibiting property of the latter probably being essential in order to express the inhibitory activity (53). Furthermore, it has been claimed that the use of dextran to aid red cell sedimentation seems to interfere with the test.

(iv) *The way in which the test is read.* In the direct test, Rosenberg and David found the best correlation to skin test reactivity when the inner dense zone of cell migration, and not the outer halo of cells, was used for measurement, suggesting that only mononuclear cells, and not polymorphs, are inhibited from migrating. Ng and Alexander also showed that PPD inhibits the migration of mononuclear cells much more consistently than that of buffy coat cells. Kowalczyk and Zembala go one step further; they find that, within the population of purified mononuclear cells, it is the T-cells, and not the B-cells or the monocytes, that are inhibited by PPD. Supernatants of mononuclear cells stimulated by PPD also inhibited the migration of T-cells, but not that of monocytes (for ref. see 41). Other authors evaluate the test with respect to migration of polymorphs (see 4). This difference between various evaluation methods is probably of fundamental importance because the migration inhibitory principles acting on monocytes, on T-lymphocytes in mononuclear cell preparations, and on polymorphs are probably not identical. Thus, mononuclear cell migration inhibitory activity is not identical with classic MIF, which according to Rocklin (74) inhibits migration of both purified monocytes and guinea pig macrophages. Moreover, human ConA-induced monocyte migration inhibitory activity is not inhibited by DFP or SBTI, whereas human

ConA-induced polymorph migration inhibitory activity is blocked by DFP (5, 77). It has been convincingly shown that lymphoid cells play an essential role in antigen-induced polymorph migration inhibition; in the absence of lymphoid cells, no migration inhibition is seen.

B. Migration under agarose gels

Based on the method described by Carpenter for use with guinea pig lymphoid cells, Clausen developed the "leukocyte migration agarose test" for use with human cells (see 4). Peripheral blood leukocytes (PBL $2.2 \times 10^6/\text{ml}$) are incubated at 37°C for 30–60 min with or without the appropriate antigen, and the cells are placed in small holes made in an agarose gel in plastic Petri dishes. Polymorphs migrate out circularly from each hole between the gel and the plastic surface. The migration is inhibited in the presence of specific antigen. In the indirect method, the effect of cell-free supernatants from antigen-(or mitogen)-stimulated lymphocyte cultures are examined on the migration of non-

antigen-sensitive polymorphs or after removal of mitogen. Clausen has shown that pulsing cells with active supernatants are sufficient to produce migration inhibition in the indirect test. The migration agarose test has received considerable attention and there are a vast number of reports where minor modifications are given and technically important factors are discussed (for ref. see 7, 36, 87, 88, 91). It is not yet clear how the leukocyte migration agarose test reflects a state of delayed type hypersensitivity of the cell donor (for ref. see 4, 7, 88). Clausen has reported that there is no quantitative correlation between skin reactivity and degree of migration inhibition *in vivo*; on the other hand, incubation for 1 h at 37°C of cells from PPD-negative individuals with plasma from PPD-positive individuals does not render the cells reactive.

As in all kinds of migration inhibition tests, a proper statistical evaluation of the data is necessary also in the migration under agarose technique (see 7, 88). A major source of variation in the test seems to be the incubation procedure with the antigen (7).

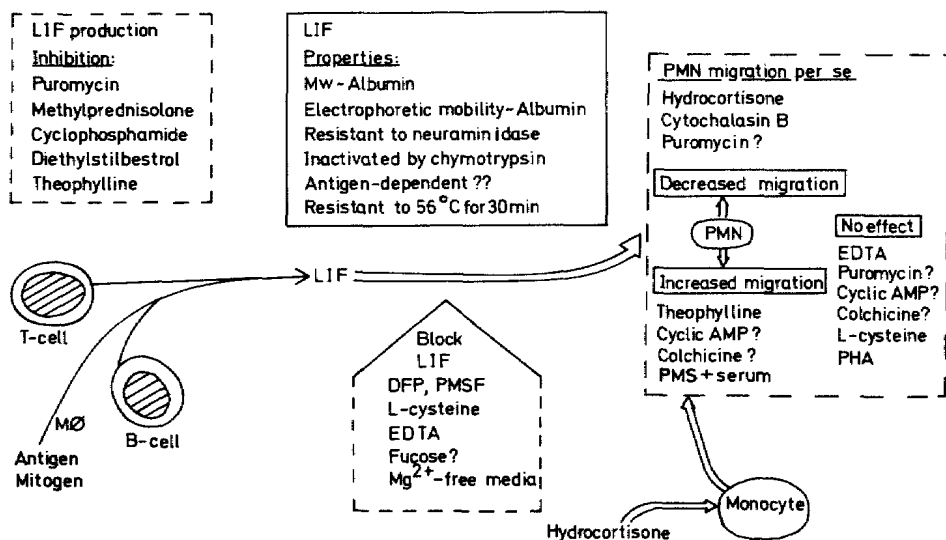


Fig. 4. A schematic representation of the properties of LIF and of various factors that influence its production and biological effect.

Recently, the agarose droplet assay has also been adopted for use in human systems by McCoy et al. and by Laverne and Harrington (see 47), and the leukocyte migration fibrinolysis technique (see 12) has been developed and found in most respects to be comparable to the capillary tube and agarose techniques.

The migration in agarose gel technique has been carried back to the guinea pig system, where it has been found to correlate to delayed type skin reactivity and to macrophage migration inhibition although the soluble inhibitory factors are not identical (see 36).

2. Leukocyte migration inhibition factor (LIF)

A. Factors influencing the polymorph migration per se

The migration of PMNs, as such, evaluated either by the capillary tube or by the migration under agarose technique, has been shown to be influenced by the following agents:

Enhancement was observed in the presence of theophylline (10^{-3} M), of $\text{PGE}_{1,2}$ (5 $\mu\text{g}/\text{ml}$) (see 51), and of the polymorph migration stimulator (PMS) described by Stevenson (review 81). Stevenson showed that hydrocortisone stimulates human monocytes to produce a factor, PMS, which enhances the migration of polymorphs in the capillary tube technique. Hydrocortisone, as such, is slightly inhibitory for polymorph migration. This situation seems to have a counterpart *in vivo*, as unseparated leukocytes from patients treated with steroids have an enhanced capacity for migration *in vitro*, whereas purified polymorphs from such populations migrate less well than corresponding cells from untreated individuals. PMS is generated by monocytes only by steroids with glucocorticoid activity and only under conditions permitting protein synthesis. Serum is not required for the production of PMS, but for the expression of its activity. PMS is stable at 56°C for 1 h, and it withstands lyophilisation

and storage at 4°C for 1 week. The apparent molecular weight (Sephadex G-100) is given at 15,000–12,000. The PMS-activity is sensitive for treatment with proteases, but not for treatment with RNase.

Bendtsen has analogously observed increased migration of polymorphs in the presence of methylprednisolone.

Inhibition of polymorph migration has been observed in the presence of cytochalasin B down to 0.01 $\mu\text{g}/\text{ml}$; this effect was blocked by cyclic AMP and by theophylline (10^{-3} M) (51).

No or only a slight effect has been observed in the presence of L-cysteine, EDTA, cyclic AMP, colchicine, vinblastine.

The action of puromycin (it is either inhibitory or without effect) is controversial.

B. Production and properties of LIF. Effect of LIF on the migration of PMNs. Enhancement and inhibition of the effect of LIF

Weisbart and co-workers in a series of papers examined the properties of PPD- and coccidioidin-induced LIF-activity. 10^6 PBL per ml are cultured for up to 7 days in medium supplemented with 10% FCS (at shorter culture times, LIF activity is sometimes counteracted by a simultaneously produced migration enhancing principle). The LIF activity is antigen dependent; supernatants that have been diluted 50-fold and have lost their activity regain it at the addition of antigen. In their work, Weisbart et al. test LIF-activity on cells from donors which are not sensitive to the appropriate antigen. The PPD-effect in these experiments is not due to mitogenicity because cells from PPD-negative individuals do not produce LIF. The LIF-activity migrates together with albumin at polyacrylamide gel electrophoresis. It is not abolished by treatment at 56° for 30 min (see 86–89).

Hoffman et al. use Streptokinase-Streptodornase (SKSD) and coccidioidin as antigens. They find that, in this system,

cytophilic antibodies or antigen-antibody complexes do not inhibit the polymorph migration. The production of LIF requires more than 25% lymphocytes in the culture. The obtained LIF-activity is eluted in the albumin region at Sephadex G-100 gel filtration. This LIF-activity does not inhibit the migration of human mononuclear cells, but it does inhibit guinea pig polymorph migration (for ref. see 36).

Chess and co-workers showed that both T-cells and B-cells (contaminated with less than 1% T-cells) produce LIF (capillary tube technique) at antigenic stimulation; both activities are eluted from Sephadex G-100 at the position of albumin (for ref. see 89). However, Fimmel found no LIF-production connected to PPD stimulation of B-cell enriched cells (19).

Hoffman et al. (for ref. see 36) have reported on the production of guinea pig LIF. 24×10^6 PBL/ml were cultured in serum-free media for 48 h with 500 $\mu\text{g/ml}$ OA. The cell-free supernatant was dialysed against H_2O and lyophilised. This LIF-activity withstands treatment at 56° for 30 min and treatment with neuraminidase but not with chymotrypsin. The apparent molecular weight is 70–150,000, the activity is not of antibody nature. Lymph node cells seem to be better producers of LIF than are spleen cells or blood leukocytes. This LIF-activity is not identical to MIF. Thus it is conclusively shown within one and the same species that the migration inhibition activities produced by lymph node cells affecting (i) polymorph migration under agarose and (ii) macrophage migration out of capillary tubes are different.

2. Mitogen-induced LIF

According to Arvilommi and Räsänen, mononuclear blood cells (probably B-cells) apparently without any stimulation (including trivial ones such as treatment of blood with heparin or Ficoll) can produce LIF at culture for 3 days in 10% heat-inactivated horse serum supplemented with conventional anti-

biotics. This LIF production is sensitive to puromycin (for ref. see 69). The same authors also showed that F_c - or C3-receptor stimulation brought about with rosetting techniques did not lead to LIF-production. The degree of LIF-production induced by LPS varies with the preparation of mitogen and condition of culture (67). Leucoagglutinin and ConA were found to induce LIF-synthesis in T-cells only, whereas Poke weed mitogen (PWM) stimulated both T- and B-cells and protein A and β_2 -microglobulin B-cells only to produce this mediator. T- and B-cell LIF showed similar characteristics. Both were eluted in the albumin region at Sephadex gel filtration; with both, slight loss of activity was observed after 30 min at 56° , and the activity of both was blocked by *N*-acetyl-glucosamine but not by fucose (69). On the other hand, Weisbart et al. (86) found that the LIF response to ConA and protein A stimulation was produced in collaboration between T-cells and either B-enriched cells or monocytes. Neither cell population was found capable of producing MIF alone. Recently, it was also shown (68) that a number of bacteria induced LIF-production polyclonally in B- but not in T-cells.

LIF produced by culturing human blood lymphocytes with ConA have been partially characterized by Horvat, Havemann and co-workers and by Rocklin and Rosenberg (for ref. see 77). The activity is irreversibly blocked by treatment with DFP for 30 min; human MIF acting on human monocytes is not affected by DFP. The LIF-activity exhibits esterolytic activity. Pretreatment with high concentrations of DFP or SBTI blocks PMNs capacity to respond to LIF, but pretreatment with low concentrations of DFP renders the PMNs more susceptible to the action of MIF than are untreated cells.

In a series of reports (for ref. see 5), Bendtzen has performed an extensive characterization of ConA-induced LIF (assayed with the leukocyte migration agarose technique). 2.5×10^6 Ficoll-Hypaque purified lymphocytes per ml are cultured in

serum-free medium for 22 h with 50–80 $\mu\text{g/ml}$ ConA for optimum LIF production. ConA was added to control culture supernatants after cell removal and the mitogen and salts were removed from both control and stimulated culture supernatants by Sephadex gel filtration in volatile buffer. LIF production in this system is blocked in the presence of methyl prednisolone (0.025–2.5 $\mu\text{g/ml}$), puromycin (0.1–10 $\mu\text{g/ml}$), diethylstilbestrol (0.01 $\mu\text{g/ml}$), and theophylline 10^{-4} – 10^{-5} M). LIF-activity is partially but not completely blocked by the serine esterase and protease inhibitors DFP and PMSF (10^{-3} M) at pulse treatment for 30 min at 37° followed by dialysis. The histidine specific protease inhibitors TLCK (inhibitor of trypsin and plasmin) and TPCK (inhibitor of chymotrypsin) did not block LIF-activity, whereas eserine (an inhibitor of acetylcholine esterase) showed a varying degree of inhibition (from 11 to 83% inhibition of LIF-activity depending on preparation). A preparation of LIF blocked by treatment with eserine can be reactivated by the oxim 2-PAM. Some arginine amides, and some phosphodiesterases showed substrate properties for the esterase activity of LIF (i.e. these agents counteracted the LIF-activity of the preparation or its inhibition by PMSF). The LIF-activity was irreversibly destroyed by treatment with L-cysteine (10 mM) or 2-mercaptoethanol or in the continuous presence at test of 1 mM EDTA or puromycin (1–10 $\mu\text{g/ml}$).

The LIF-activity of Bendzen withstands treatment for 1 h at 56°C, and can be gel-filtered on Sephadex in volatile buffers suitable for lyophilisation, but apparently the activity is not quite stable at –20°C. The apparent molecular weight is similar to that of albumin. The activity of nonconcentrated LIF-rich supernatants is reversibly inhibited by 0.1 M fucose but not by other examined sugars. Concentrated and purified LIF-activity is only weakly inhibited by fucose even at 0.33 M.

Stimulation of PMNs with LIF-rich supernatants does not affect the intracellular con-

centration of cyclic AMP, but LIF-treated PMNs partially escape migration inhibition in the presence of dibutyryl cyclic AMP (10^{-5} M or higher concentration).

Bendzen purified this LIF-activity about 70 times with a yield of about 60–80% in one step by affinity chromatography with L- α -fucose-Sepharose or about 40 times with almost complete yield by adsorption of contaminating proteins to an immobilized rabbit antiserum to human serum proteins. He has also produced a rabbit antiserum to his purified LIF-preparation (containing 0.9 μg protein per ml of original supernatant); this antiserum was shown to block both ConA- and PPD-induced LIF-activity.

The LIF-activity induced by ConA was shown by Rocklin (capillary tube technique) not to be inhibited by fucose but by *N*-acetylglucosamine. Treatment of PMNs with neuraminidase increases their response to LIF (75). The activity is inhibited by benzoyl arginine ethyl ester (a substrate for trypsin) (BAEE); BAEE protects LIF-activity from inactivation by DFP, and purified LIF-rich preparations hydrolyse BAEE (78). ConA-induced leukocyte inhibitory factor (LIF) activity is reduced when ambient pH falls below 7.2 (91).

PHA-induced LIF-activity has been characterized by Lomnitzer and co-workers (51). These authors culture 2×10^6 blood mononuclear cells or 4×10^6 tonsillar lymphocytes per ml in medium supplemented with 10% FCS for 72 h. PHA-stimulation (10–20 $\mu\text{g/ml}$) is performed by pulse treatment followed by washing, or PHA is allowed to reside in cultures; PHA does not inhibit the migration of purified polymorphs used by the authors in the capillary tube technique to assay LIF. In this system, LIF-activity is partially sensitive to treatment at 56°C for 30 min. It is connected to a component with a molecular weight less than 50,000 (membrane filtration). LIF-production is suppressed by cyclic AMP, theophylline, and $\text{PGE}_{1,2}$ at high concentrations. The LIF-activity is blocked in the absence of Mg^{2+} , but is not affected by

colchicine. LIF-activity induced by PHA at culture conditions similar to those of Lomnitzer et al. has also been described by Räsänen & Arvilommi (67).

Cell-free supernatants obtained from human mixed leukocyte cultures also express LIF-activity (see 4). Maximum activity is seen 3–4 days after initiation of cultures; this LIF-activity is produced also in cultures treated with mitomycin C. If both cell populations are treated with puromycin, there is no LIF-production, but Górski et al. (28) showed that a one-way test for LIF-production is possible in cultures where only one of the cell populations has been pretreated with puromycin.

C. Antigen-induced and mitogen-induced enhancement of the migration of human leukocytes

There are several reports where an unequivocal polymorph migration stimulating effect at the addition of antigen has been observed in the direct capillary tube or agarose techniques (e.g., 7). The basis for this effect is obscure, but the nature of the antigen could be a contributing factor. Weisbach et al. (86) have reported on the

partial purification by polyacrylamide gel electrophoresis of a leukocyte migration enhancing factor (coined MEF) obtained from antigen-stimulated peripheral blood cells. 10^6 cells/ml were cultured in medium containing 10% horse serum and approximately 5 $\mu\text{g/ml}$ PPD or coccidioidin. The temporal pattern of emergence of the stimulating activity varied between individuals. The culture supernatants were not dialysed, but were diluted in fresh medium before test. The MEF activity causes a rather weak degree of stimulation (MI in the region of 1.10–1.20). The activity migrates similar to a β -globulin in polyacrylamide gel electrophoresis and withstands treatment for 30 min at 56°C.

V. PRODUCTION BY ACTIVATED MACROPHAGES/MONONUCLEAR CELLS/ POLYMORPHS OF PROTEOLYTIC AND ESTEROLYTIC ENZYMES

Fibroblasts, transformed by oncogenic virus or chemical carcinogens, produce and secrete activator(s) of the circulating proenzyme plasminogen (PA). Thioglycollate-elicited peritoneal macrophages from mice have the same capacity; when appropriately harvested,

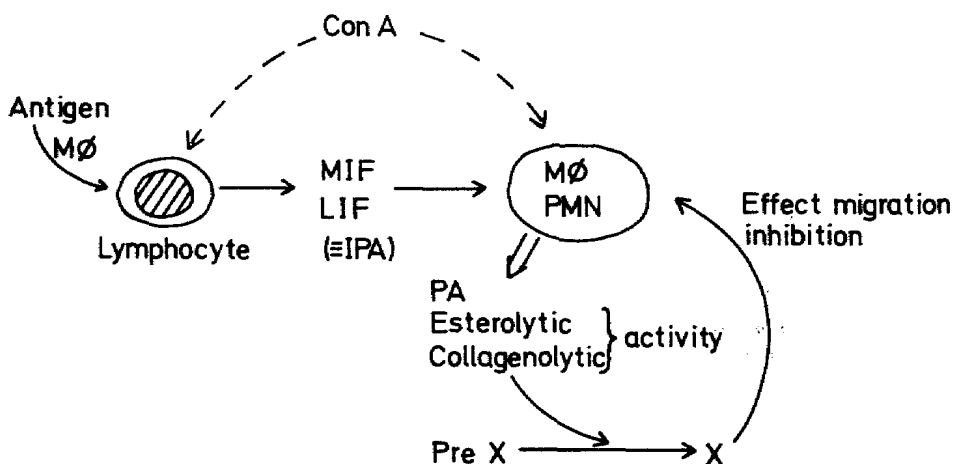


Fig. 5. A hypothetical representation of the involvement of macrophage/polymorph produced proteolytic/esterolytic activity in the migration inhibition phenomenon.

they produce about 100 times more PA activity than normal peritoneal cells. This PA activity is produced by young cells which have recently arrived at the peritoneal cavity. Lysozyme is not produced or secreted to any greater extent by the elicited cells. The PA activity is blocked by dithiothreitol and by DFP (2×10^{-4}) but not by SDS. The apparent molecular weight is reported to be about 48,000 for a major component; a smaller component of about 28,000 molecular weight has also been observed. The PA production/secretion can be regulated pharmacologically. Glucocorticoids, cholera toxin, colchicine and vinblastine at very low concentrations inhibit the PA secretion without affecting other functions of the macrophage. Isoproterenol and PGE show some inhibitory capacity, whereas theophylline, cytochalasin B histamine, indomethacin, and PGF_2 do not affect PA secretion. Endotoxin- and mineral oil-elicited peritoneal macrophages do not produce/secrete PA until the cells are further activated by stimulation with latex, aggregated α -globulin, or immune complexes. The degree of PA secretion is determined by the degree of triggering; non-digestible latex particles promote PA secretion for a longer time than digestible triggers. Macrophages obtained from BCG immunized animals, challenged with PPD i.p., produce 5–10 times more PA than cells from similarly sensitized animals challenged with an irrelevant antigen. The PPD-induced stimulation of PA production by macrophages is T-cell dependent; BCG-sensitized peritoneal cells after challenge with PPD release a soluble inducer of macrophage PA. Also supernatants from antigen-stimulated mouse lymph node and spleen cells, but not from control cultures, induce PA secretion from proteose-peptone induced macrophages. Macrophages can also be induced to synthesize and secrete PA by treatment with ConA and lymphokine-rich supernatants from ConA-stimulated spleen cell cultures. These agents induce normal macrophages to initiate PA production and also enhance

ongoing PA production/secretion in endotoxin-activated cells (for ref. see 27).

As mentioned above, supernatants from cultures of SV40 transformed 3T3 cells, but not medium from non-transformed serum cultures, inhibit the migration of guinea pig macrophage (for ref. see 30, 64). MIF-like-activity is also produced if supernatants from serum-free cultures of SV40-3T3 cultures are incubated with fresh frozen guinea pig serum; the factor in the medium thought to generate the MIF-activity is a plasminogen activator. Human urokinase (which exhibits PA activity) also generates MIF-activity at incubation with serum (for ref. see 30). As mentioned above, supernatants from mixed leukocyte cultures contain an inducer of PA activity that cannot be separated from MIF (59). Supernatants from cultures of antigen- or ConA-stimulated guinea pig lymph node cells express PA-activity. The experimental conditions allowing release of this PA have been reported to be identical with those necessary for release of MIF. No linear relation was observed between degree of migration inhibition and degree of PA-activity in the supernatants (52 but cf. 30).

Human blood monocytes and to an even higher degree polymorphs also produce a PA activity. The polymorph production of PA (approx. molecular weight 60,000) is stimulated by ConA and is pharmacologically regulated by, e.g., glucocorticosteroids, theophylline, and cholera toxin. At more intense stimulation, the polymorphs also secrete a plasminogen independent fibrinolytic activity with properties similar to those of elastase. This activity is inhibited by DFP and SBTI, but not by TLCK or TPCK (see 29). Similar elastolytic and also collagenolytic activity can be detected in high concentrations in supernatants from macrophage cultures (27).

Thus evidence is beginning to accumulate that products of antigen- or mitogen-stimulated lymphocytes stimulate the macrophages/polymorphs to produce, among others, PA, esterolytic, and collagenolytic activities. It is also clear that ConA directly

could stimulate polymorphs to produce such enzymes. These macrophage-produced enzymes, possibly by acting on serum components, could then conceivably generate the principles that finally effect the migration inhibition. Fig. 5 gives a schematic representation of such a *hypothetical* chain of events which is now beginning to emerge (30). Therefore, unless proper precaution is observed, macrophage/polymorph produced enzymes could falsely be apprehended and characterized as MIF/LIF, although they are not lymphocyte derived. It is also clear that serum protease/esterase inhibitors play important roles at the evaluation of MIF/LIF activity: 1) because they affect the macrophage response to MIF; 2) because they might regulate the activity of macrophage/polymorph produced proteases/esterases.

VI CONCLUDING REMARKS

The production, properties, and biological effects of soluble *in vitro* cell migration inhibitory activities are surveyed. The evidence indicates the existence of a number of factors that exhibit this effect. These factors differ with respect to cell type used as indicator, cell type used at its production, and physico-chemical properties; the total migration inhibitory activity in a single system can be due to the combined effect of more than one factor. Evidence is also accumulating, in both animal and human systems, that stimulation of lymphocytes induces production of factors which in turn induce the release from macrophages/polymorphs/monocytes of plasminogen activator (PA) activity. The phase of migration inhibition is then conceivably a non-immunologic event incorporating the action of PA on serum component(s).

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Abbreviations:

BAEE	Benzoyl arginine ethyl ester
BGG	Bovine gamma globulin
CFI	Chemotactic factor inhibitor
ConA	Concanavalin A
DFP	Diisopropyl fluorophosphate
DNP	Dinitrophenol
EACA	ϵ -amino caproic acid
FCA	Freund's complete adjuvant
HPRO	Horse radish peroxidase
HSF	Histamine-induced suppressor factor
IPA	Inducer of plasminogen activator
IRA	Immunoregulatory α -globulin
MDP	Muramyl dipeptide (<i>N</i> -acetylmuramyl-L-alanyl-D-isoglutamine)
MEF	Migration enhancement factor
MIF	Migration inhibitory factor
MIFIF	Migration inhibitory factor inhibitory factor
MØ	Macrophage
LIF	Leukocyte migration inhibitory factor
LNC	Lymph node cells
LPS	Lipopolysaccharide
OA	Ovalbumin
2-PAM	Pralidoxime methansulphonat
PBC	Peripheral blood leukocytes
PEC	Peritoneal exudate cells
PMN	Polymorphonuclear
PMS	Polymorph migration stimulator
PMSF	Phenyl methyl sulphonyl fluoride
PPD	Purified protein derivative of tuberculin
PWM	Poke weed mitogen
SBTI	Soybean trypsin inhibitor
SDS	Sodium duodecylsulphate
SKSD	Streptokinase streptodornase
SV	Simian virus
TLCK	<i>N</i> - α -tosyllysine chloromethyl ketone
TPCK	<i>L</i> -1-tosylamide-2-phenylethyl chloromethyl ketone

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