ALTERATIONS OF MACROPHAGE FUNCTIONS BY MEDIATORS FROM LYMPHOCYTES*

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Metchnikoff maintained that the macrophage is the body's chief defense against particulate foreign matter and many chronic infections (1). Later workers have demonstrated that the macrophage responds to certain infections with an adaptive increase in its defensive capacities (2, 3). Recently, Mackaness and his colleagues have shown that the enhancement of macrophage function during infection has an immunologic basis (4) and involves the lymphocyte (5). The mechanism of interaction between macrophage and lymphocyte during the expression of cellular immunity is not yet known.

As described by Mackaness, the activated macrophages obtained from animals during a certain stage of infection are more spread out on glass, more heavily endowed with mitochondria and lysosomes, and more phagocytic than their normal counterparts (6). Most important, such cells have an enhanced bactericidal capacity which is effective even against organisms antigenically unrelated to those infecting the host (4). As cellular immunity fades with time, it can be recalled in an accelerated manner only by repeated presentation of the original antigenic stimulus (7). The capacity to develop activated macrophages can be transferred with sensitized lymphocytes, but only if the recipient is also challenged with the specific sensitizing antigen (5). These points underscore that cellular immunity, though nonspecific in its expression, is specific in its elicitation, and that the nonspecific component is expressed by the macrophage, while the specific component resides with the lymphocyte. Moreover, Mackaness has shown that the development of cellular hypersensitivity[1] parallels that of cellular immunity[2] (8).

As with cellular immunity, cellular hypersensitivity is characterized by nonspecific and specific components. Using isotopically labeled leukocytes and cell transfer techniques, McCluskey et al. (9) demonstrated that the vast majority of cells infiltrating the site of a dermal cellular hypersensitivity reaction were not specifically sensitized. On the other hand, these workers and others (10–12) found that labeled leukocytes

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[1] Cellular hypersensitivity refers to delayed hypersensitivity and models thereof in vitro.

[2] Cellular immunity refers here to acquired cellular resistance to infection.

from sensitized donors were always present in small numbers in the infiltrates. Furthermore, in vitro inhibition of macrophage migration, a phenomenon correlated with cellular hypersensitivity (13, 14), could be achieved even when 97.5% of the peritoneal exudate cells employed were from an unsensitized donor, as long as the remainder included lymphocytes from a sensitized animal (15). Thus, in vivo and in vitro, cellular hypersensitivity involves both sensitized and unsensitized cells.

The following model arises from analysis in vitro of the specific and nonspecific components of cellular hypersensitivity. Sensitized lymphocytes, stimulated by the specific antigen, secrete soluble mediators; these are nonspecific in the sense that they can exert their characteristic effects on cells from unsensitized donors and in the absence of antigen. Since the demonstration of migration inhibition factor (MIF)[3] (16, 17), more than 10 soluble factors from lymphocytes have been described (18). Three are assayed by their effects on macrophages. These are MIF, macrophage chemotactic factor, which is chemically distinct from MIF (19), and macrophage aggregation factor (20), which may be identical with MIF.

It is of obvious interest to determine whether the soluble mediators of cellular hypersensitivity in vitro, especially MIF and chemotactic factor, induce alterations in macrophage function similar to those seen in cellular immunity. Such evidence would suggest a mechanism for activation of macrophages in cellular immunity and strengthen the hypothesis that the same mechanism operates in both cellular immunity and cellular hypersensitivity.

In this study, supernatants from antigenically stimulated lymphocytes were examined for their effects on the following functions of macrophages: adherence to the culture vessel, spreading, motility, phagocytosis, glucose oxidation, and protein synthesis.

Materials and Methods

Sensitization of Animals.—*o*-Chlorobenzoyl bovine γ-globulin (OCB-BGG) emulsified with complete adjuvant (Difco Laboratories, Inc., Detroit, Mich., H37Ra) was injected into each footpad and the dorsal nuchal dermis of 500 g male Hartley guinea pigs (total, 125 mg of OCB-BGG). Preparation of the antigen has been described (21).

Culture of Lymphocytes.—14 days after sensitization, draining lymph nodes were removed and the lymphocytes prepared for culture as described (21). One-half of the lymphocyte suspension was cultured for 24 hr with 100 μg/ml of OCB-BGG and the cell-free supernatant referred to as the MIF-rich supernatant. The other half of the lymphocyte suspension was cultured without antigen and the cell-free supernatant referred to as the control supernatant. In all studies using fractionated supernatants, antigen was added to the control supernatant before fractionation. When the unfractionated supernatant was employed, it was dialyzed against Eagle's minimal essential medium (MEM), adjusted to contain 100 μg of streptomycin/ml, 100 units of penicillin/ml, and 15% normal guinea pig serum by volume and filtered (Milli-

[3] *Abbreviations used in this paper:* BCG, Calmette-Guérin bacillus; HBSS, Hanks' balanced salt solution; HMPS, hexose monophosphate shunt; Krebs-G, Krebs-Ringer phosphate buffer in glucose; MEM, Eagle's minimal essential medium; MEM-S, culture medium without supernatants or fractions; MIF, migration inhibition factor; NADPH, nicotinamide adenine dinucleotide phosphate; OCB-BGG, *o*-chlorobenzoyl bovine γ-globulin; TCA, trichloroacetic acid.

pore HAWP-013-00, 0.45 μ). The same concentrations of antibiotics and serum were used in all other incubation media described below. Culture medium without supernatants or fractions will be referred to as MEM-S.

Fractionation of Supernatants.—To remove antigen and antibody, fractions were dialyzed, lyophilized, and chromatographed on Sephadex G-100 columns (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) as detailed previously (21). Fraction III contained material eluted with an albumin marker, and Fraction IV (25,000–55,000 mol wt) was somewhat broader than that previously described (21). The fractions were reconstituted in MEM-S and filtered (Millipore HAWP-013-00, 0.45μ). (Fractions prepared in this fashion contain macrophage chemotactic factor [19] and lymphotoxin[4] as well as MIF.) Fractions were reconstituted to a 2.5- to 14-fold concentration compared to the volume of the original supernatant. Approximately 40% of the original MIF activity is recovered in each of these fractions (21).

Harvest of Macrophages.—Unimmunized male Hartley guinea pigs weighing approximately 500 g were injected intraperitoneally with 20 ml of sterile 1% sodium caseinate (Difco) in isotonic saline (22). 4 days later the animals were exsanguinated under ether anesthesia, and injected intraperitoneally with 150 ml of Hanks' balanced salt solution (HBSS). The cell suspensions were drained through a trocar, washed twice in cold HBSS, resuspended in MEM, pooled, and adjusted to contain 4–6 $\times$ 10^6 cells/ml plus antibiotics and serum. The exudates contained 85–95% mononuclear cells. Certain lots of caseinate elicited largely polymorphonuclear exudates and were unsuitable.

Preparation of Macrophage Monolayers.—Sterile technique was used throughout. Plastic Petri dishes (35 $\times$ 10 mm, Falcon Plastics, Los Angeles, Calif., for tissue culture, No. 3001) were placed in 100 mm Petri dishes, which served as carriers. (Results were the same if glass dishes were used instead of plastic.) 1 ml of the cell suspension was dispensed to each dish and the dishes were incubated at 37°C in 5% CO_2 in air for 45 min, after which, using sterile forceps, each dish was emptied, rinsed vigorously in three successive beakers of HBSS, drained on a towel, and replenished with 1 ml of the medium to be tested. Each medium was usually tested in triplicate. The order of plating maximized the dispersal of the members of each triplicate. The dishes were incubated at 37°C in 5% CO_2 in air for up to 3 days. Dishes incubating longer than 24 hr received, at 24 and 48 hr, 0.1 ml of a supplemental solution containing normal guinea pig serum, MEM, glucose, essential amino acids, and glutamine (23) in order to rule out possible effects of unequal rates of utilization of nutrients. It was occasionally necessary to adjust the pH of the dishes at 48 hr with several drops of isotonic $NaHCO_3$. Microscopic examination of 3-day monolayers revealed that 100% of adherent cells were capable of phagocytosis; they were considered to be macrophages.

Measurement of Cellular Adherence.—At the conclusion of each experiment, the dishes were emptied, vigorously rinsed in six successive beakers of isotonic saline, and air-dried. Slight variations in the amount of rinsing did not affect the number of adherent cells. The contents of the dishes were dissolved overnight in 2.0 ml of 0.5 N NaOH and centrifuged. An 0.2 ml sample of the supernatant was removed for protein determination by the method of Lowry et al. (24) using a bovine serum albumin standard. In addition, immediately after the 45 min plating period in each experiment, three dishes were rinsed for measurement of the adherent cell protein. This indicated the starting amount of adherent cell protein and demonstrated that it usually varied from dish to dish by 3% or less. This method of measuring cellular adherence was chosen to avoid the subjectivity of microscopic enumeration of cells distributed unevenly on a dish. Milligrams of cell protein rather than cell number was preferred for comparing metabolic and phagocytic activities of monolayers in different media so that differences in these activities would not merely reflect possible differences in cell size. Microscopic examination confirmed that increased protein measurements did represent increased numbers of adherent cells.

[4] S. Rosenberg, M. Henrichon, H. Remold, and J. R. David. Unpublished observations.

Phagocytosis.—Phagocytosis was studied by the method of Michell et al. (25) using ^{14}C-acetyl starch granules from *Amaranthus caudatus* seeds (average diameter 2.58 μ), and heat-inactivated, ^{14}C-labeled *Mycobacterium tuberculosis hominis* H37Rv. Preparation of these particles has been described elsewhere (25–27). Particles were added to monolayers for precisely timed periods. The dishes were then rapidly emptied, rinsed, and NaOH added as described above. One sample of the hydroxide solution was used to determine adherent cell protein; another sample was added to Bray's solution for liquid scintillation counting. The specific activity of the particles was determined and results expressed as milligrams of particles (dry weight) taken up both per monolayer and per milligrams of adherent macrophage protein. The relationship between uptake and particle dose was determined, and all subsequent experiments were performed with the dose of particles that gave optimal uptake by cells in MEM-S. Thus, for starch, the dry granules were suspended in MEM and 0.1 ml containing 5.0 mg added to each monolayer. For mycobacteria, the desiccated bacteria were suspended in MEM in a glass homogenizer with a motor-driven teflon pestle, centrifuged to remove clumps, and 0.2 ml containing 6.5–7.0 mg added to each monolayer. Particle uptake was found to be a linear function of time for a certain period, after which little or no further uptake took place. Measurements of uptake made during the former period reflected initial rates of uptake; during the latter period, measurements reflected extent of uptake. It was appropriate to make the measurements of rate of uptake 5–10 min after the addition of starch particles, and 20 min after the addition of mycobacteria. Measurements of extent of uptake were made 30 min after the addition of starch particles and 2–5 hr after the addition of mycobacteria.

Unlike the starch particles, the mycobacteria gave a reaction for protein. From the radioactivity contained in each monolayer, the amount of protein due to the mycobacteria was calculated and subtracted from the total protein on the dish. This gave the amount of macrophage protein in the monolayer. Alternatively, the mean protein content of several monolayers in the absence of mycobacteria was used as a measure of the macrophage protein in monolayers in the presence of mycobacteria. In special experiments, the adherent macrophage protein values of the same monolayers were calculated by both methods; the results differed by 5% or less.

Glucose Oxidation.—The oxidation of ^{14}C-labeled glucose to $^{14}CO_2$ by macrophage monolayers was studied by the method of Michell et al. (25) for neutrophils. After incubation in supernatant fractions or MEM-S, dishes were emptied, rinsed in three beakers of serum- and antibiotic-free Krebs-Ringer phosphate buffer pH 7.4 (28) made 2 mM in glucose (Krebs-G), and drained. 1 ml of Krebs-G was then added to each dish. When it was desired to test the immediate effects of supernatant fractions, they were reconstituted directly in Krebs-G and added to monolayers which had been plated 45 min previously in MEM-S and rinsed in Krebs-G. The Petri dishes were then fitted with lids modified as follows. A hole was punched through the lid 1 cm from the edge using a hot No. 18 needle, and the hole covered with Parafilm. A 1 cm square of Whatman No. 4 filter paper was placed in the center of the inside of the lid and impregnated with 0.05 ml of 1 N NaOH, which held the filter paper in place by capillarity when the lid was inverted. 0.1 ml of glucose-1-^{14}C (1 μCi, specific activity 7.08 mCi/mM, New England Nuclear Corp., Boston, Mass.) or glucose-6-^{14}C (7–10 μCi, specific activity 4.6 mCi/mM, New England Nuclear) in isotonic saline was added rapidly with gentle agitation of the dish. The dishes were incubated at 37°C in air for 20 min. The Parafilm was then lifted and 3 drops of 2 N HCl were added rapidly through the hole in the lid using a syringe with a No. 20 needle coated on the outside with silicone grease. The Parafilm was replaced and the dish gently agitated and returned to the incubator for 15 min. The filter paper containing the evolved CO_2 was removed, the inside of the lid wiped dry with a second square of filter paper, and both pieces placed in a vial with Bray's solution for liquid scintillation counting. All operations outside the incubator were performed on a 37°C warming plate. 1 ml of 1 N NaOH was added to the Krebs-G in each dish and the protein content determined. In each experiment, three dishes containing MEM-S with antibiotics, but no macrophages,

were carried through the entire procedure. The addition of acid to the medium in these dishes caused the release as $^{14}CO_2$ of approximately 0.01% of the glucose-1 label and 0.001% of the glucose-6 label. These background values were subtracted from the counts per minute recorded for the other dishes. Assays were performed with sterile technique.

Macrophage Protein Synthesis.—The test medium was aspirated from each macrophage monolayer after 3 days of incubation, and replaced with 1 ml of Earle's balanced salt solution containing 3% fetal bovine serum which had been dialyzed against Earle's solution, plus 100 units of penicillin/ml, 100 μg of streptomycin/ml, and 0.25 μCi of ^{14}C-labeled reconstituted protein hydrolysate (Schwarz BioResearch Inc., Orangeburg, N.Y.) per milliliter (29). After 15–60 min the dishes were rinsed and analyzed for protein content. The protein in one sample, together with several drops of dilute fetal bovine serum as carrier protein, was precipitated with 10% trichloroacetic acid (TCA) and collected on a Millipore filter (DAWG-025-00, 0.65 μ). The filter was washed with TCA, dried, and dissolved in 10 ml of Spectrofluor (New England Nuclear) for liquid scintillation counting. (Dissolution of the monolayer in NaOH in the usual fashion reduced the amount of TCA-precipitable protein by about 39%; this fractional reduction was the same for different total protein values and therefore does not affect the interpretation of the results in which protein synthesis of two cell populations is expressed as a ratio.) The same supernatant fractions tested on macrophages in the above manner were incubated at the same concentration and for the same time period with mouse L cells (Microbiological Associates Inc., Bethesda, Md.). Lymphotoxin activity in these fractions was assayed by Dr. Steven Rosenberg according to the method of Granger and Williams (29), using the same labeling medium as above.

Time-Lapse Cinemicrography.—1–2 $\times$ 10^6 macrophages were injected into each of two Sight chambers (Sight Instruments Co., Long Beach, Calif.) and cultured in MIF-rich and control fractions. The area of the glass cover slip comprising the chamber floor was 5 cm^2. 4-hr observations were recorded on 16 mm color film at approximately 2–9, 24, 48 and 72 hr of culture, at an exposure rate of 16 frames/min, using a Nikon inverted phase-contrast microscope with a DLL40 objective and a Sage time-lapse apparatus.

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 (30). Areas of migration were recorded at various times from 2 to 72 hr.

RESULTS

Effect of Unfractionated Supernatants on Cell Adherence.—More adherent cells were observed when macrophages were incubated in MIF-rich than in control supernatant for 24 hr. In 10 experiments, the adherent cell protein in the MIF-rich supernatant averaged 177% of that in the control supernatant (Fig. 1 *a*). Statistically, this difference was highly significant ($P < .0005$, Student's t test for paired means). In order to test the effect of antigen, the following media were tested in four experiments in addition to the supernatants (Fig. 1 *b*): MEM-S alone, MEM-S with antigen, and control supernatant with antigen added after the removal of the lymphocytes. When OCB-BGG was added to MEM-S in an amount equal to that in the stimulated supernatant, the adherent protein was significantly greater than that in MEM-S alone ($P < .0025$). Antigen also increased adherence in the control supernatant above that of control supernatant alone ($P < .0025$). However, the adherent cell

protein in the MIF-rich supernatant was still significantly greater than in control supernatant plus antigen ($P < .025$). In a single experiment, no effect on cell adherence was observed after only 2½ hr incubation in unfractionated supernatant.

Thus, the unfractionated MIF-rich supernatant promoted macrophage adherence after 24 hr in culture, but antigen alone, and possibly antigen–antibody complexes, contributed to this effect. For this reason subsequent experiments

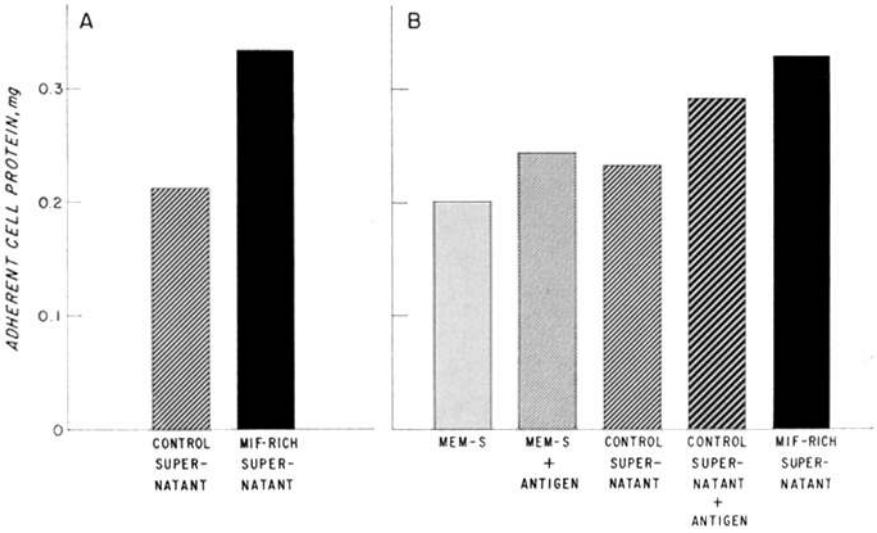

FIG. 1. Effect of unfractionated supernatants on macrophage adherence after 24 hr of incubation. (*A*) Mean adherent protein values from 10 experiments. Values in each experiment were obtained from triplicate monolayers for each determination. (*B*) Mean adherent protein values from four experiments, showing effect of antigen. For statistical significance, see text.

were performed with Sephadex fractions rich in MIF but free of antigen and antibody.

Effect of MIF-Rich Fractions on Macrophage Adherence.—More macrophages adhered to the culture vessel when incubated in MIF-rich fractions for 3 days than in control fractions. This effect was observed in 29 of 30 experiments, using material from nine separate fractionations. Results from the 30 experiments are summarized in Table I.

No difference in adherence was detected at 5 hr of culture (3 experiments) or 24 hr (18 experiments); in one of 3 experiments at 48 hr of culture, increased adherence in the MIF-rich fraction was observed compared to the control. Peritoneal exudates which contained approximately 50% polymorphonuclear leukocytes adhered poorly in all three media after 3 days and were not used.

Studies on Phagocytosis.—The rate of phagocytosis of heat-killed mycobacteria and of starch granules by macrophage monolayers in MEM-S was dependent on the dose of particles, as shown in Fig. 2. With starch granules, the rate and extent of phagocytosis in MEM-S were much greater than with

TABLE I

*Adherent Macrophage Protein after 3 Days' Incubation**

Medium	μg protein‡
MIF-rich fraction	191 ± 14
Control fraction	97 ± 9
Culture medium	56 ± 6

* Data from 30 experiments (triplicate monolayers per determination).

‡ Starting amount adherent protein 434 ± 19 (SE).

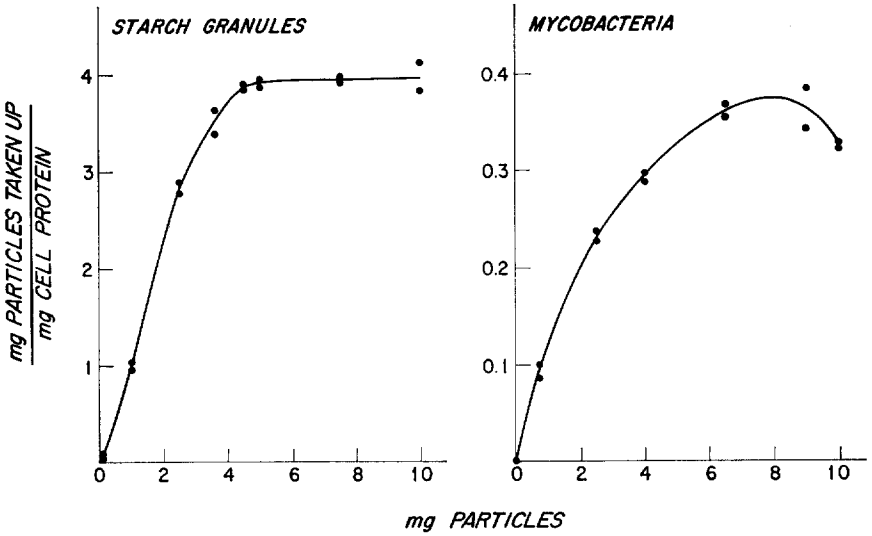

FIG. 2. Effect of particle dose on rate of phagocytosis by macrophage monolayers in MEM-S. Phagocytosis was measured 8 min after the addition of starch granules or 20 min after the addition of mycobacteria.

mycobacteria (Fig. 3). Experiments were performed to learn if MIF-rich media might affect the phagocytosis of either particle.

Macrophage monolayers incubated for 3 days in MIF-rich fractions phagocytized mycobacteria more than three times faster than monolayers in control fractions and nearly nine times faster than those in MEM-S (Table II). To a large extent these differences reflected the greater number of adherent cells in MIF-rich fractions. When the rates of uptake were corrected for the amount

of adherent macrophage protein, uptake in MIF-rich fractions was 145% of that in control fractions and 206% of that in MEM-S. In an additional experiment measuring extent of phagocytosis of mycobacteria, the stimulation in the MIF-rich fraction was even more pronounced (Table III).

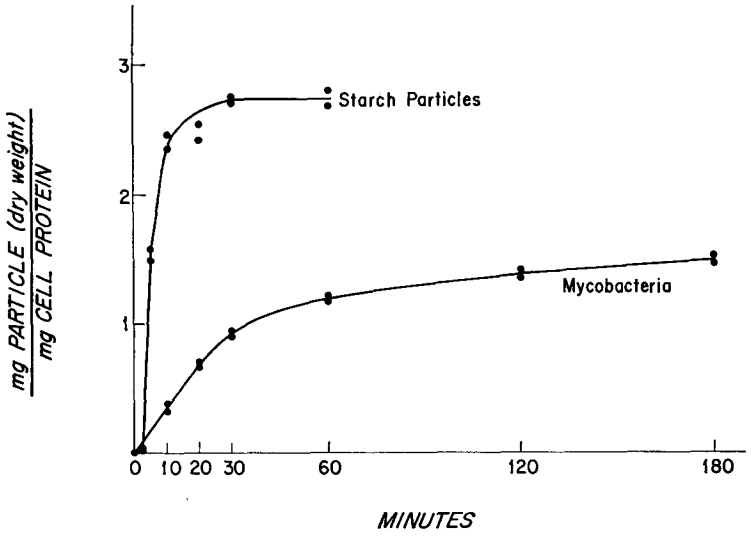

FIG. 3. Time course of phagocytosis by macrophage monolayers in MEM-S after the addition of 5.0 mg of starch granules or 2.5 mg of mycobacteria. There was a 2-min lag before starch uptake commenced. With maximal uptake 26% of the added starch granules and 19% of the added mycobacteria were phagocytized.

TABLE II

*Rate of Phagocytosis after 3 Days' Incubation**

Medium	Particle uptake			
	μg/monolayer/min		μg/mg cell protein/min	
	Mycobacteria	Starch	Mycobacteria	Starch
MIF-rich fr.	22.4 ± 5.1 (4)	82.3 ± 16.7 (6)	67.8 ± 14.4 (4)	347 ± 40.0 (6)
Control fr.	6.8 ± 2.8 (4)	36.4 ± 8.2 (6)	46.6 ± 10.8 (4)	285 ± 27.8 (6)
MEM-S	2.6 ± 1.6 (4)	29.2 ± 8.1 (6)	32.9 ± 9.1 (4)	308 ± 41.6 (6)

* Phagocytosis measured 20 min after addition of mycobacteria (6.5–7.0 mg) or 7 min after addition of starch (5.0 mg). The standard error of the mean is shown, with the number of experiments in parentheses. The mean value from triplicate monolayers was used for each experiment.

The rate of phagocytosis of starch granules was also stimulated in MIF-rich fractions after 3 days of incubation, although to a lesser extent than with mycobacteria. Whole monolayers in MIF-rich fractions took up starch 226% as fast as in control fractions and 282% as fast as in MEM-S. When corrected for the amount of adherent cell protein, the uptake in MIF-rich fractions was

122% as fast as in control fractions, a statistically significant difference ($P < 0.025$, t test for paired means). An additional experiment measuring extent of starch phagocytosis after 3 days of culture gave similar results.

No stimulation of rate or extent of phagocytosis of starch (four experiments) or mycobacteria (six experiments) was evident after only 24 hr of incubation in MIF-rich and control fractions. Furthermore, no stimulation of phagocytosis of starch was observed when macrophages were incubated in unfractionated supernatants for 24 hr, the period at which increased cell adherence in these media was evident.

If a significant proportion of macrophages, engorged with particles, should detach from the dish and no longer be counted in the monolayer, an artifact would be introduced into the above results. This was excluded by the finding with both types of particles that the adherent cell protein in monolayers engaged in phagocytosis was consistently slightly greater than that in matched monolayers to which no particles were added, under the time conditions employed.

TABLE III

*Extent of Phagocytosis after 3 Days' Incubation**

Medium	Particle uptake			
	μg/monolayer		μg/mg cell protein	
	Mycobacteria	Starch	Mycobacteria	Starch
MIF-rich fr.	253.0	1042	1901	5320
Control fr.	13.9	261	635	4830
MEM-S	9.6	242	509	4650

* Phagocytosis measured 2 hr after addition of mycobacteria (6.7 mg) or 30 min after addition of starch (5.0 mg). Values are means from triplicate monolayers.

Glucose Oxidation.—Because the act of phagocytosis (31) and the administration of certain membrane-active agents (32) stimulate glucose oxidation by fresh mononuclear cells, it was of interest to determine whether MIF-rich media might affect this aspect of macrophage metabolism. Glucose carbon-1 oxidation by macrophage monolayers was directly proportional to the amount of adherent cell protein, over the range of protein values customarily found after 3 days of incubation (Fig. 4).

Table IV summarizes the results of the seven experiments performed after 3 days of culture. It can be seen that glucose carbon-1 oxidation was dramatically stimulated in the MIF-rich fraction compared to control in every experiment. On the average, the rate of glucose carbon-1 oxidation by macrophages in MIF-rich fractions, corrected for the amount of adherent cell protein, was 378% of that in control fractions and 608% of that in MEM-S. The absolute

values of glucose carbon-1 oxidation varied somewhat from experiment to experiment.

In three experiments, oxidation of glucose carbon-1 to CO_2 was studied after 0, 5, 24, 48, and 72 hr of incubation to learn how early it might be stimulated. The results clearly showed that glucose oxidation was stimulated in MIF-rich

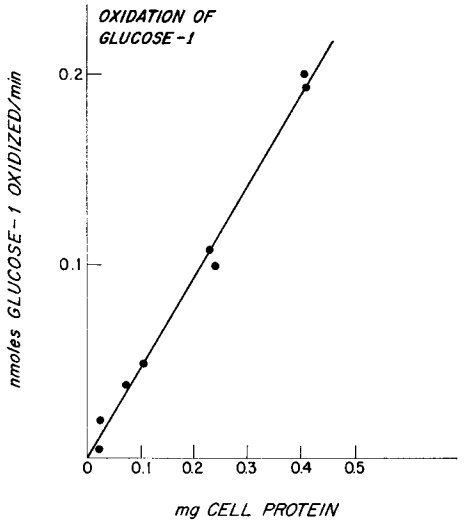

FIG. 4. Dependence of glucose-1-^{14}C oxidation upon amount of macrophage protein in the monolayer. $^{14}CO_2$ was collected after 20-min incubation of macrophages with labeled glucose. Macrophages were preincubated in MEM-S for 1½ hr.

TABLE IV

Glucose Carbon-1 Oxidation after 3 Days' Incubation

Exp.	Fraction*	Conc.*	Mean cpm*			Mean nanomoles glucose carbon-1 oxidized/mg cell protein/min*			Per cent glucose carbon-1 oxidation/mg/min‡	
			MIF-rich fractions	Control fractions	MEM-S	MIF-rich fractions	Control fractions	MEM-S	MIF-rich/control	MIF-rich/MEM-S
76	G27, Fr. III	6.4×	6,750	1189	676	1.87	0.65	0.50	289%	378%
79	G26, Fr. III	10.5×	24,664	1555	375	9.04	1.17	0.68	774%	1331%
91	Gcn, Fr. IV	2.5×	1,584	260	162	0.45	0.21	0.15	211%	293%
92	Gcn, Fr. IV	4.0×	1,617	681	184	1.44	0.62	0.41	234%	357%
93	G34, Fr. IV	5.0×	3,271	817	163	1.24	0.48	0.26	257%	472%
95	G34, Fr. IV	5.0×	991	301	261	2.65	1.16	0.70	229%	379%
100	G35, Fr. IV	3.5×	1,194	425	158	0.95	0.44	0.20	213%	485%
Mean ± SE			5,724	747	283	2.52 ±1.12	0.68 ±0.14	0.41 ±0.08	315% ±77%	527% ±136%

* Each datum is the mean from triplicate macrophage monolayers.

‡ The data for MIF-rich fractions are given as a percentage of the values for control fractions or MEM-S in each experiment.

fractions compared to the controls after 3 days of incubation but not at earlier periods (see Fig. 5). Moreover, the rates of oxidation of glucose-1-^{14}C to $^{14}CO_2$ in MIF-rich fractions on the 3rd day represented an absolute increase above the rates in the same media at earlier periods and not merely a decrease occurring

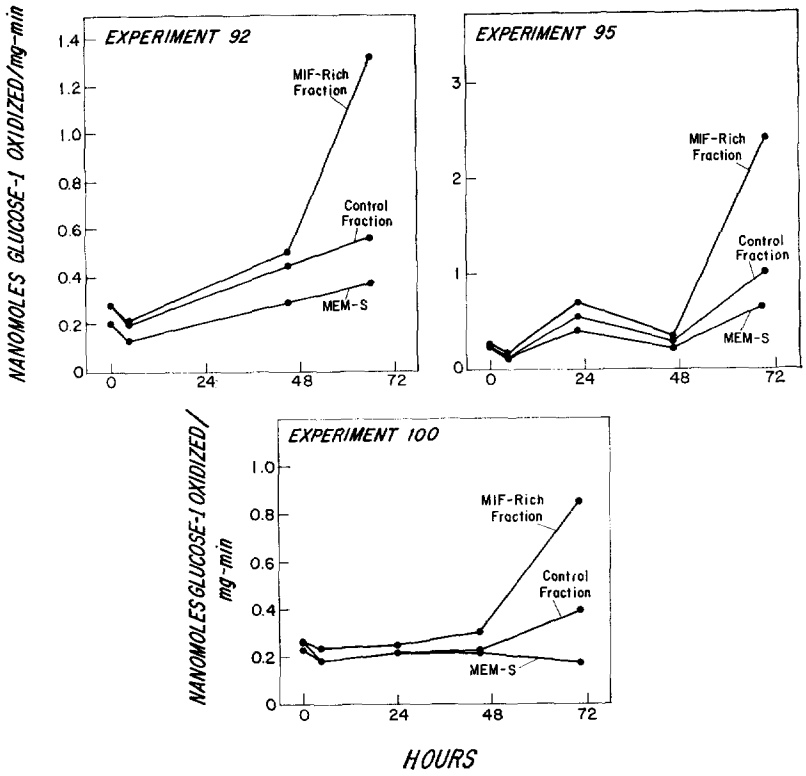

FIG. 5. Oxidation of glucose-1-^{14}C to $^{14}CO_2$ after 20 min incubation with macrophage monolayers in the presence of supernatant fractions or buffer alone (0 hr) or after preincubation in fractions or MEM-S for approximately 5, 24, 48, or 72 hr. Each point represents the mean of values from triplicate monolayers. Each experiment used fraction IV from a different Sephadex G-100 fractionation.

to a lesser extent than in controls. The baseline rate of glucose oxidation in MEM-S over the 3-day period corresponds closely to that measured in untreated mononuclear cells by a different method (22).

Oxidation of glucose carbon-1 by guinea pig mononuclear cells is increased 9-fold during phagocytosis (22). Hence it was important to determine whether significant amounts of phagocytizable matter might be formed during 3 days of culture in the MIF-rich fractions. To test this possibility, media from 3-day

old cultures which themselves demonstrated the typical effect were aspirated and placed directly on newly plated monolayers for 1 hr. In two experiments, fresh monolayers receiving MIF-rich medium, control medium, or MEM-S from 3-day old cultures all exhibited the same unstimulated rate of glucose-1 oxidation.

Next, CO_2 production after 3 days in supernatant fractions was compared using glucose labeled either on the first or the sixth carbon to determine how much of the observed glucose oxidation represented metabolism through the direct oxidative pathway (hexose monophosphate shunt, or HMPS). The amount of oxidation of glucose carbon-6 was quite small, so that nearly all the observed oxidation of glucose-1-^{14}C could be attributed to HMPS. $^{14}CO_2$ production from glucose-6-^{14}C was not significantly greater than in cell-free dishes in one experiment, despite the greater specific activity of glucose-6-^{14}C used (see Materials and Methods); in the second it amounted to 5.5% of the oxidation of glucose carbon-1, which corresponds to value obtained for resting (5.0%) and phagocytizing cells (4.4%) by another method (22). Moreover, in the latter experiment the rates of oxidation of the first and sixth carbons were increased in the MIF-rich fraction by almost the same proportion, as previously observed during phagocytosis (22). Subtracting the rate of oxidation of glucose carbon-6 (assumed to be equal in magnitude to the oxidation of glucose carbon-1 by all paths other than HMPS) from the rate of oxidation of glucose carbon-1, the HMPS in the cells incubated in the MIF-rich fractions in the two experiments was calculated to be 211% and 256% of that in control fractions.

Protein Synthesis.—The incorporation of ^{14}C-labeled amino acids into TCA-precipitable material of macrophages was measured after 3 days of incubation in supernatant fractions in six experiments (Fig. 6). The amount of radioactivity incorporated into the macrophage monolayers was more than twice as great after incubation in the MIF-rich fraction as in the control fractions.

When the radioactivity of the washed macrophage monolayers was corrected for the total amount of adherent protein on each dish, macrophage protein synthesis per milligram of adherent cell protein in the MIF-rich fractions was not significantly different from that in the control fractions. Thus the increased incorporation of amino acids by the monolayers in the MIF-rich fractions merely reflected a greater amount of adherent cellular material.

It has been reported that mouse lymphotoxin cytolyzes mouse peritoneal macrophages (33). The MIF-rich fractions employed in this study contained lymphotoxin when assayed on mouse L cells.[5] To contrast the effects of these media on guinea pig macrophages and on L cells, four of the supernatant fractions tested on macrophages as described above were also incubated at the same concentration and for the same time period with L cells (Fig. 6). The radio-

[5] S. Rosenberg, M. Henrichon, H. Remold, and J. R. David. Unpublished observations.

activity incorporated into the whole L cell culture in the MIF-rich fractions averaged one-third of that in the control fractions. This reflected killing of L cells by lymphotoxin in the MIF-rich fractions. As usual, inhibition of migration was demonstrated with the MIF-rich fractions when assayed at the same concentrations on samples of the same macrophage preparations.

Thus, at the concentrations tested, supernatant fractions with demonstrated MIF activity killed L cells (exhibited lymphotoxin activity) but promoted

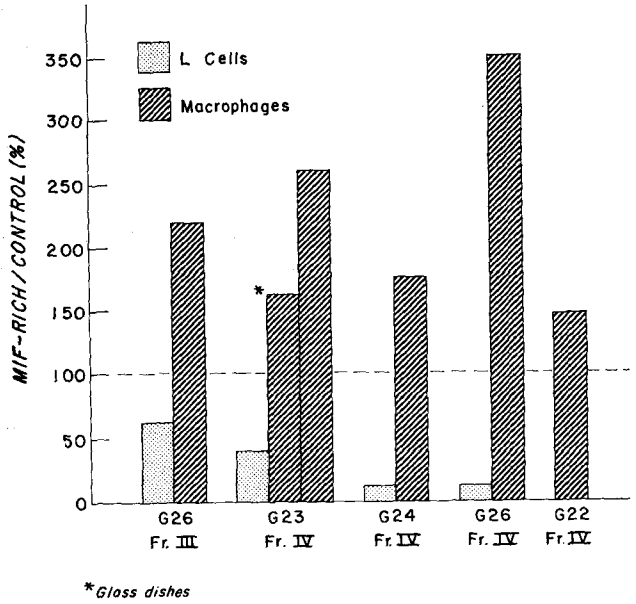

FIG. 6. Protein synthesis by whole macrophage monolayers or L cell cultures incubated in the same supernatant fractions at the same concentrations for 3 days. Results are expressed as ratio of incorporation of ^{14}C-labeled amino acids into protein in MIF-rich fractions to incorporation in control fractions. Values above 100% represent increased macrophage adherence in MIF-rich fractions; below 100%, death of L cells in the same fractions.

macrophage adherence. No consistent effect on the rate of protein synthesis per milligram of adherent macrophage protein was demonstrated on the 3rd day of culture.

Time-Lapse Cinemicrography.—Macrophages in both the MIF-rich and control fractions were almost all spread out after 45 min in the cine chambers. Between 2 to 9 hr of culture, at a time when matched capillary chambers exhibited maximal migration inhibition in the MIF-rich fractions, there was no consistent effect on cell spreading or motility in the cine chambers. By 24 hr cells in both media tended to be much more rounded-up, again with no detectable difference between the two media. In three films between 48 and 72

hr, numerous cells in the MIF-rich fractions became markedly spread out, while most cells in the control fractions remained rounded-up. At the periphery, the spread-out cells exhibited extensive, sometimes vast areas of undulating cytoplasm devoid of visible organelles (ruffled border). Many phase-dense cytoplasmic granules were seen nearer the center. The spread cells projected long pseudopods and moved rapidly. Rounded-up cells in the control fractions exhibited small areas of ruffled border and occasionally spread out to a limited extent, only to return to the rounded-up state. This behavior indicated that they were alive. Their complement of granules seemed smaller when rendered visible during spreading. The rounded-up cells moved less rapidly.

Migration Inhibition.—With oil-induced peritoneal exudate macrophages, migration inhibition is commonly recorded after 18–24 hr of culture. With the casein-induced macrophages used in this study, however, migration inhibition in MIF-rich fractions was usually maximal at 5–10 hr and decreased thereafter. In fact, by 48 or 72 hr, the area of migration of cells in MIF-rich fractions was equal to that in control fractions. The absolute areas of migration increased in all chambers over a 3-day period, but did so at different rates. In three experiments, the mean rate of increase of area of migration, measured over the interval from 0 to 5 hr of culture, was for the MIF-rich fractions 22%, 35%, and 78% of that in control fractions. In contrast, when measured at the termination of the experiment, the mean rate of increase of area of migration in MIF-rich fractions during the preceding interval of observation was 153%, 147%, and 174% of that in control fractions. Thus, inhibition of migration reflected an early, relative decrease in the rate of migration of macrophages in MIF-rich compared to control fractions. This was followed by a relative increase in the rate of migration of macrophages in the MIF-rich fractions, resulting in eventual equalization of the areas of migration.

DISCUSSION

The results presented here indicate that certain macrophage functions can be enhanced by a soluble factor or factors elaborated by antigen-stimulated lymphocytes. The observed effects were not due to antibody (including cytophilic antibody), antigen, or antigen–antibody complexes, because these were excluded from the chromatographic fractions used. Nor could the effects be due to antigen-dependent MIF (34), as antigen was excluded.

Increased macrophage adherence, membrane ruffled border activity, phagocytosis, and direct hexose monophosphate oxidation appeared only after 3 days of incubation in MIF-rich fractions. The significance of this latent period in vitro is not yet known. However, it is of interest that a period of 2–3 days was sometimes found by Mackaness and his colleagues to be required for the development of enhanced nonspecific bactericidal capacity in mouse peritoneal macrophages. For example, macrophage bactericidal capacity was enhanced

only 2–3 days after normal mice received Calmette-Guérin bacillus (BCG) plus lymphocytes from BCG-immunized donors (5).

When whole supernatants were employed, more macrophages remained adherent after 24 hr in the MIF-rich supernatant than in the control. This finding resembles that of Mooney and Waksman (35), who incubated rabbit macrophages for 24–48 hr in whole supernatants from antigen-stimulated lymph node cells and observed more macrophage adherence and spreading than after incubation in supernatants of lymph node cell cultures from unsensitized donors. In the study cited, one of the antigens used was itself slightly stimulatory for macrophages. Antigen alone was also slightly stimulatory in the present study; this occasioned the use of antigen-free fractions.

After 3 days of culture in fractionated supernatants, twice as much macrophage protein remained adherent to the culture vessel in MIF-rich as in control fractions. After only 1 day, no relative increase in adherence was yet apparent, although whole MIF-rich supernatants gave increased adherence at this time. This discrepancy in timing suggests that cell adherence may be influenced by different substances in the two preparations. Certain substances, present in the whole supernatant but not in the fractions, are known to be biologically active in leukocyte culture systems. The promotion of adherence by antigen has been noted above. Antigen–antibody complexes increase leukocyte spreading (36), phagocytosis (37), and respiration (38), and may affect adherence as well. Such complexes were probably present in the MIF-rich supernatant (39). Addition of antigen to the control supernatant or basic culture medium does not control for effects of complexes, since there is little antibody in the control supernatant (39) and none in the basic culture medium. Several other reports (40, 41) of increased macrophage adherence or survival in culture systems containing antigen and sensitized lymphocytes are consistent with the observation reported here with whole supernatants but are equally difficult to interpret.

When MIF-rich and control supernatants are obtained with cells from the same pool of lymphocytes, chromatographed to provide fractions of an appropriate molecular weight, and reconstituted in the same media, it is possible to argue that the increased cell adherence in MIF-rich compared to control fractions is due to substance(s) of the molecular weight of MIF which are released by antigen-stimulated lymphocytes. Effects of other stimulatory substances (42, 43), if present, are automatically taken into account.

Extension of membrane-bound ruffled borders and spreading of the cell bodies of macrophages were greatly promoted by incubation in MIF-rich fractions for 3 days. These phenomena may be expressions of an increase in cell stickiness that was perhaps also manifest as increased resistance to being rinsed from the culture vessel. Mackaness observed that macrophages activated in vivo spread out more rapidly on glass than normal cells and covered a greater area when spread (6); in adoptive transfer experiments, increased macrophage

spreading was dependent on the presence, in vivo, of lymphocytes from sensitized donors (5).

Different conclusions regarding the effect of sensitized lymphocytes on macrophage spreading were reached by workers using other systems. Fauve and Dekaris (44) found inhibition of macrophage spreading; however, they used cells washed off the unstimulated peritoneum, cultured in the presence of antigen, and observed for less than an hour. Salvin and his colleagues incubated sensitized exudates on agar in which antigen was incorporated (45, 46) or incubated normal exudates on agar in which antigen-containing lymphoid supernatants were incorporated (46), and found decreased macrophage spreading and motility. Possible reactions of the macrophages to the antigen in the underlying agar make these results difficult to compare to ours.

The increased phagocytic capacity of macrophages incubated in MIF-rich fraction was due to a substance(s) whose molecular weight (25,000–55,000) distinguishes it from other materials produced by the host and known to stimulate phagocytosis when tested with polymorphonuclear leukocytes in vitro or the reticuloendothelial system in vivo. Such substances include opsonins, complement and certain of its cofactors (47), C-reactive protein (48), leucophilic globulin (49), bradykinin (50), and certain steroids (51, 52) and fatty acids (53).

The most dramatic effect of MIF-rich fractions on macrophages was the 2- to 7-fold increase in oxidation of the first carbon of glucose compared to that seen in cells incubated in control fractions. The increase was due to stimulation of the hexose monophosphate shunt. The effect was attendant upon changes taking place within the macrophages and not in the external medium, such as the formation of phagocytizable particles, because the medium from monolayers exhibiting the typical effect failed to stimulate glucose oxidation by fresh monolayers after 1 hr of incubation. Two classes of materials have previously been reported to stimulate leukocyte glucose oxidation, and both do so very rapidly upon being introduced into the leukocyte culture. These are phagocytizable particles (31), or certain membrane-active substances which may stimulate pinocytosis (54), such as endotoxin, deoxycholate, digitonin (32), and dilute anti-macrophage serum (55, 56). MIF-rich fractions exert their effect on glucose oxidation only after 3 days of incubation. The MIF-rich fractions therefore appear to act by a different mechanism from the materials listed above, one involving prolonged intracellular processes that culminate in activation of certain metabolic pathways.

Speculation as to the function of the activated HMPS in macrophages treated with MIF-rich fractions centers on two biochemical processes. Both utilize reduced nicotinamide adenine dinucleotide phosphate (NADPH), which is produced by the HMPS, and regenerate its oxidized form (NADP), the level of which appears to regulate HMPS activity (57). The first process is

synthesis of membrane lipids. In phagocytosis, where the plasma membrane is expended in the formation of phagosomes and presumably regenerated, increased incorporation of precursors into phospholipids has been demonstrated (22). Likewise, the stimulation of HMPS activity in macrophages treated with MIF-rich fractions may reflect increased turnover or even net synthesis of membrane involved in locomotion (58), spreading, and ruffled border activity.

Alternatively, increased HMPS activity in macrophages incubated in MIF-rich media might be related to enhanced microbicidal capacity. The oxidation of reduced pyridine nucleotides, possibly from the HMPS, might produce hydrogen peroxide (59–61), which has been clearly shown by Klebanoff (62) to be implicated (with a peroxidase) in the bactericidal capacity of polymorphonuclear leukocytes. It is also possible that an increase in HMPS activity might be a sequel to increased H_2O_2 production, perhaps from the oxidation of NADH (63). Recently, hydrogen peroxide-generating mechanisms, the activities of which increase during phagocytosis, have been reported in guinea pig alveolar macrophages (64) and mouse peritoneal macrophages (65). Macrophages cultured from human peripheral blood appear to possess an O_2-dependent, KCN-insensitive bactericidal mechanism, and this mechanism can be stimulated by a redox agent which enhances O_2 consumption (66). It is intriguing to speculate that stimulation of HMPS activity in macrophages in MIF-rich media may reflect activation of a peroxide-forming mechanism which is potentially microbicidal.

The effect of MIF-rich media on macrophage microbicidal capacity is a question of great import, since enhanced macrophage microbicidal activity is the hallmark of cellular immunity. A hint that a stimulatory effect may exist is provided by the recent report of Patterson and Youmans (67), who found that lymphocytes from *M. tuberculosis*-immunized mice, when cultured in vitro with the same organism, produced supernatants which inhibited the growth of this organism in normal macrophages. Unfortunately, the use of the same agent as immunogen and test organism does not elucidate whether macrophage bactericidal capacity was enhanced nonspecifically, as in cellular immunity. Moreover, antibiotics were present during the assay for bactericidal capacity. Studies are now being pursued concerning the bactericidal capacity of macrophages treated with MIF-rich media in the absence of antigen, antibody, and antibiotics.

In conclusion, the present findings demonstrate that alterations in macrophage function and metabolism can be brought about by a factor released from antigenically stimulated lymphocytes. These results support the hypothesis that cellular immunity and cellular hypersensitivity are mediated by common mechanisms. The chemical nature of the macrophage activating factor and its relationship to MIF and other mediators remain to be determined.

SUMMARY

Sensitized lymphocytes were incubated in vitro with the specific antigen Supernatants from these cultures were chromatographed on Sephadex G-100 columns. Supernatant fractions containing MIF, chemotactic factor, and lymphotoxin, but free of antigen and antibody, were incubated with normal peritoneal exudate macrophages. Macrophage adherence, phagocytosis, spreading, motility, and direct hexose monophosphate oxidation were enhanced, while protein synthesis was unaffected. Thus, antigen-stimulated lymphocytes secrete a factor or factors which enhance certain macrophage functions. Implications for models of cellular immunity and cellular hypersensitivity are discussed.

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