

## CHARACTERIZATION OF A LYMPHOCYTE FACTOR WHICH ALTERS MACROPHAGE FUNCTIONS\*

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(Received for publication 4 August 1972)

It was reported previously that certain macrophage functions could be enhanced by a factor from antigen-stimulated lymphocytes (1, 2). Stimulated lymphocyte supernatant fractions, incubated for 3 days with normal guinea pig macrophages, resulted in increased adherence of macrophages to the culture vessel, spreading, motility, phagocytosis, and glucose carbon-1 oxidation. These changes will be referred to as "macrophage activation," without implying that the functional alterations are necessarily equivalent to those accompanying activation of macrophages in vivo.

Does the factor responsible for macrophage activation correspond to one of the previously recognized mediators of cellular hypersensitivity known to affect macrophages? In the present report, the macrophage activating factor was compared physicochemically with migration inhibitory factor (MIF)<sup>1</sup>, chemotactic factor for monocytes, and, in addition, with lymphotoxin. These studies exploited the fact that guinea pig MIF behaves like a glycoprotein in terms of its buoyant density and sensitivity to neuraminidase (3), while chemotactic factor (4) and lymphotoxin (5) do not. The macrophage activating factor could not be distinguished from MIF by the methods employed. In addition, the significance of the 3 day culture period apparently required for activation in vitro was subjected to kinetic analysis. The reversibility of activation, the dose-dependent effect of activating factor, and the time-course of activation in unfractionated supernatants were examined.

### *Materials and Methods*

*Preparation of Lymphocyte Supernatant Fractions.*—MIF-rich and control supernatants were prepared from *o*-chlorobenzoyl bovine gamma globulin-sensitized

\* Supported in part by U. S. Public Health Service grants AI-7685-07 and National Institutes of Health Contract 71-2172, and a grant from the John A. Hartford Foundation.

† Recipient of a Cancer Research Scholar Award from the American Cancer Society.

<sup>1</sup> *Abbreviations used in this paper:* HMPS, hexose monophosphate shunt; MEM, minimal essential medium containing 100 µg streptomycin and 100 U penicillin/ml; MEM-S, MEM with 15% guinea pig serum; MIF, migration inhibitory factor; OCB-BGG, *o*-chlorobenzoyl bovine gamma globulin.

tized) guinea pig lymph nodes using OCB-BGG as antigen (3); these were concentrated and fractionated on Sephadex G-100 as previously described (6, 7). Each fraction was concentrated by vacuum dialysis, diluted with Eagle's minimal essential medium containing 100  $\mu$ g streptomycin and 100 U penicillin per ml (MEM), filtered (Millipore 0.45  $\mu$ m), and made to contain 15% normal guinea pig serum. All media described below were similarly sterilized and made to contain 15% normal guinea pig serum. The concentration of Sephadex fractions in the culture medium was expressed in "ml-equivalents/ml," arbitrarily defined as the volume of the original supernatant divided by the volume of the test medium prepared from each fraction.

*Isopycnic Centrifugation.*—MIF-rich and control Sephadex G-100 fractions containing substances of mol wt 35,000–55,000, representing 113–130 ml-equivalents of original supernatant were subjected to centrifugation at 39,000 rpm in CsCl as previously described (3). Aliquots of 0.1–0.2 ml were then collected from the bottom of each tube and were pooled to give six fractions of MIF-rich material and six fractions of identical density of the control material. The density ranged from 1.20 to 1.56 g/ml. Each fraction was tested at 8–27 ml-equivalents/ml in MEM with serum.

*Treatment with Neuraminidase.*—Lyophilized powder corresponding to 50 ml of MIF-rich or control supernatants was dissolved in 3 ml of 0.1 M sodium acetate buffer pH 5.1, clarified by centrifugation, and stirred for 1 h at 37°C with 0.7 ml of water containing 0.0525 U (83  $\mu$ g) of neuraminidase from *Clostridium perfringens* (Worthington Biochemical Corp., Freehold, N. J.). Lyophilized portions of the same supernatants were treated identically except that the added water contained no enzyme. Each preparation was then fractionated on Sephadex G-100 columns and fractions of  $K_d$  0.09–0.22 were pooled (see legend, Fig. 1). The Sephadex G-100 fractions were concentrated by ultrafiltration, dialyzed against MEM, and tested at 8 ml-equivalents/ml.

It has been shown that fractions prepared in this manner contain no neuraminidase (3). It was necessary to exclude neuraminidase from the test media before incubation with macrophages because a preliminary experiment demonstrated a marked, dose-dependent stimulation of cell adherence by neuraminidase in MEM with 15% guinea pig serum (MEM-S) after 2–3 days of culture.

*Assay for Macrophage Activating Factor.*—Macrophage monolayers prepared from casein-induced peritoneal exudates from normal Hartley guinea pigs were cultured in plastic Petri dishes in triplicate with MIF-rich and control supernatant fractions and MEM-S as described previously (2). Oxidation of [1-<sup>14</sup>C] glucose to <sup>14</sup>CO<sub>2</sub> by the macrophage monolayers and the protein content of the same monolayers were measured as before (2). Measurements of the DNA content of matched monolayers by a fluorometric method (8) indicated that the ratio micrograms of DNA to milligrams of cell protein was the same for macrophages incubated in MIF-rich or control fractions or MEM-S<sup>2</sup>. Milligram of cell protein was therefore regarded as a measure of the number of adherent cells.

When the media were to be reutilized, they were gently aspirated from the Petri dish with a Pasteur pipette, centrifuged to remove cellular debris, and frozen at –20°C. Before use, the media were dialyzed overnight against MEM and filtered (0.45  $\mu$ m).

*Migration Inhibition Assay.*—Samples of macrophages used in each experiment were packed in capillary tubes and incubated with samples of the MIF-rich and control media used in the same experiment according to methods described previously (7).

## RESULTS

*Sephadex G-100 Fraction.*—The elution of macrophage adherence-promoting activity from Sephadex G-100 columns was the same as that observed with MIF

<sup>2</sup> Remold, H. G., and A. Mednis. Unpublished observations.

prepared with the same antigen (6, 7). As seen in Fig. 1 (kindly contributed by Alice Vreeland), peak adherence of cells to dishes resulted from incubation in fraction IVa, mol wt approximately 35,000–55,000, and significant adherence from fraction III, with which albumin elutes. At 5.0 and 2.5 ml-equivalents/ml, the MIF-rich fraction IVa gave 0.157 and 0.72 mg adherent cell protein, respec-

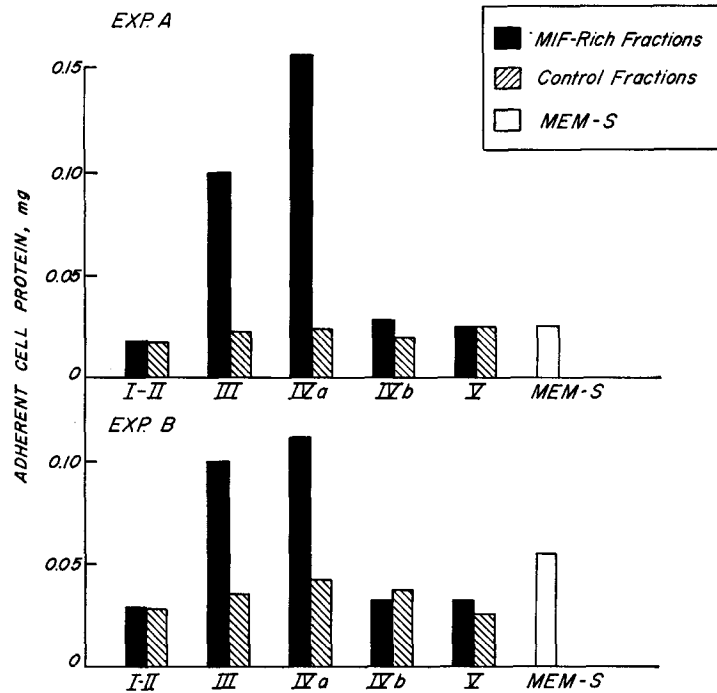


FIG. 1. Elution pattern of cell adherence-promoting activity from Sephadex G-100 columns. Fractions were incubated for 3 days with triplicate macrophage monolayers in each experiment. Fractions I and II, which were pooled, represented the void volume and contained large proteins such as OCB-BGG and other immunoglobulins. Fraction III ( $K_d = 0.09-0.19$ ) contained molecules eluting with albumin. Fraction IVa ( $K_d = 0.19-0.33$ ), eluting after albumin and before chymotrypsinogen, contained substances of mol wt 35,000–55,000. Fraction IVb ( $K_d = 0.33-0.45$ ) eluted with chymotrypsinogen (mol wt 25,000) and fraction V ( $K_d = 0.45-0.70$ ) contained substances of lower molecular weight which eluted with lysozyme.

tively, whereas the control fraction IVa gave 0.024 and 0.019 mg. No cell adherence-promoting activity was found in the other fractions.

*Isopycnic Centrifugation.*—The buoyant density of macrophage activating factor proved to be the same as that of MIF (3), but denser than that measured for chemotactic factor (4) or lymphotoxin (5). Cell adherence-promoting activity and hexose monophosphate shunt-stimulating (HMPS-stimulating) activity were determined in three experiments, summarized in Fig. 2 A and B, and were distributed exclusively in fractions C ( $\rho_{25} = 1.427-1.366$ ) and D ( $\rho_{25} = 1.366-$

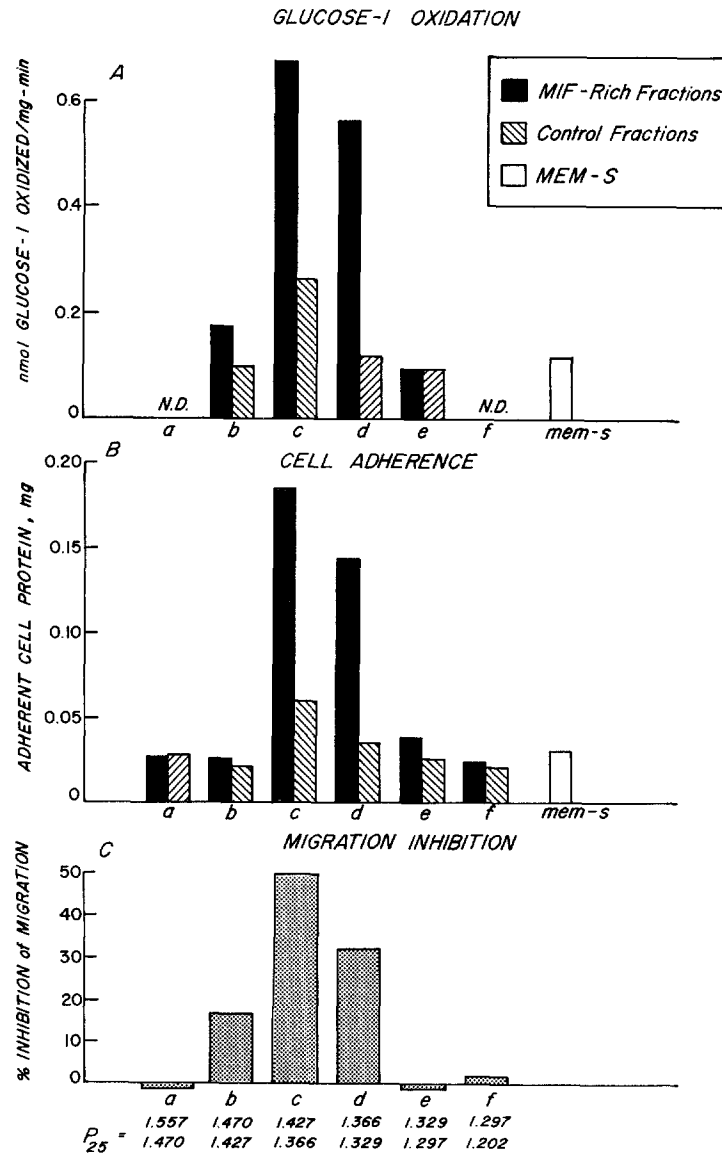


FIG. 2. Isopycnic centrifugation. Effect of fractions from CsCl density gradient centrifugation of Sephadex G-100 fraction IVa, on (A) glucose carbon-1 oxidation and (B) cell adherence, in three experiments, after 3 days' incubation with triplicate macrophage monolayers, and (C) migration inhibition (quadruplicate capillaries). The fractions are defined by their densities in grams per milliliter at 25°C as shown in (C).

1.329). MIF was assayed in one of these experiments (Fig. 2 C) at 15 ml-equivalents/ml and also showed activity in fractions C and D; tested at 8 ml-equivalents/ml, MIF was detected only in fraction C. It was previously reported that MIF peaked in a fraction with  $\rho_{25} = 1.412\text{--}1.352$  g/ml, with a range of 1.452–1.312 (3). Essentially no activating factor could be detected in the denser or lighter zones. On similar gradients, lymphotoxin activity is found in a lighter zone, at  $\rho_{25} = 1.350\text{--}1.250$  (5), and chemotactic factor also bands with pure proteins (4).

*Neuraminidase Treatment.*—Since MIF is sensitive to neuraminidase (3), while chemotactic factor (4) and lymphotoxin (5) are not, it was of interest to determine whether neuraminidase affected macrophage activating factor. MIF-rich and control lymphocyte supernatants were tested for macrophage adherence and HMPS activity after incubation with and without neuraminidase and subsequent fractionation. The results of two experiments are presented in Fig. 3. The activity of macrophage activating factor was completely destroyed by neuraminidase with regard to both cell adherence and HMPS activity. The data demonstrate that terminal sialic acid residues are necessary for the effect of the activating factor on both cell adherence and HMPS activity. This distinguishes the activating factor from chemotactic factor and lymphotoxin, but not from MIF. The susceptibility to neuraminidase and the buoyant density of the activating factor are consistent with a glycoprotein composition (9).

*Do MIF and Activating Factor Persist in Culture with Macrophages?*—To answer this question, the test media were cultured with macrophages for 7, 24, 48, and 72 h and then centrifuged, dialyzed, filtered, and retested on fresh macrophages for 72 h. At this time, cell adherence in the MIF-rich fraction was enhanced to approximately the same extent no matter how long the fraction had been preincubated with macrophages. The persistence of activating factor was compared with the persistence of MIF. As shown in Table I, there appeared to be as much activating factor and as much MIF present in the MIF-rich fraction after 3 days of incubation with macrophages as there had been initially. Finally, media which had been incubated with macrophages for 3 days were assayed for HMPS stimulation of fresh cells after another 3 days of culture. The cells in the MIF-rich fraction oxidized 0.46 nmol glucose-1 per milligram cell protein per minute, compared with 0.22 in the control fraction. Thus, the activating factor could not be distinguished from MIF on the basis of its persistence in culture and appeared to exert its effect without being markedly consumed by the monolayers.

*Is Activating Factor Required in the Latter Part of the 3 Day Culture?*—Since activating factor is present throughout the culture period with macrophages, it might exert its effects at any time. To determine whether the presence of activating factor was required during the latter part of culture, we treated macrophages with MIF-rich and control fractions for the first 7, 24, and 48 h of culture and then replaced the culture media with MEM-S. On the 3rd day of culture, we

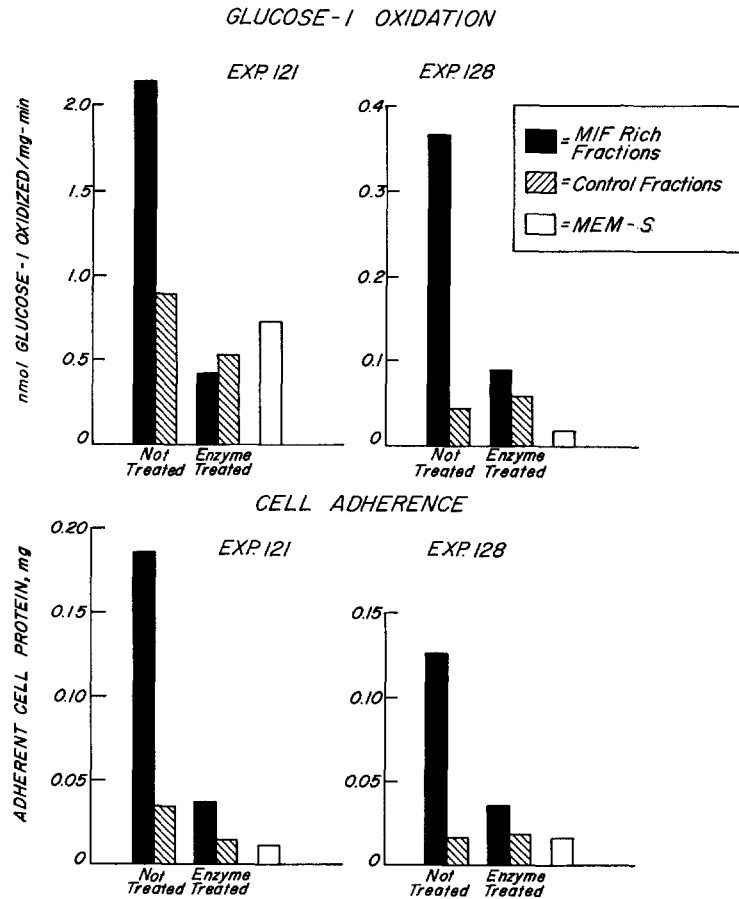


FIG. 3. Effect of treatment of supernatants with neuraminidase, followed by fractionation on Sephadex G-100 columns to remove the enzyme, on glucose carbon-1 oxidation and cell adherence by macrophage monolayers after 3 days of culture. Data are means from triplicate monolayers.

TABLE I  
*Persistence of Activating Factor and MIF\**

	Cell adherence†			Inhibition of migration %
	MIF-rich fraction	Control fraction	MEM-S	
Initial experiment at 3 days	0.147	0.040	0.019	39.5
Reincubated for 3 more days with fresh cells	0.151	0.035	0.026	40.5

\* Values are means from three monolayers or eight capillaries. Casein-induced strain 2 exudate cells were used to test cell adherence and oil-induced Hartley exudate cells to test migration for these experiments.

† Milligram cell protein.

assayed all the monolayers for glucose-1 oxidation and cell adherence, as well as monolayers which had remained in the initial test media for the full 3 day period (Fig. 4). Only the latter demonstrated activation. Thus, an essential interaction between macrophages and substances in the MIF-rich fraction occurred in the last 24 h before activation was expressed. It was not yet clear, however, whether this late-acting substance was the same as the activating factor added at the beginning of culture.

*Is the Factor Required Late in the Culture the Same As That Added Initially?*—The fact that the MIF-rich medium had to be present in the last 24 h of 3 day culture in order for activation to occur suggested the following hypothesis: The activating factor was altered during incubation with macrophages, or induced the elaboration of a new substance by macrophages, and this altered or newly elaborated product quickly brought about activation. If this were so, the MIF-rich fractions removed from macrophage monolayers after 3 days of culture

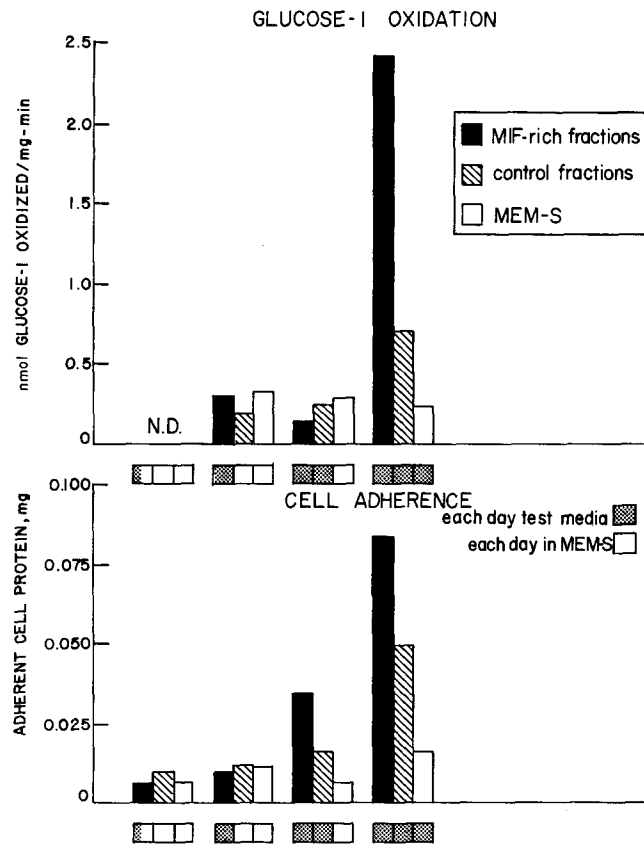


FIG. 4. Inability to enhance glucose carbon-1 oxidation or cell adherence by pulsing with fractions in the early part of culture. Media were aspirated at the times indicated and replaced with MEM-S for the remainder of the 3 day culture. Data are means from triplicate cultures.

should bring about activation of fresh macrophages in less than 3 days. This hypothesis was proven incorrect by an experiment which showed that fresh macrophages were activated only after 72 h in such media, and not after only 1, 24, or 48 h.

A similar conclusion was reached in another way. If the substance required in the final 24 h of culture were released into the MIF-rich medium during the first 48 h, then replacing the MIF-rich fractions with fresh MIF-rich fractions at 48 h should prevent the appearance of activation at 72 h, just as replacement with MEM-S at 48 h blocked the appearance of activation at 72 h (Fig. 4). In fact, replacing the MIF-rich fraction with fresh MIF fraction on the 2nd day of culture resulted in even greater cell adherence on day 3 than if the media were left unchanged. (The cell protein adherence, measured after 3 days, in MIF-rich, control, and MEM-S-containing dishes, was 112, 35, and 66  $\mu\text{g}$  respectively when fractions were not changed; it was 190, 72, and 70  $\mu\text{g}$  at this time when the fractions had been replaced with fresh fractions at 2 days.) This demonstrated that the substance required during the latter part of 3 day culture in order for activation to come about is present in fresh MIF-rich fractions. These studies would not have detected a macrophage-derived factor produced within a few hours of the addition of MIF-rich media to the culture and thereafter exhibiting the same kinetic characteristics as seen with MIF-rich media.

*Is Activating Factor Required in the Early Part of 3 Day Culture?*—Experiments were now performed to determine whether activating factor was also required during the early part of culture. Monolayers were incubated in MEM-S for 0, 24, and 48 h and the medium was then replaced with MIF-rich and control fractions or fresh MEM-S. At 72 h, all the monolayers were assayed for activation. As seen in Fig. 5, only a 24 h exposure to activating factor was necessary to enhance cell adherence, provided the monolayers had been cultured for the previous 48 h in MEM-S. Stimulation of the HMPS required 48 h of exposure to the activating factor, provided the monolayers had been cultured for the previous 24 h with MEM-S. The same results were obtained when the experiment was repeated with test media which had already been incubated with macrophages for 3 days. These results suggest that the 3 day period required by macrophages to manifest a response to the activating factor consists of two stages. This is discussed below.

*Is Activating Factor Required to Sustain Activation Once It Has Occurred?*—It was of interest to learn whether activation, once established, might persist after removal of the MIF-rich fraction. Accordingly, monolayers were incubated in MIF-rich and control fractions for 3 days. At this time, triplicate dishes from each set were tested to demonstrate that activation had occurred. The media in the remaining dishes were replaced with MEM-S, and the monolayers were incubated further. The results of three experiments are shown in Fig. 6. The adherent cell protein of activated monolayers persisted at an elevated



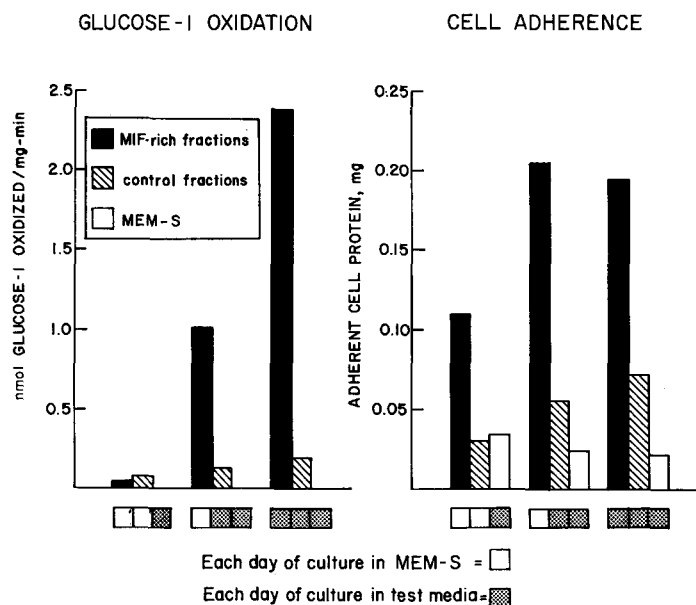


FIG. 5. Shortening of the time-course of enhancement of glucose carbon-1 oxidation and cell adherence by fractions after preincubation of the cultures in MEM-S. Cultures were incubated initially in MEM-S, which was aspirated at the times indicated and replaced with test media for the remainder of the 3 day culture. Data are means from triplicate cultures.

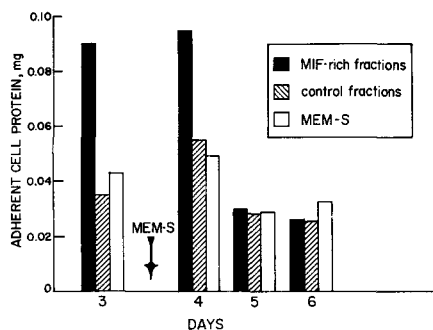


FIG. 6. Persistence of enhanced cell adherence for 24 h when fractions were removed from activated monolayers. Monolayers were incubated in test media for 3 days, at which time the test media were replaced with MEM-S and the culture continued for 1-3 days. Data are means from three experiments, each in triplicate.

level for 24 h after the removal of the test media, but fell to the same level as control monolayers by 48 h. Stimulation of HMPS in the monolayers treated for 3 days with MIF-rich fractions also persisted at least 24 h after the medium was replaced with MEM-S, though at a lower level.

*Dose-Response Relationship.*—The increments in the rates of glucose carbon-1

oxidation per milligram macrophage protein in MIF-rich fractions, compared with those rates in MEM-S, were related to the concentration of MIF-rich medium over a 10-fold range, as displayed in Fig. 7. The 11 assays shown represent experiments performed with 10 different cell preparations, using four fraction III's and six fraction IV's from a total of eight different frac-

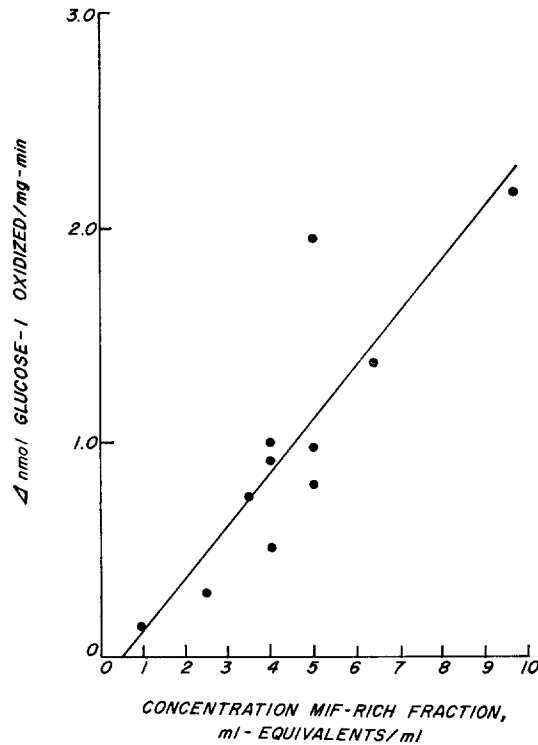


FIG. 7. Effect of the concentration of the MIF-rich fraction on rate of glucose carbon-1 oxidation by macrophages after 3 days of incubation. On the abscissa is indicated the rate of oxidation per milligram cell protein in the MIF-rich fraction less the rate in MEM-S in the same experiment, using mean values from triplicate monolayers. Except as noted in text, each point represents an experiment performed with different cells and fractions on different days. For definition of ml-equivalent, see text.

tionations (2). All experiments were included in which these fractions were used without further modification and at concentrations under 10 ml-equivalents/ml. The plot, as determined by the method of least squares, has a slope of 0.246 glucose carbon-1 oxidized per milligram cell protein per minute per "ml-equivalent per ml." The coefficient of variation,  $r = 0.86$ , implies a strong dose-response relationship.

The absolute value of adherent cell protein monolayers incubated in MIF-rich

fractions did not correlate closely with the magnitude of the HMPS response in the same experiment.

*Glucose Oxidation in Whole Supernatants.*—The increased cell adherence observed after 1 day of culture in whole supernatants stood in contrast to the absence of such an effect after 1 day in fractions (2). The increased adherence after 1 day in whole supernatant was at least partly antigen dependent and was not accompanied by an increase in phagocytic capacity (2). These observations suggested that the increased adherence after 1 day in whole supernatants might not be related to the action of macrophage activating factor.

To clarify this point, glucose carbon-1 oxidation by macrophage monolayers was assayed after 1 and 3 days of culture in whole supernatant. The typical, partly antigen dependent, effect on cell adherence was observed in whole supernatant after 1 day of culture, but there was no effect on HMPS at this time. By 3 days, the cells in the MIF-rich supernatant exhibited enhanced adherence (0.137 mg) compared with control supernatant (0.055 mg) with minimal antigen dependence (control supernatant plus antigen, 0.068 mg), but they also showed a marked increase in HMPS activity (Fig. 8). The addition of antigen to the control supernatant had no effect on HMPS at either incubation period. Thus activation, defined in terms of an increase in both cell adherence and HMPS activity, required 3 days of culture in unfractionated supernatant, just as in supernatant fractions.

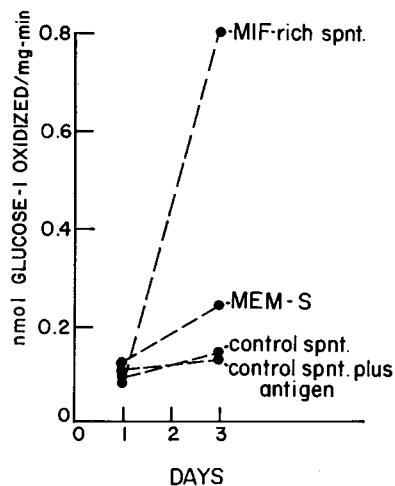


FIG. 8. Effect on cell adherence and glucose carbon-1 oxidation of whole supernatant from antigen-stimulated lymphocyte cultures, control supernatant from lymphocytes cultured without antigen, control supernatant to which antigen was added after the removal of the lymphocytes, and MEM-S. The adherent cell protein of monolayers at the beginning of the experiment was 0.329 mg. Data are means from triplicate cultures.

## DISCUSSION

The macrophage activating factor described in this study is indistinguishable from MIF in terms of its elution pattern from Sephadex G-100, buoyant density, destruction of its activity by neuraminidase, and persistence during culture with macrophages for 3 days. These studies do not prove that MIF and macrophage activating factor are identical; they do indicate, however, that the macrophage activating factor, like MIF, is physicochemically distinct from two other mediators of cellular hypersensitivity, chemotactic factor for monocytes and lymphotoxin.

Previous studies demonstrated that 3 days of incubation were required to elicit activation of macrophage monolayers (2). In the present report, kinetic studies were performed to evaluate significance of this requirement. The results indicate that the macrophage cultures were relatively refractory to the HMPS-stimulating influence of the activating factor during the first 24 h *in vitro*, but could then respond after 48 h of exposure to the activating factor. Similarly, the cultures were refractory to the adherence-promoting influence of the activating factor during the first 48 h, but could then respond after 24 h of exposure. The ability to elicit enhanced cell adherence with 24 h less exposure to activating factor than required for enhanced HMPS activity suggests that neither of these phenomena is an immediate cause of the other.

The above findings imply that a maturation process may be required before guinea pig peritoneal exudate macrophage monolayer cultures can respond to macrophage activating factor. Others have observed that mononuclear phagocytes respond differently to exogenous modulating influences at different times during their development *in vitro* or *in vivo*. Such changes may be related to the progressive maturation of mouse peritoneal macrophages (10) or horse monocytes (11) with time in culture that has been described by Cohn and his associates. Differential susceptibility of macrophages to stimulants may depend not only on the age of the cells in culture, but also on the age of the donor. Thus, peritoneal macrophages of adult mice, but not sucklings, respond to intraperitoneal injections of proteose-peptone by development of enhanced sticking to glass, spreading, phagocytosis, interferon production, and viricidal capacity (12). Finally, the age of mononuclear phagocytes before their removal from the host, or the use of an intraperitoneal irritant (which presumably draws forth a younger cell population than the resident macrophages) may have a profound influence on the responsiveness of the cells in culture (13). The resident peritoneal macrophages of the guinea pig migrate poorly on glass (14) and show poor inhibition of migration (14). In addition, they adhere poorly to plastic dishes and fail to demonstrate increased adherence or increased phagocytosis of starch particles when incubated for 24 or 72 h in MIF-rich fractions.<sup>3</sup> Presumably, the guinea pig peritoneal exudate mononuclear phago-

<sup>3</sup> Nathan, C. F., and J. R. David. Unpublished observations.

cyte more closely resembles the type predominating at the site of a cellular hypersensitivity inflammatory reaction than the apparently more effete cell which is resident in the uninfamed guinea pig peritoneal cavity.

Thus, the requirement for aging of the culture partly explains the long time-course of activation. However, even in aged cultures, activation required 1–2 days of exposure to MIF-rich fractions. Reports of monocyte or macrophage activation in unfractionated lymphocyte supernatants have involved incubations of 1–2 days (15), 3 days (16–18), or 4–10 days (19). The importance of aging of the culture in the time-course of activation in these experiments is not known.

Since we previously observed enhanced macrophage adherence after a total culture period of only 1 day in unfractionated supernatants (2), we considered the possibility that whole supernatants contain a substance, not present in MIF-rich fractions, which might hasten the response of macrophages to activating factor. However, in the present report, stimulation of HMPS in whole supernatants required 3 days of culture, just as in fractions. Presumably, the stimulation of cell adherence observed after only 1 day in whole supernatants, without a concomitant increase in HMPS or phagocytic capacity (2), was related to something other than the macrophage activating factor, possibly antigen and antibody.

In the presence of MIF, inhibition of macrophage migration is apparent within hours, in fact, as soon as migration in control chambers has proceeded far enough to allow a comparison. 1–2 days later, by the time that monolayers prepared from the same cell samples are susceptible to enhancement of HMPS activity and cell adherence, the rate of macrophage migration from capillary tubes in MIF-rich media is already greater than in controls (2). This difference in timing need not imply that migration inhibition and macrophage activation are responses to different effector molecules. Migration inhibition may be one of the earliest in a sequence of responses by macrophages to the same effector molecule, the timing of which either reflects an intrinsic capacity of the cell, or is determined by physical and chemical constraints upon cell pellets packed in capillary tubes.

It is of interest that once activated, macrophages remained so for at least 24 h after removal of activating factor. By 48 h they appeared to return to control levels. This reversibility of activation took longer than that of lysosomal hydrolases in mouse macrophages, which were increased by newborn calf serum but had already fallen to control levels by 24 h after the serum was removed (20).

The significance of macrophage activation as described here remains speculative. Other workers have found that bactericidal capacity could be enhanced *in vitro* by incubation of sensitized peritoneal exudates with the specific antigen (21, 22), and that tumoricidal activity could be enhanced by incubation of sensitized lymphocytes and normal macrophages with the specific antigen (23, 24). A soluble factor has been implicated by additional studies

showing that supernatants from antigenically stimulated lymphocytes could enhance the antibacterial (16, 17, 19) and antitumor activity (18, 25) of normal macrophages. The active moiety in the studies cited was not characterized, but may be related to the macrophage activating factor described in this report. The physicochemical and kinetic characteristics of the latter suggest that it is probably the same as previously defined mediator of cellular hypersensitivity, MIF.

#### SUMMARY

As reported previously, antigenically stimulated guinea pig lymphocytes elaborate a soluble factor which activates macrophages in the sense of promoting increased adherence, spreading, phagocytosis, and glucose oxidation through the hexose monophosphate pathway. Further studies on the characteristics and kinetics of this substance were carried out. The activating factor could not be distinguished from a previously characterized lymphocyte mediator, migration inhibitory factor (MIF), on the basis of Sephadex G-100 gel filtration, CsCl density gradient centrifugation, or sensitivity to neuraminidase. It was, however, shown to be distinct from two other lymphocyte mediators, chemotactic factor for macrophages and lymphotoxin. The kinetics of activation were further studied. The data suggest that the 3 day period required by macrophages to manifest a response to the activating factor consists of two stages. In the first, requiring 1–2 days, the macrophages are refractory to the influence of activating factor, but undergo changes which render them receptive. In the second, they respond to activating factor with increased cell adherence and glucose oxidation. Once macrophages have been activated, the effect persists in the absence of activating factor for 24 h. Finally, it was shown that activation in unfractionated supernatants followed the same time-course as that in more purified fractions. The data suggests that the activating factor is the same as MIF and that, *in vitro*, macrophages respond to this substance with migration inhibition before they become sensitive to its activating influence.

The authors wish to express their appreciation for the excellent technical assistance of Alice Vreeland, Roberta David, and Alma Mednis.

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