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Research Article

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Activation of Human Blood Monocytes by Products of Sensitized Lymphocytes

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ABSTRACT Studies were carried out to determine whether products of activated human lymphocytes altered human monocyte function. Supernatants from sensitized human lymphocytes stimulated by specific antigen were cultured with monolayers of human monocytes. Such monocytes exhibited enhanced adherence to their culture vessels and increased glucose carbon-1 oxidation after 2–3 days of incubation. The substance responsible for these effects was found to elute from Sephadex G-100 gel columns in a fraction with 23,000 mol wt, the same fraction containing human migration inhibitory factor.

INTRODUCTION

In the course of studies on patients with cutaneous anergy, we were surprised to find that lymphocytes from some patients functioned normally with respect to antigen-induced thymidine incorporation and production of migration inhibitory factor (MIF)[1] despite completely negative skin tests (1, 2). This finding suggested that the defect in these patients was not the result of abnormal lymphocyte function but might reside elsewhere, possibly with some other cell type involved in cellular immune reactions. Therefore, experiments were undertaken to evaluate human monocyte function as it relates to cellular hypersensitivity.

Recent studies in animals have shown that macrophage function may be enhanced in vitro after exposure of these cells to the products of activated lymphocytes (3, 4). Such macrophages demonstrated increased cell adherence, ruffled membrane activity, enhanced phagocytosis, and glucose oxidation through the hexose monophosphate shunt pathway (HMPS). Furthermore, studies show that the factor responsible for these effects on macrophages can not be distinguished from MIF (5).

In order to determine whether human monocytes could be similarly activated, they were incubated in vitro in the presence of unfractionated or partially purified supernatant preparations obtained from stimulated and unstimulated sensitive lymphocytes. After appropriate intervals, cell adherence and glucose carbon-1 oxidation were determined. The results indicate that human monocyte function may be enhanced in vitro by soluble products of lymphocyte activation. The material responsible for this effect is eluted from Sephadex G-100 columns in the same region as human MIF.

METHODS

Preparation of soluble products from activated human lymphocytes. 120 ml of heparinized blood was drawn from donors exhibiting cutaneous delayed-type hypersensitivity to streptokinase-streptodornase (SK-SD) and sedimented with dextran as previously described (6). The buffy coat was obtained by collecting the leukocyte-rich plasma and centrifuging at 250 *g* for 8 min at room temperature. After resuspending the cells 8 ml of TC-199 (Microbiological Associates, Inc., Bethesda, Md.), 4-ml aliquots were carefully layered into 12-ml conical centrifuge tubes containing 3 ml of a Ficoll-Hypaque solution (7). The lymphocytes and monocytes were separated from the red cells and granulocytes by centrifugation at 400 *g* for 40 min at 20°C. After centrifugation, the mononuclear cells layered at the TC-199-Ficoll interface were carefully removed with a Pasteur pipette and transferred to another 12 ml centrifuge tube. The cells were washed three times in TC-199 and the cell count and differential determined in a hemocytometer at 400 × magnification. The volume of the cell suspension was adjusted to contain 10×10^6 lymphocytes per ml in TC-199 containing 100 U penicillin and 100 μg of streptomycin and without serum (TC-199-P&S). One-half of the cell suspension was cultured in the absence of antigen (resulting in a control supernatant) and the other half in the presence of 50 U/ml of SK-SD (resulting in a MIF-rich supernatant); both were incubated in a 5% CO_2-air atmosphere at 37°C. After 48 h of incubation, the

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[1] *Abbreviations used in this paper:* HMPS, hexose monophosphate shunt pathway; MIF, migration inhibitory factor; SK-SD, streptokinase-streptodornase.

cells were removed by centrifugation and antigen in the original amount was added to the supernatant obtained from the control culture. Both supernatants were dialyzed against fresh TC-199-P&S for 24 h, sterilized by Millipore filtration, and made to contain 20% normal pooled human serum. These supernatants were subsequently added to monolayers of blood monocytes.

Preparation of monocyte monolayers. Mononuclear cell preparations were obtained using the above Ficoll-Hypaque separation technique. The mononuclear cell suspension was adjusted to $3–5 \times 10^6$ monocytes per ml in TC-199-P&S made to contain 20% fetal bovine serum. 1 ml of cell suspension was pipetted into each petri dish (35×10 mm, Falcon Plastics, Oxnard, Calif.) and the cells incubated for 1 h at 37°C in a 5% CO_2-air atmosphere. After decanting the nonadherent cells, the dishes were washed once in fresh TC-199-P&S containing 20% fetal bovine serum and 1 ml of test supernatant was added to each dish (in triplicate). The dishes were then incubated for 48 or 72 h under the above atmospheric and temperature conditions.

Fractionation of supernatants. Lyophilized supernatants (equivalent to 100 ml of starting material) obtained from SK-SD-stimulated human spleen lymphocytes were applied to 2.5×100 cm columns containing Sephadex G-100 as previously described (8). Aliquots from these supernatants were tested and found to contain MIF activity. The effluent material was pooled into five fractions: fraction I-II contained material eluting from the void volume of the columns to the beginning of the elution of the albumin; fraction III (K_d 0.09–0.19) contained material eluting with albumin; fraction IVb (Kd 0.33–0.45) contained material eluting with molecules the size of chymotrypsinogen and is the region where peak MIF activity is found (8); fraction IVa contained material between the albumin and chymotrypsinogen markers. The pooled fractions were dialyzed against distilled water and lyophilized. Before use the fractions were reconstituted to one-fifth the original volume in medium TC-199-P&S, made to contain 20% pooled human normal serum, and sterilized by Millipore filtration. 1 ml of each fraction was added to the dishes in triplicate.

Measurement of cell adherence. At the conclusion of each experiment, the dishes were emptied, rinsed in six successive beakers of isotonic saline, and air dried. The contents of the dishes were dissolved overnight in 1 ml of 1 N NaOH, and a 0.5 ml sample of the supernatant was removed for protein determination by the method of Lowry et al. (9). In other experiments, the deoxyribose nucleic acid (DNA) content per dish was determined using the method of Kissane and Robbins (10). The enhancement of cell adherence was calculated from the following formula:

$$\% \text{ Enhanced cell adherence} = \left[\frac{\mu g \text{ protein or DNA in dishes containing MIF-rich supernatants}}{\mu g \text{ protein or DNA in dishes containing control supernatants}} \times 100 \right] - 100$$

Glucose oxidation. The oxidation of [1-^{14}C]glucose to $^{14}CO_2$ by macrophage monolayers was determined as previously described (4). After incubation in unfractionated or fractionated supernatants and medium TC-199, the dishes were emptied, rinsed in three beakers of Krebs-Ringer phosphate buffer pH 7.4 containing 2 mM glucose, and drained (4). 1 ml of Krebs buffer was then added to each dish. The petri dishes were fitted with modified lids. A hole was punched through the lid 1 cm from the edge using a hot #18 needle, and the hole was covered with parafilm. A 1 cm square of Whatman #4 filter paper was placed in the center of the inside of the lid and impregnated with 0.05 ml of 1 N NaOH using a 50 lambda automatic pipetter. 1 μCi (in 0.1 ml) of [1-^{14}C]glucose (specific activity 6.57 mCi/mM, New England Nuclear Corp., Boston, Mass.) was added rapidly with gentle agitation of the dish. The dishes were incubated on a 37°C warming plate for 30 min. The parafilm was then lifted and 0.05 ml of 2 N HCl were added rapidly through the hole in the lid. The parafilm was replaced and the dishes were gently agitated and incubated another 15 min. The filter paper containing the evolved $^{14}CO_2$ was removed, the inside of the lid wiped dry with a second square of filter paper, and both pieces placed in a vial with 10 ml Brays solution and counted in a liquid scintillation counter with quench correction. 1 ml of 1 N NaOH was added to the Krebs buffer in each dish, and the protein content was determined.

Protein and DNA synthesis. Because cell adherence was being determined by methods which measure cell protein and DNA, it was important to determine whether the observed increased cell adherence was due to increases in protein synthesis and DNA synthesis per se. For that reason protein and DNA synthesis was measured in monocyte monolayers incubated for 3 days in the presence of control and MIF-rich Sephadex G-100 fraction IVb. On the third day the monolayers were pulsed for 4 h with 1 μCi/dish of [C^{14}]leucine and 1 μCi/dish [^{3}H]thymidine. The adherent cells were removed by adding 1 ml of Versene (Microbiological Associates, Inc., Bethesda, Md.) to each dish for 15 min and gently mixing and aspirating the cell suspension with a Pasteur pipette. The cells were then placed in test tubes, washed twice in normal saline, and precipitated with 5% trichloroacetic acid. The precipitates were dissolved in 10 ml of scintillation fluid, and the radioactivity was determined in a Packard Tri-Carb counter. Uptake of [C^{14}]leucine was calculated per microgram macrophage DNA per dish and uptake [^{3}H]thymidine per microgram macrophage protein per dish.

RESULTS

Effect of unfractionated supernatants on monocyte adherence. More human monocytes were found adherent to the dishes after incubation for 2 days in MIF-rich

TABLE I

Effect of Supernatants on Monocyte Adherence

No. of experiments	Supernatant	Protein*	Increase in MIF
		μg	%
Adherent macrophage protein			
13	MIF	38.7±5.6	41%‡
	Control	27.5±4.8	
Adherent macrophage DNA			
18	MIF	28.7±2.8	35%‡
	Control	21.3±2.6	

* Per dish.
‡ $P < 0.05$.

supernatants than in dishes incubated with control supernatants. The results are summarized in Table I. In 13 experiments there was a mean of 41% more protein in monocyte monolayers incubated with MIF-rich supernatants compared to the controls; in other experiments there was a 33% increase in DNA content in dishes in the experimental group. This was statistically significant at $P < 0.05$.

Effect of unfractionated supernatants on glucose oxidation. [1-C^{14}]glucose oxidation to $^{14}CO_2$ was increased in monocytes exposed to MIF-rich supernatants for 2 days compared to those in control supernatants. The results of 13 experiments are shown in Fig. 1. The nanomoles glucose carbon-1 oxidized per μg cell protein per min in 13 experiments was 0.54±0.09 in monolayers containing MIF-rich supernatants and 0.31±0.06 in those containing control supernatants ($P < 0.05$), a mean increase of 74%.

Specificity and kinetics. The results observed thus far indicated that soluble products of antigen-stimulated sensitized lymphocytes altered monocyte function. In order to investigate further the specificity of this response, supernatants were prepared from lymphocyte donors who lacked delayed cutaneous hypersensitivity to SK-SD, and the effect of these supernatants on human monocyte function was assessed. Supernatants prepared from SK-SD-sensitive lymphocytes were assayed in parallel. The supernatants prepared from antigen-stimulated SK-SD-negative lymphocytes did not alter either cell adherence or glucose oxidation of monocytes obtained from either SK-SD-positive or SK-SD-negative subjects. However, the supernatants from antigen-stimulated SK-SD-positive lymphocytes enhanced adherence and glucose oxidation whether the monocytes were obtained from SK-SD-positive or SK-SD-negative subjects (see Fig. 2). The increase in monocyte cell adherence in cultures incubated with supernatants from SK-SD-negative lymphocytes stimulated by antigen was 1% compared to their controls; the increase in monocyte cell adherence in cultures incubated with super-

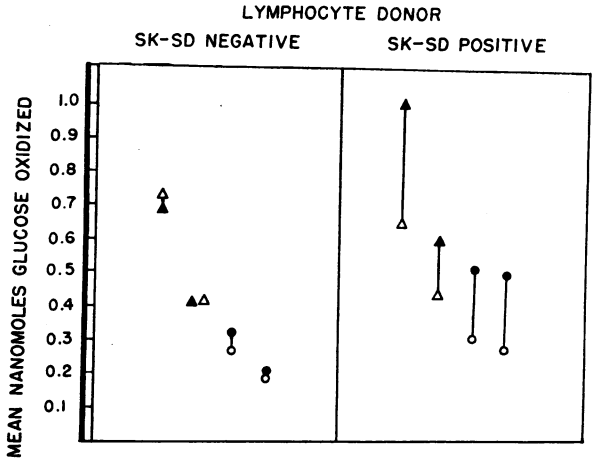

FIGURE 2 Requirement for antigen specificity in the elaboration by lymphocytes of the factor which alters monocyte function. Lymphocytes were obtained from SK-SD-positive and SK-SD-negative donors and cultured with and without SK-SD. The resultant supernatant from antigen-stimulated cultures (solid symbols) and unstimulated cultures (open symbols) were then incubated 2 days with monocytes. The triangles represent the monocytes obtained from SK-SD-negative donors. It can be seen that the supernatant from antigen-stimulated lymphocytes from SK-SD-positive donors enhanced glucose carbon-1 oxidation compared to its control regardless of the source of monocytes. The supernatants from antigen-stimulated lymphocytes of SK-SD-negative donors had no effect.

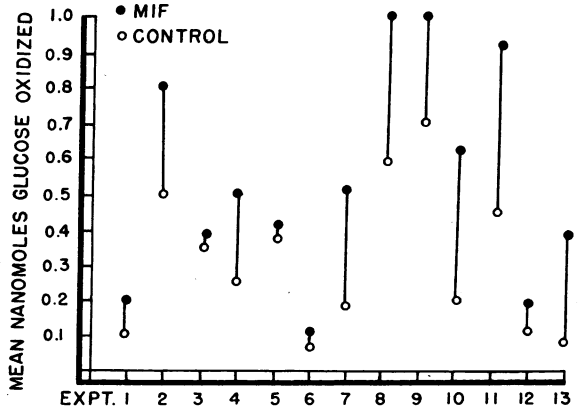

FIGURE 1 Glucose carbon-1 oxidation by human monocytes. Monolayers incubated for 2 days in the presence of MIF-rich supernatants from SK-SD-stimulated sensitive lymphocytes (solid circles) or control supernatants (open circles) were assayed for $^{14}CO_2$ release.

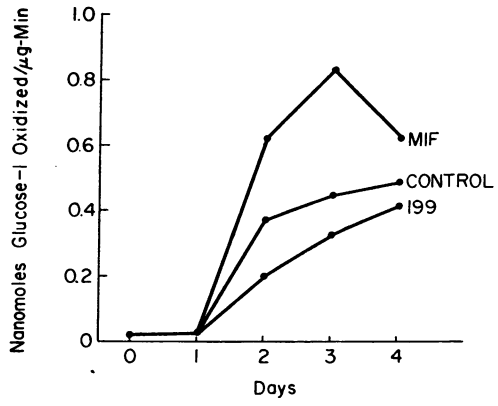

FIGURE 3 Kinetic studies. The effect of MIF-rich and control supernatants and culture medium itself (TC-199) on glucose carbon-1 oxidation by human monocytes. Data expressed in nanomoles glucose oxidized per microgram cell protein per min.

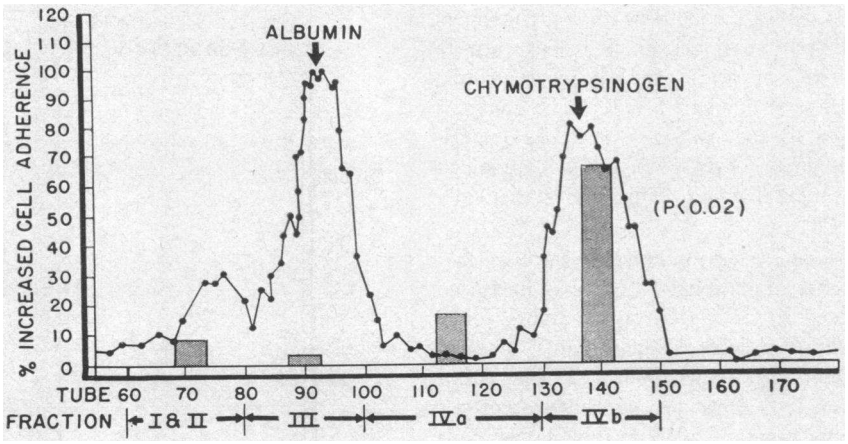

FIGURE 4 Elution pattern of adherence-promoting activity from Sephadex G-100 columns. Per cent increase cell adherence of monocytes incubated for 3 days in fractions from supernatants of antigen-stimulated lymphocyte cultures compared to control fraction is plotted as bars. Note only fraction (IVb) eluting at position of the chymotrypsinogen marker is active.

natants from SK-SD-positive antigen-stimulated lymphocytes was 26% in these experiments.

The kinetics of glucose carbon-1 oxidation and cell adherence were monitored over 4 days and measured at times 0, 24, 48, 72, and 96 h. Results of one experiment are shown in Fig. 3. It can be seen that no significant difference in glucose oxidation occurred until 2 days of incubation, and the peak effect of MIF-rich supernatants occurred at 3 days. In this experiment the adherent cell protein found in the monolayers at 0 time was 258 μg/dish. After 3 days, when maximum glucose oxidation was seen, there was 50 μg, 37 μg, and 25 μg/dish in cultures incubated with MIF-rich supernatants, control supernatants, and culture medium, respectively. This illustrates that the increase in cell adherence in the monolayers incubated with MIF-rich supernatants is a relative rather than an absolute one. This is similar to findings previously described using guinea pig MIF and macrophages (4).

Sephadex G-100 fractionation. MIF-rich and control supernatant preparations were filtered on Sephadex G-100 columns and the fractions assayed for cell adherence and glucose oxidation on monocyte monolayers. Monocyte adherence-promoting activity was only found in fraction IVb; this is where the chymotrypsinogen marker (23,000 mol wt) also elutes (see Fig. 4). Simi-

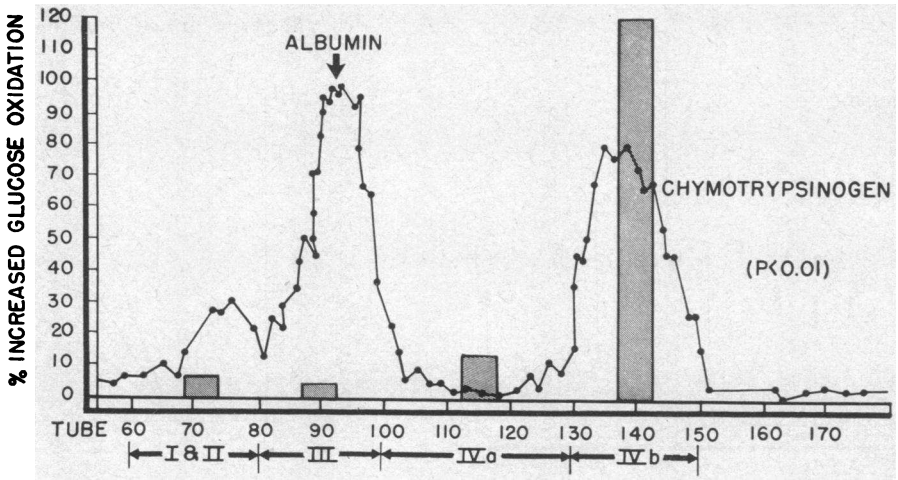

FIGURE 5 Elution pattern of glucose carbon-1-promoting factor from Sephadex G-100 columns. Percent increase glucose carbon-1 oxidation of monocytes incubated 3 days in fractions from supernatants obtained from antigen-stimulated lymphocyte cultures compared to control fractions is plotted as bars. Note only fraction (IVb) eluting at position of the chymotrypsinogen marker is active.

larly, only this fraction, IVb, caused significant increase in glucose carbon-1 oxidation (see Fig. 5). It is of note that this is the same fraction where human MIF elutes (8).

Protein and DNA synthesis by monocytes. Experiments were carried out to see if protein or DNA synthesis of monocytes was affected by incubation for 3 days in active Sephadex G-100 fraction IVb. The results of three experiments are presented in Table II and show that no significant increase in incorporation of either [^{14}C]leucine or [^{3}H]thymidine per cell was seen despite the finding, as expected, that there was a mean increase of 51% more cells in the MIF-rich fraction IVb-treated cultures than the corresponding controls.

TABLE II

Assessment of Protein and DNA Synthesis by Human Monocyte Monolayers

Sensitivity of monocyte donor	[^{14}C]leucine* incorporation		
	MIF	Control	% MIF / Control
(1) SK-SD+	634±118	584±88	108%
(2) SK-SD−	1,046±78	1,004±135	104%
(3) SK-SD−	639±32.7	591±26.7	108%
Sensitivity of monocyte donor	**[^{3}H]thymidine‡ incorporation**		
	MIF	Control	% MIF / Control
(1) SK-SD+	302±26	253±39	119%
(2) SK-SD−	447±40	430±73	104%
(3) SK-SD−	279±13.9	251±17.9	111%

* Mean cpm±SE adherent cell DNA per dish.
‡ Mean cpm±SE adherent cell protein per dish.

DISCUSSION

The results of the studies presented here demonstrate that supernatants obtained from antigen-stimulated human lymphocytes have the capacity to enhance certain human monocyte (macrophage) functions in vitro. These include increased adherence of human monocytes to their culture vessels and increased glucose carbon-1 oxidation. Increased cell adherence was demonstrated by measuring either the amount of cell protein per dish or the DNA content per dish, as this was more quantitative than morphologic evaluation. It should be noted that the increased adherence was relative; the monolayers lost cells daily and the attrition was reduced in monolayers containing MIF-rich supernatants. This effect was not seen at 1 h or 24 h, but occurred at 2 days and was maximum at 3 days.

Glucose oxidation per cell increased during the 3 day culture period when monolayers were incubated in culture medium or control supernatants. This latter effect may be associated with the maturation of the monocytes into macrophages. The increase in glucose oxidation was significantly enhanced by supernatants from antigen-stimulated sensitized lymphocytes. As the culture supernatants were known to contain antigen and antibody, possibly in complex form, it was particularly important to determine whether these substances were responsible for the observed effects on macrophage function. Supernatants were filtered over Sephadex G-100 columns, and the eluate was divided into fractions which were tested for their capacity to increase adherence and glucose oxidation in blood monocytes. Only one of the fractions (fraction IVb, of approximately 23,000 mol wt) of the eluate reproduced the effects observed with unfractionated supernatants. This is the only fraction where human MIF is found (8). Fractions which contained antibody (fractions I and II) or the antigen SK-SD (fraction III) were inactive.

Further experiments showed that the elaboration of the monocyte-activating material was antigen-specific. Incubation of lymphocytes from SK-SD-negative individuals with SK-SD yielded supernatants which were inactive when tested on monocytes. The results were the same whether the monocyte monolayers consisted of cells from SK-SD-positive or SK-SD-negative individuals. On the other hand, the supernatants obtained from stimulated SK-SD-positive lymphocytes enhanced adherence or glucose carbon-1 oxidation of the monocyte monolayers regardless of the SK-SD sensitivity of the monocyte donor.

The active fraction did not cause an increase in either protein or DNA synthesis, as measured by the incorporation of [^{14}C]leucine or [^{3}H]thymidine into precipitable cell material. Although the enhanced survival of monocytes in MIF-rich supernatants compared to control supernatants might be interpreted as the effect of a "conditioned medium," it should be emphasized that this effect is intimately related to a specific immunologic event, and that the activating principle is only present in the Sephadex fraction which also contains MIF.

The results given here indicate that human monocyte functions can be altered by products of activated lymphocytes. This may be one of the early steps in the lymphocyte-macrophage interaction in cellular immunity directed against certain microorganisms or tumors in vivo. Studies of this type may prove useful in assessing monocyte function vis-á-vis cellular immunity in various disease states, particularly if used in conjunction with other measures of monocyte responsiveness to lymphocyte mediators (such as their inhibition of migration or chemotaxis) and with the methods for assessing lymphocyte function. It will also be of interest to determine whether lymphocyte mediators alter the phagocytic or bactericidal capacities of human monocytes or affect certain of their membrane receptors such as those for

IgG or C_3 (11–13). Such information on the various aspects of monocyte and lymphocyte function should help to define the underlying defects in immunodeficiency states of unknown etiology.

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