ALTERATION OF SOME FUNCTIONAL AND METABOLIC CHARACTERISTICS OF RESIDENT MOUSE PERITONEAL MACROPHAGES BY LYMPHOCYTE MEDIATORS*

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Several decades ago correlation between host resistance to certain types of infections and alteration of functions of macrophages (activation of macrophages) was established (1–4). The process by which macrophages become activated involves interaction of specifically sensitized T lymphocytes with the appropriate antigen (5, 6). Activated macrophages exhibit their enhanced microbicidal and tumoricidal actions nonspecifically (7–9).

It has been shown that stimulated lymphocytes produce several mediators that affect macrophages in vitro. The production of one of these mediators, i.e., migration inhibitory factor (MIF)[1] has been correlated with delayed hypersensitivity (10, 11). The question whether MIF itself mediates the activation of macrophages has still not been definitively proven. This stems partly from the fact that MIF, as a molecular entity, has not yet been obtained, and the various criteria to define a fully activated macrophage have not yet been established.

Activated macrophages from animals infected with various intracellular parasites show, in addition to the functional changes mentioned above, a number of metabolic and morphologic changes (12–14). Such changes have also been observed in macrophages obtained by elicitation with a number of agents, e.g., caseinate, thioglycollate, and endotoxin (13–16). However, these elicited cells do not show such enhanced functions as killing of microorganisms or tumor cells and, therefore, we will refer to them in this paper as "elicited" cells.

In a previous report, Nathan et al. (17, 18) established that when guinea pig macrophages are incubated with fractions containing MIF, an enhancement is observed in cell maintenance, phagocytosis, spreading, motility, and oxidation of glucose-carbon-1 to CO_2—presumably via the hexose monophosphate shunt pathway. Furthermore, such cells had an increased bacteriostatic activity against *Listeria* (19) and enhanced anti-tumor cytotoxicity (20).

Experiments with normal mouse peritoneal macrophages and murine lymphokines were clearly needed to augment data from the guinea pig system, not only because the latter system must employ elicited cells (which are stimulated), but also because of the recent availability of murine MIF in appreciably large amounts, and in a partially purified form (21). In addition, there is a growing list of biochemical criteria for classifying mouse macrophages as activated, elicited, or normal (12–15).

The above-mentioned studies thus offer us the methodology to explore the effect of partially purified lymphocyte supernates on normal mouse macrophages. In the present report we shall employ resident peritoneal mouse macrophages obtained by

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[1] *Abbreviations used in this paper*: Con A, concanavalin A; KRP, Krebs-Ringer phosphate; MEM, Eagle's minimal essential medium; MIF, migration inhibitory factor; P, penicillin; S, heat-inactivated fetal calf serum; St, streptomycin.

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simple lavage from normal mice, and describe the conditions under which these cells in culture reach a state that can be compared to that of the *Listeria*-activated cell, at least by certain biochemical criteria. We also suggest the possibility of a dissociation between MIF activity and the factor(s) that cause certain metabolic changes.

Materials and Methods

Animals. Male CD-1 (Charles River Breeding Laboratories, Wilmington, Mass.), and C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Maine) weighing between 18 and 25 g were used.

Reagents. Adenosine monophosphate triethyl ammonium salt-^{32}P, and D-glucose-[1-^{14}C] were purchased from New England Nuclear, Boston, Mass. Bovine serum albumin (essentially fatty acid-free), adenosine-5′-monophosphoric acid, *p*-nitrophenyl phosphate, disodium salt (Sigma 104 phosphatase substrate), and concanavalin A (Con A) were all purchased from Sigma Chemical Co., St. Louis, Mo.

Harvesting of Macrophages. Normal mice were sacrificed by cervical dislocation, and injected i.p. with 10 ml of Krebs-Ringer phosphate solution (KRP). The cell suspensions were aspirated, pooled, and washed three times in KRP. The cells were resuspended in culture medium which consisted of Eagle's minimal essential medium (MEM) containing 100 U/ml of penicillin (P), 100 μg/ml streptomycin (St), 1% of 200 mM glutamine solution, and 15% heat-inactivated fetal calf serum (S). (All of these were purchased from Microbiological Associates, Walkersville, Md.). The suspensions were adjusted to contain 1×10^7 cells/ml. The cells present were >95% mononuclear cells. When elicited cells were employed, the mice were injected intraperitoneally with 2.5 ml of sterile (1.2%) sodium caseinate (Difco Laboratories, Detroit, Mich.) in isotonic saline. 4 days later, the animals were sacrificed and the procedure was carried out as described. Manipulations were performed on ice.

Preparation of Macrophage Monolayers. Sterile technique was used throughout. 1 ml of the cell suspension was dispensed to each plastic Petri dish (35 × 10 mm, model 3001; Falcon Plastics, Div. BioQuest, Oxnard, Calif.). The dishes were incubated at 37°C under 5% CO_2 in air for 2 h, after which each dish was emptied, and rinsed vigorously in four successive beakers containing sterile isotonic saline solution. They were drained and replenished with 2 ml of culture medium or MIF-containing and control fractions from the Sephadex G-100 chromatography that were reconstituted to a given concentration in culture medium and filtered (HAWP-013-00, 0.45 μ; Millipore Corp., Bedford, Mass.).

The dishes were incubated at 37°C under 5% CO_2 in air for up to 72 h (16). Dishes incubated longer than 24 h received, at 24 and 48 h, 0.1 ml of a supplemental solution containing fetal calf serum (10%), MEM, glucose, essential amino acids, and glutamine (22).

Production and Sephadex G-100 Fractionation of Murine MIF. C57BL/6 mouse murine MIF was produced as previously described (21). Briefly, spleen cells were prepared and resuspended in culture medium to 1–2.4 × 10^7 cells/ml. The suspension was divided into two equal parts and Con A (2 μg/ml) was added to one part. The other half served as control. The cells were incubated for 48 h at 37°C in an atmosphere of 10% CO_2, 7% O_2, and 83% N_2. The suspension was centrifuged at 18,000 *g* for 20 min at 4°C, and the supernates were collected. Con A was added to the control supernate. Preparations were then dialyzed against 0.15 M NaCl and then against distilled water, and lyophilized. The equivalent of 100–350 ml of starting material was redissolved in 3.0 ml of phosphate buffer 0.1 M, pH 7.4, centrifuged at 100 *g* for 5 min, and applied to 2.5 × 100-cm columns containing Sephadex G-100. The columns were calibrated with appropriate markers. The effluent material was divided into fractions as previously described (21). The fractions were concentrated (by vacuum dialysis) 100-fold in relation to the original supernatant volume, and stored at −20°C.

Murine MIF Assay. .The Sephadex fractions were tested for activity using a capillary tube MIF assay (23) with modifications made for use in the mouse system as previously described (21). Briefly, peritoneal exudate cells from C57BL/6 normal mice were induced by injection of 3 ml of Marcol oil (Humble Oil & Refining Co., Houston, Tex.) i.p. 4 days before collection. The exudates were harvested, and after washing three times in Hanks' balanced salt solution, the cells were resuspended in MEM-P-St containing 15% heat-inactivated guinea pig serum.

Drummond capillary tubes (20 μl; Drummond Scientific Co., Broomall, Pa.) were filled with the cell suspension, sealed, and centrifuged at 250 *g* for 5 min. The capillaries were cut and placed in Mackaness-type chambers. The chambers were filled with the fractions to be tested, sealed, and incubated for 12–24 h at 37°C, and the percent inhibition of migration was calculated using the following formula:

$$\% \text{ inhibition} = \left(1.0 - \frac{\text{average area of migration in Con A fraction}}{\text{average area of migration in control fraction}}\right) \times 100,$$

in which the average area of migration was determined from six capillaries, and 20% inhibition (with $P < 0.05$) was considered significant inhibitior of migration.

Assay of 5′-Nucleotidase. The AMPase assay was based on the observation by Crane and Lipmann (24) that charcoal adsorbs adenosine phosphates but not inorganic phosphate. The procedure used was similar to the one reported by DePierre and Karnovsky (25). The standard assay mixture contained 1 mM ^{32}P-AMP in KRP and 1 mM *p*-nitrophenylphosphate to eliminate appearance of ^{32}P due to nonspecific phosphatases. The monolayer was incubated with the mixture for 30 min at 37°C. At the end of this period, the supernatant fluid was removed with a Pasteur pipette and the monolayer was washed with 1 ml of KRP, which was added to the first supernate and kept at 0°C. These were mixed and treated with Norit-trichloroacetic acid (2 ml; 10% Norit in 10% trichloroacetic acid). The charcoal was removed by filtration through Whatman no. 1 filter paper (Whatman Inc., Clifton, N. J.). ^{32}P in an aliquot of the filtrate was then determined. A control without cells was routinely performed.

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. (26), essentially as it was performed by Nathan et al. (17).

Measurement of Cellular Maintenance. At the conclusion of each experiment, the dishes were washed three times and left to air-dry. The contents were dissolved overnight in 2.0 ml of 0.5 N NaOH, and an aliquot was removed and proteins were determined according to Lowry et al. (27). The standard was bovine serum albumin, essentially fatty acid-free. Microscopic examination confirmed that protein measurements correlated with numbers of adherent cells. We shall refer to these observations under the heading of "cell retention" or "cell maintenance," indicating continued cell adherence to dishes.

Results

Effect of Fractionated Lymphocyte Supernates on Normal Mouse Macrophages in Vitro. Several supernates from Con A-stimulated and control spleen cell cultures were chromatographed on Sephadex G-100. The eluted fluid was divided into several fractions and assayed for MIF as described previously (21). The distributions of the MIF activity in the different fractions for all the supernatant fractions used in these experiments are shown in Table I.

MIF-rich fractions containing peak MIF activity (fractions III and IV), and control fractions, were pooled and reconstituted in complete culture media. Macrophage monolayers were exposed to these fractions for varying periods. The effects on the oxidation of glucose 1-^{14}C are shown in Fig. 1. It can be seen that this function was stimulated two- or threefold in the MIF-rich fractions compared to the control fractions. It also can be seen that the stimulation is present as early as 24 h.

When maintenance of the cell monolayer was studied, more adherent cells were observed after macrophages had been incubated in MIF-rich than in control fractions. The effect was significant in that in eight experiments with 5 × concentrated preparations monitored at 48 h, the increment above control values (i.e., incubation in supernatant fractions from unstimulated lymphocytes) was 63 ± 4%.

When 5′-nucleotidase activity was followed up to 72 h, (Table II) it was observed that there was a normal increase of the enzyme when the cells were cultured, as

TABLE I

MIF Activity of Four Sephadex G-100 Fractionated Spleen Cell Supernatant Fluids

Fraction	Concentration		
	5 ×	2.5 ×	1.25 ×
I	0	0	0
II	20 ± 5	7 ± 10	0
III	31 ± 5	36 ± 11	46 ± 2
IV	27 ± 3	39 ± 4	54 ± 2
V	16 ± 8	15 ± 14	0
VI	5 ± 8	4 ± 5	0
VII	0	0	0
VIII	0	0	0

Dilutions of the preparations were examined to establish the fraction with the peak activity. Concentrations are expressed in nominal terms compared with the original filtrate. Means for the results from the four preparations, and average deviations from the means are given in each case. The values for each fraction are expressed as percent of total recovered activity.

previously described (15, 16). When MIF-rich fractions were present in the culture medium, a decrease in the rate of appearance of the enzyme was observed, based on specific activity with respect to cell protein. One experiment is given in Table II. The data for all three experiments were statistically examined in two ways. First, each zero time value was set at 100 and later values were normalized. The ratios of values obtained in the presence of lymphocyte supernates to those with MEM-S were calculated, i.e. B/A[2] and C/A at each time period (Table II). At 24, 48, and 72 h, the means and standard errors of these ratios were 99 ± 8, 89 ± 9; 91 ± 4, 70 ± 6; 101 ± 12, and 69 ± 1, respectively. At 48 h, the ratios for C/A versus B/A were different at a P value <0.05; if all data at 48 h and later are considered, P is <0.01. Secondly, when the titers of 5′-nucleotidase were plotted for all experiments compared to values with MEM-S, it was clear, with confidence limits of 90–95%, that the slope for the line for change with time of C was about one-third that for B, i.e., the rate of acquisition of the enzyme was diminished by the presence of the MIF-rich lymphocyte supernatant fraction. The use of ratios with values for MEM-S as the denominator took account of variability between the different batches of test macrophages, and sharpened the focus on the differences that resulted from incubation of macrophages in control or MIF-rich fractions.

Correlation of MIF Activity With Other Effects of Fractions on Macrophages. When the supernates were analyzed for MIF activity (Table I), the peak was generally found in the fraction that eluted with the 67,000 dalton marker (21). When the effects on glucose carbon-1 oxidation and 5′-nucleotidase were determined for all fractions of one preparation, it was found that the most significant activity was present in fraction III, whereas cell retention was enhanced by fractions II, III, and IV, as can be seen in Fig. 2.

Concentration Dependence. Since MIF activity shows a concentration dependence, it

[2] "A" are results for MEM-S, "B" are those for control lymphocyte supernatant fractions, and "C" are those for MIF-rich fractions.

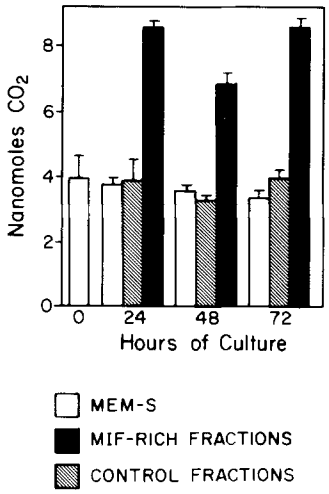

FIG. 1. Effects of MIF-rich fractions on glucose carbon-1 oxidation of normal mouse peritoneal macrophages. Preparations from three different spleen cell culture supernates were studied; the data from one representative experiment are presented here. Fractions III and IV (Sephadex G-100 chromatography) were pooled and prepared at a final concentration of 5 × that of the original supernate. The results are presented as nmol of CO_2 produced/mg cell protein/h. Each point represents the mean value from triplicate monolayers. The vertical dispersion bars represent 1 SD. The data from all three experiments were examined statistically. 0 time values were set at 100%, and percentage data for 24, 48, and 72 h could be combined as follows: MEM-S, 98.1 ± 7.7%; control-fractions, 93.8 ± 7.5%; MIF-rich fractions, 255.6 ± 26.6%. (Means ± SE; n = 8).

was relevant to establish if the effect on glucose carbon-1 oxidation, AMPase, and cell adherence showed the same dependence. The results are shown in Fig. 3. It can be observed that there was a significant concentration-dependent loss of cells from the monolayers when they were incubated in the presence of fractions from normal spleen cell supernates. This loss was not observed when the cells were incubated with the MIF-rich fractions. In fact, when the higher concentrations of the MIF-rich fraction were used, a significant increase of retained cells was observed. A clear concentration dependence was observed when glucose-1-^{14}C oxidation or ecto-AMPase activity were studied.

Effects of MIF-Rich Fractions on Different Mouse Peritoneal Macrophage Preparations. To establish if any requirement exists regarding the type of peritoneal macrophage which responds, the MIF-rich and normal control fractions were applied to macrophages obtained in three different ways. First, resident macrophages were harvested. Second, cells were elicited with sodium caseinate. Third, cells were obtained by lavage of the peritoneal cavity of mice that had received, 18 h earlier, an i.p. injection of a fraction containing a lymphocyte-derived chemotactic factor. The latter had a mol wt of 37,000 daltons and was isolated by Sephadex G-100 chromatography of supernates of Con A-stimulated lymphocyte cultures. This procedure yielded at least twice the amount of cells compared to that obtained with control fractions.[3] By Wright and peroxidase staining, these cells were indistinguishable from peritoneal macrophages obtained by lavage, i.e., they were peroxidase-negative. They showed glucose 1-^{14}C

[3] We are greatly indebted to Dr. M. Meltzer for assays of chemotactic factor(s) and for information concerning this method of cell harvesting.

TABLE II

Effect of MIF-Rich Fractions from Spleen Cell Culture Supernates on 5'-Nucleotidase of Normal Peritoneal Mouse Macrophages

Culture	Hours of culture			
	0	24	48	72
A MEM-S	2282 ± 52	2859 ± 50	4304 ± 60	5506 ± 136
B Control fractions		2619 ± 105	4183 ± 121	6226 ± 141
C MIF-rich fractions		2179 ± 110	3245 ± 122	3730 ± 170

Three experiments were performed, each in triplicate. A representative experiment is given; a statistical treatment of all experiments is found in the text. Fractions III and IV pooled and adjusted to a final concentration of 5 × the original supernate were used. The 5'-nucleotidase activity is expressed as nmol of AMP hydrolyzed/mg of cell protein/30 min. Each number is the mean of triplicate monolayers, ± SD.

oxidation levels similar to those of the latter cells. The 5'-nucleotidase levels were about two-thirds of those of cells obtained by simple lavage.

The results shown in Table III indicate that the resident macrophage is the cell type that undergoes the most dramatic changes when exposed to fractions from stimulated lymphocyte supernates. No effect of the MIF-rich fractions was seen on the low 5'-nucleotidase level of caseinate-elicited cells, and the effect on the cells elicited with chemotactic factor was not dramatic, perhaps because these cells have such high levels of enzyme to start with. The effect on glucose-1-^{14}C conversion to $^{14}CO_2$, a doubling, at least, in cultured resident macrophages, and chemotactic factor-elicited macrophages, was at best very slight in casein-elicited cells. On the other hand, an effect on cell retention was observed with all three types of cultured peritoneal macrophages, when MIF-rich fractions are compared to normal fractions.

To determine whether the fractions which may stimulate caseinate-elicited cells were different from those that stimulate resident macrophages, the full spectrum of Sephadex G-100 fractions was analyzed on both these cell types. The results presented in Table IV again indicate that with regard to glucose-1-^{14}C conversion to $^{14}CO_2$, the resident cell is more susceptible to the MIF-rich fractions from stimulated lymphocytes. The effect on cell retention with the caseinate-elicited macrophages showed that they responded to all of the fractions II-VI in the preparation used. There was some indication of a peak effect with fraction IV. However, when resident cells were examined for this function, a peak effect was observed with fraction IV. It appears that the elicited cells were unresponsive with respect to glucose oxidation, but more sensitive than resident cells in the second context (cell retention) to small amounts of factor spread throughout the fractions. It should be noted that the particular batch of MIF-rich supernate used in this experiment showed MIF activity in all fractions at 5 × concentration, and even when diluted to 1/16 of the concentration of original supernate, it still exhibited activity in fraction IV.

Effect of Storage on MIF-Rich Fractions. After several months of storage at −20°C, the fractionated supernates were re-examined with respect to their effect on the stimulation of glucose carbon-1 oxidation. This effect was found to have been lost, whereas the effect on cell retention was maintained. Table V presents these data for two preparations. MIF activity was shown to be present and was as potent within the limits of measurement as had been observed 7 mo earlier.

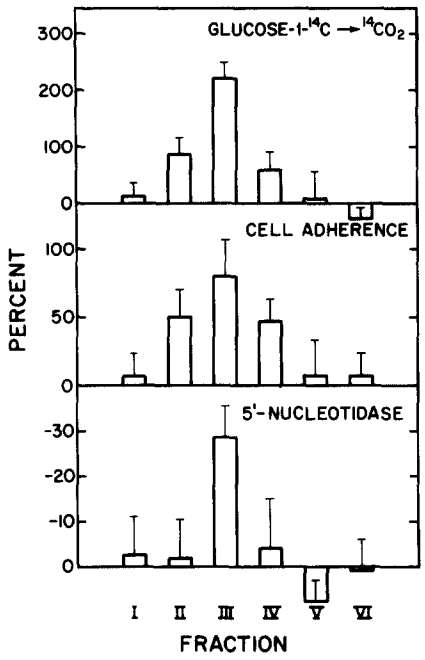

FIG. 2. Effect of fractions from Sephadex G-100 chromatography of spleen cell supernates on normal mouse peritoneal macrophages. Glucose carbon-1 oxidation, 5′-nucleotidase, and cell retention were studied. Fractions from a single supernatant preparation at 2.5 × concentration were used. The data are based on monolayers incubated with the fractions for 48 h. The bars refer to percent increase or decrease with respect to control levels set to 100. The vertical dispersion bars are standard errors.

Discussion

The results presented establish the effect of MIF-rich fractions on some metabolic aspects of mouse peritoneal macrophages, i.e. oxidation of glucose carbon-1 to CO_2, and 5′-nucleotidase activity, and on cell retention.

Nathan et al. (17, 18), using elicited peritoneal macrophages from guinea pigs incubated in the presence of guinea pig MIF-rich lymphocyte supernates or Sephadex G-100 fractions, reported a significant stimulation of glucose oxidation after 3 days of incubation, but not at earlier periods. We found a similar increase when the murine system was used; however, we were able to observe the effect (increased $^{14}CO_2$ production from glucose-1-^{14}C) by 24 h of culture. The levels reached at this time (two- to threefold stimulation) did not change much upon further culture. When resident mouse macrophages were exposed to different concentrations of MIF-rich fractions, a concentration dependence was observed. However, even when a fivefold concentration (compared to that of the original supernate) was used, we were not able to observe stimulation to levels comparable to those reported during activation in vivo (10–15-fold) (28, 13). The data presented in Fig. 3 were analyzed on the assumption that the overall phenomenon (glucose carbon-1 oxidation to CO_2), including possible saturation of receptors for lymphocyte mediators, shows hyperbolic saturation kinetics. Thus a Lineweaver-Burk plot yielded a value for V_{max} of 50 nmol of glucose oxidized per mg cell protein per h. This amount represents a 10-fold stimulation compared to the control cells. The magnitude of this effect is comparable

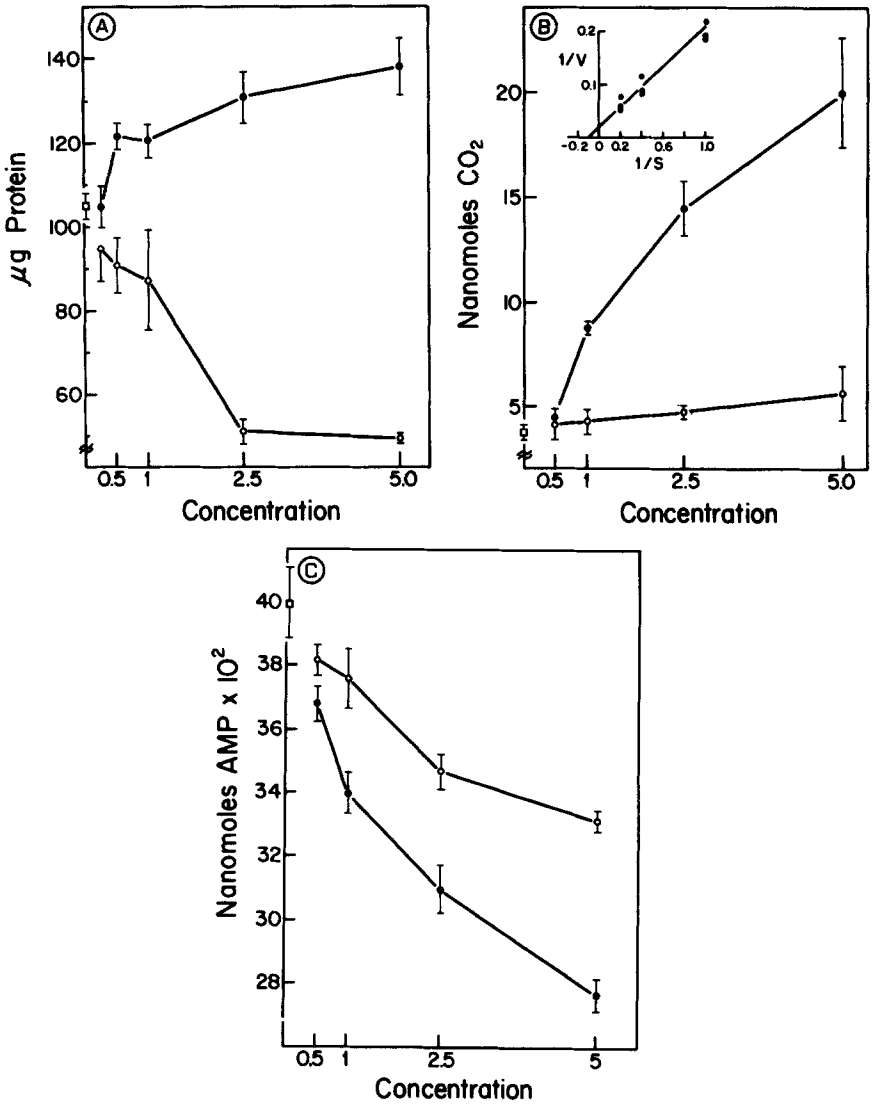

FIG. 3. Concentration dependence of effects of MIF-rich fractions on normal mouse peritoneal macrophages. The values given refer to cells at 48 h of culture. Effects on cell retention expressed as μg cell protein/monolayer (A); glucose carbon-1 oxidation as nmol CO_2 produced/mg cell protein/h (B); and 5′-nucleotidase as nmol AMP hydrolyzed/mg cell protein/30 min (C) are shown. Fraction III of one supernate of a stimulated lymphocyte population was used. Bars represent 1 SD of triplicate plates. Insert of (B) shows a Lineweaver-Burk plot of the data shown. Concentrations are nominal and refer to the original spleen cell culture supernate as 1. (○), Control; (●), MIF-rich; (□), MEM-S.

to the situation in vivo, i.e., cells activated with *Listeria monocytogenes* (13, 28). The amount of mediator that would be required to achieve the maximum effect (50 nmol and above) is far in excess of the concentration of mediator in the culture media. Thus, the "apparent Km", i.e., concentration to achieve only half maximal stimulation, would require a 10-fold concentration of the original lymphocyte supernates. One interpretation of this apparent requirement for a very high concentration of

TABLE III

Effect of MIF-Rich Fractions on Resident, Caseinate-Elicited, and Chemotactic-Factor-Elicited Mouse Peritoneal Macrophages

Variable	Macrophage type		
	Resident	Caseinate-elicited	Chemotactic factor-elicited
(a) Glucose-1-^{14}C oxidation*			
MEM-S control	3.1 ± 0.6	5.1 ± 0.5	5.1 ± 1.3
Normal fraction III	4.9 ± 0.8	4.7 ± 1.3	4.7 ± 1.7
MIF-rich fraction III	10.5 ± 0.4	7.3 ± 0.8	14.7 ± 3.9
Normal fraction IV	4.0 ± 0.9	4.8 ± 0.8	—
MIF-rich fraction IV	13.6 ± 1.3	5.3 ± 0.8	—
(b) 5′-Nucleotidase‡			
MEM-S control	2,208 ± 190	224 ± 15	3,075 ± 192
Normal fraction III	2,108 ± 174	226 ± 20	3,008 ± 110
MIF-rich fraction III	1,504 ± 90	204 ± 22	2,690 ± 89
Normal fraction IV	2,324 ± 150	200 ± 11	—
MIF-rich fraction IV	1,462 ± 70	201 ± 18	—
(c) Cell Retention§			
MEM-S control	137 ± 6	174 ± 10	92 ± 4
Normal fraction III	80 ± 6	122 ± 15	78 ± 9
MIF-rich fraction III	160 ± 11	205 ± 7	105 ± 3
Normal fraction IV	92 ± 2	150 ± 6	—
MIF-rich fraction IV	149 ± 7	190 ± 7	—

The MIF used was at 5 × original concentration. The values given were taken at 48 h of culture. Data represent means of triplicate plates. Standard deviations are given. The zero time values for glucose-1-^{14}C oxidation, 5′-nucleotidase, and cell adherence were: resident macrophages, 4.7, 1,300, and 1,090, respectively; caseinate-elicited cells, 12.9, 25, and 1,160, respectively; chemotactic factor-elicited cells, 5.0, 884, and 244, respectively, i.e., sparser monolayers of the last mentioned cells were used.

* nmol $^{14}CO_2$/mg cell protein/h.

‡ nmol AMP hydrolyzed/mg cell protein/30 min.

§ μg cell protein/plate.

lymphocyte factors is that a cell-to-cell (macrophage-lymphocyte) interaction is required for maximal effects, as suggested by Fowles et al. (19) for enhancement of macrophage bacteriostasis, and by Farr et al. (29) for the stimulated secretion of mitogenic protein by cultures containing T lymphocytes and macrophages. Though this kind of analysis in complex biological systems should be viewed with caution, the observed results indicate that further investigation of the possibility is warranted.

When the effect on 5′-nucleotidase was studied, the typical increase with time, exhibited by control cells under normal culture conditions, was observed (15, 16). When MIF-rich fractions were added to the culture media, lower levels of enzymatic activity at a given time were displayed compared to the controls. This observation can be correlated with the report by Edelson and Cohn (15) that when endotoxin-elicited mouse peritoneal macrophages, which have 5′-nucleotidase levels similar to *Listeria*-activated macrophages (13), were cultured, an elevated rate of enzyme degradation with an essentially normal rate of synthesis was found, leading to lower levels of ectoenzyme compared to normal cells at any given time. It is possible that a similar mechanism pertains to the decrease in the overall rate of acquisition of 5′-nucleotidase when resident cells are cultured in the presence of MIF-rich fractions; i.e., the latter

TABLE IV

Effect of Supernatant Fractions from Stimulated Lymphocytes on Glucose-1-^{14}C Oxidation by, and Retention of, Normal Resident and Sodium Caseinate-Elicited Mouse Peritoneal Macrophages

Fraction	Glucose-1-$^{14}C \rightarrow CO_2$		Cell retention	
	Resident	Caseinated-elicited	Resident	Caseinate-elicited
I	1.1 ± 0.13	1.0 ± 0.09	0.9	1.4 ±0.07
II	1.1 ± 0.18	1.1 ± 0.09	1.1 ± 0.16	2.1 ± 0.08
III	3.2 ± 0.17	1.0 ± 0.08	0.9 ± 0.11	1.7 ± 0.09
IV	1.3 ± 0.12	0.9 ± 0.08	1.9 ± 0.06	2.4 ± 0.08
V	1.4 ± 0.12	1.0 ± 0.11	1.6 ± 0.06	1.7 ± 0.09
VI	1.0 ± 0.13	0.8 ± 0.12	1.4 ± 0.08	1.8 ± 0.08
VII	0.9 ± 0.31	1.0 ± 0.06	1.4 ± 0.10	1.6 ± 0.10

Entries represent ratio of value obtained with fraction from stimulated lymphocytes to that with control fraction (unstimulated lymphocytes). A single MIF-rich preparation was used at 5 × concentration. The measurements were taken at 48 h of culture. The fractions I–VII were obtained by Sephadex G-100 chromatography (see text).

may stimulate degradation, which has been associated with elevated pinocytosis activity (15).

Both activated macrophages and stimulated macrophages show increased adherence to the plates, and spreading (30). When mouse peritoneal macrophages are maintained in complete culture media, a consistent loss of cells is seen with time. From the results presented, it can be observed that when control fractions are added to the culture media, an enhanced loss of adherent cells occurred, suggesting that perhaps a toxic substance is present in the supernates. Since supernates from Con A-stimulated lymphocytes and control supernates are treated in exactly the same manner, it is logical to assume that whatever substance is responsible for cell loss in culture should be found in both preparations. The significantly higher cell retention found when MIF-rich fractions were added to the macrophages, and the even more dramatic effect observed when concentrated fractions were used, indicates the presence of a factor in these preparations that enhances maintenance of the monolayers.

Several experiments reported here suggest that cell retention can be correlated with MIF activity, but not necessarily with the metabolic changes caused by lymphocyte supernate fractions. When the full spectrum of fractions from some preparations was analyzed (Tables I and IV, Fig. 2), MIF activity and cell maintenance were observed to be broadly distributed, whereas in the same experiments the effects on glucose oxidation and 5′-nucleotidase were limited to fewer fractions, with a peak in fraction III (see below). It is of interest that when similar fractions of the same preparation were tested for their ability to enhance tumoricidal activity of mouse macrophages, activity was found in fractions III, IV and V, with a clear peak in IV (M. Meltzer, personal communication).

In most studies in vitro where macrophages are involved, peritoneal cells are employed that are elicited with one of several agents (mineral oil, thioglycollate, sodium caseinate, peptone, etc.). The large yield that can be achieved dictates this stratagem. We and others (13,1 5, 31) have shown by means of different biochemical criteria, that the "elicited" cell is considerably different from the normal "washout"

TABLE V

Effect of Spleen Cell Fractions that Had Been Stored at −20°C

Preparation	Glucose 1-$^{14}C \rightarrow {}^{14}CO_2$		Cell adherence	
	0 h	48 h	0 h	48 h
MEM-S control	100	167 ± 9.2, 151 ± 5.2	100	36 ± 4.6, 36 ± 9.2
Normal control Fraction IV		190 ± 9.8, 161 ± 4.6		37 ± 13.9, 24 ± 3.5
MIF-rich Fraction IV		196 ± 9.8, 153 ± 5.8		58 ± 8.7, 51 ± 4.0

The preparations used were "aged" 38 or 210 days. They were used at 5 × original supernatant concentration. There was no significant loss of MIF activity per se during storage, within the precision of the method. Data at 0 time were set at 100, and the values for each of the two experiments taken at 48 h of culture were normalized to the 0 time value. Standard errors of the means of three determinations are given in each experiment.

peritoneal resident macrophage. With this consideration in mind, we tested the sodium caseinate-elicited cell in our system in vitro, with all the fractions of lymphocyte supernate (Table IV). No significant effect on glucose oxidation could be observed in any of the fractions, whereas resident cells showed the expected effect with one fraction. The fractions that increased maintenance of resident cells in culture and possessed MIF activity again did not coincide exactly with the fractions exhibiting an effect on glucose oxidation. It should be noted that the resting glucose 1-^{14}C oxidation of the elicited macrophages is greater than that of resident cells, and appears to be rather insensitive to further change. A significant difference with elicited cells as compared to resident cells was found when cell retention was estimated. The effect was rather scattered through all the fractions. This observation could be explained if the MIF activity for the particular supernatant fraction used was rather high, which was indeed the case—it was the most active filtrate we used (Results). This result points further to the possibility that elicited cells, in contrast to resident cells, show greater sensitivity to the supernatant factor that is responsible for changes in cell adherence. Furthermore, when one uses the most sensitive cells, it would appear that in the fractionation of the particular supernate used for the experiment of Table IV, the column may have been "overloaded". In general terms, the data reported here mirror the observation by other workers that the resident macrophage population of the unstimulated guinea pig peritoneal cavity was less responsive to MIF than oil-elicited peritoneal macrophages (32, 33).

When peritoneal macrophages induced by injection of the animal with a fraction containing chemotactic factor were examined, a response similar to that observed with resident cells was found, suggesting that perhaps this chemotactically elicited cell might be a more logical target for the activating factor.

During the development of the experiments reported here, it was observed that the fractionated supernates lost their ability to stimulate glucose carbon-1 oxidation after several months of storage. A specific experiment was therefore performed to re-examine the relevant supernatant fraction of particular preparations. It was shown that although the material had become ineffective in stimulating glucose oxidation by resident mouse peritoneal macrophages, the effect on cell maintenance remained, and MIF activity was still present. These results are further indications that MIF activity and enhancement of cell adherence are expressions of a property of these supernatant fractions different from that concerned with some metabolic functions,

or that the latter are indeed sensitive only to much greater concentrations of lymphocyte factors.

Summary

Resident mouse peritoneal macrophages were incubated in Sephadex G-100 fractions of supernates from concanavalin A-stimulated lymphocytes. A significant effect of the lymphocyte supernatant fractions containing mediators on macrophage 5′-nucleotidase, glucose-1^{14}C oxidation, cell maintenance, and migration is reported. The 5′-nucleotidase was depressed to an extent similar to that seen in activated macrophages obtained from *Listeria*-infected mice. On the other hand, glucose-1-^{14}C oxidation was enhanced, but not to the same degree as seen in the counterparts in vivo. Whereas migration inhibitory factor (MIF) and cell adherence-augmenting activity were found in a number of adjacent fractions, the metabolic effects were found predominantly in a single fraction. Resident peritoneal macrophages or those elicited by the injection of a lymphocyte-derived chemotactic factor were more responsive with respect to the biochemical changes than caseinate-elicited macrophages. On the other hand, caseinate-elicited macrophages appeared to be more sensitive with respect to the effects of mediator(s) on cell retention. A possible dissociation between MIF and cell-adherence augmenting activity, on the one hand, and the entities that stimulate glucose-1-^{14}C oxidation is reported, based on fractionation studies, and loss of the latter activity upon storage of lymphocyte supernates.

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