THE MODULATION OF LYMPHOCYTE FUNCTIONS BY MOLECULES SECRETED BY MACROPHAGES

I. Description and Partial Biochemical Analysis*

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Mononuclear phagocytes are thought to play a regulatory role in immune induction, although there is no general consensus on their exact mode of action. Phagocytes are capable of multiple functions—uptake, endocytosis, catabolism and retention of foreign molecules, activation by extracellular events, secretion of enzymes and other regulatory molecules, etc. As a result of these diverse functions, it would not be surprising for phagocytes to influence the immune process at different stages of its development and evolution. One of the explanations for the role of macrophages in inductive events is that it takes up and presents antigen molecules in an appropriate manner, serving as an antigen-focusing cell for T-B cell interactions (1-3). This is the explanation that appeals more to us based on the superior immunogenicity of macrophage-associated antigen and its counteraction by free soluble materials (4, 5). However, other experiments strongly suggest that phagocytes contribute in some other way to inductive events apart from simple presentation of antigen: (a) in the guinea pig, macrophage associated antigen stimulates T-cell proliferation best when the macrophage and the T-cell share their histocompatibility background—this indicates that in this system T-lymphocyte proliferation requires some other unknown factor besides antigen presentation (6, 7); (b) antigens bound to macrophages stimulated by adjuvants are more immunogenic than bound to unstimulated macrophages despite the fact that antigen handling is identical in both cells (8); (c) viability of lymphocytes in cultures is increased by the presence of macrophages (9); and (d) factors that enhance or support lymphocyte function have been found in macrophage cultures (10-15). Of particular interest is the observation of Gery, Gershon, and Waksman reported in this journal (10, 11) of an activity in cultures of human macrophages stimulated by endotoxin that enhanced the response of thymocytes to lectins. We have recently confirmed and extended this observation using murine macrophages stimulated by peptone (16). Upon removal from the cultures of a small molecular weight inhibitor of DNA and protein synthesis (17), an activity was found that

stimulated proliferation of spleen and thymocytes without the need of addition of lectins. This paper reports on this phenomenon. We have confirmed that culture fluids from macrophages contain activities that stimulate lymphocyte proliferation and influence B-cell differentiation. These activities are only secreted by macrophages and not by unstimulated lymphocytes, are not restricted in action to cells of the same species, and are in molecules that do not contain antigens of the *H-2* complex.

Materials and Methods

Culture Fluids. Peritoneal exudate cells (PEC)[1] were harvested from mice injected 3 days previously with 1.5 ml of 10% proteose peptone solution (Difco Laboratories, Detroit, Mich.), intraperitoneally. The mice were killed and their peritoneal cells harvested after an injection of 3 ml of minimal essential medium (MEM, Microbiological Associates, Bethesda, Md., catalogue no. 12-683) containing 10 U of heparin per ml and 5% vol/vol heat-inactivated fetal calf serum (Associated Biomedics, Inc., Buffalo, N. Y.). The cells were centrifuged (500 g, 10 min), washed with same medium (not containing heparin), and suspended at a density of 10^7 per ml in RPMI 1640 (Associated Biomedics) with 5% fetal calf serum, 0.5 mM Na pyruvate, 2 mM L-glutamine, 1% Na bicarbonate, 50 U of penicillin, and 50 μg of streptomycin. (In some experiments fetal calf serum was omitted and replaced by 200 μg per ml of lactalbumin hydrolyzate [Mann Research Laboratory, New York].) The cells were planted on 35 × 10 mm dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) at 37°C in 5% CO_2 in air incubator for 2 h, washed vigorously three times, and incubated for 24-72 h. Culture fluids were harvested, centrifuged, and dialyzed 72 h against 1640 medium, changing the dialysate three to four times. This procedure is necessary for removing the low molecular weight inhibitor of DNA and protein synthesis (17).

In one experiment the PEC monolayer after the first 2 h of culture was treated with anti-θ serum and guinea pig serum, as a complement source, in order to kill and eliminate any possible dish-adherent T cells. The anti-θ serum, raised in AKR mice by immunization with CBA thymocytes, had been previously standardized. The PEC monolayer was incubated in 1 ml of MEM containing 10% anti-θ serum or normal mouse serum at 4°C for 30 min; then the cells were washed three times, incubated at 37°C for 45 min in medium containing 20% guinea pig serum, as a complement source, washed again, and allowed to culture for 24 h.

Culture fluids were also obtained from 3T3 cells obtained from Dr. Thomas Benjamin of our department and from a line of normal mouse embryo cells.

Mice. Most experiments were done in A/St mice from West Seneca Laboratories, Buffalo, N. Y., of either sex, of 8-10 wk of age at the time of initiation of the experiments. Nu/nu athymic mice, obtained from the colony of Dr. Gabriel Michael, University of Cincinnati Medical School, were used at 10-12 wk of age. In occasional experiments the donors of PEC were Swiss outbred mice obtained from Charles River Laboratories, Boston, Mass.

Culture of Thymocytes and Spleen Cells. Thymocytes and spleen cells were harvested by conventional procedures and cultured at a density of 5 × 10^6 or 10^6 per ml, respectively, in 1640 medium, as described above, with 5% fetal calf serum. The cells were cultured in 12 × 75 mm tubes (Falcon Plastics, catalogue no. 2054) for 72 h in 37°C incubated with 5% CO_2 in air. 12-15 hours before termination, the cultures received 1 μCi of [^{3}H]thymidine (2 Ci/mmol from New England Nuclear Company, Boston, Mass.). Trichloracetic acid insoluble material from cultured cells was collected on glass fiber filters in a sampling manifold. Radioactivity was measured in a liquid scintillation spectrometer. The basis of the experiments consisted of evaluating the incorporation of [^{3}H]thymidine of the cells cultured in medium containing various amounts of PEC culture fluids. The time of culture was always 72 h—pilot experiments indicated this to be the optimal time to see effects. All results are means of triplicate cultures expressed as counts per minute (cpm) plus or minus the value of one standard error of the mean. In some experiments we express the ratio of cpm of experimental

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[1] *Abbreviations used in this paper:* DFP, diisopropylfluorophosphate; F, fluorescein; KLH, keyhole limpet hemocyanin; PEC peritoneal exudate cells; PFC, plaque-forming cells; PHA, phytohemagglutinin; RGG, rabbit gamma globulin.

tubes over cpm of control tubes not containing the PEC fluid-E/C value. In many experiments phytohemagglutinin (PHA) (Difco Laboratories) was also added to the cultures.

Antibody Response In Vitro. The methodology of Mishell and Dutton (18) was applied for the study of the response of fluorescein (F) as a hapten. This compound behaves as a typical hapten, the immune response depending on T-cell cooperation via an appropriate carrier protein (J.-M. Kiely and E. R. Unanue, unpublished). We followed the exact methodology reported by Mishell and Dutton employing a cell density of 10^7 per ml. In all experiments we report the total plaque-forming cells (PFC) per culture at the fourth day, which was the peak time of the response. Each result represents the arithmetic mean of three culture dishes.

The PFC response to F was assayed by conventional plaque technique using F conjugated to sheep red blood cells (SRBC). The technique of conjugation of SRBC to F was as follows: a solution of 0.5 mg per ml (in bicarbonate buffer, 0.125 M, pH 9.2) of fluorescein isothiocyanate was freshly prepared, filtered to eliminate small undissolved particles, and slowly added to an equal volume of 20% SRBC (also in bicarbonate buffer). The suspension was stirred with a magnet for 40 min at ambient temperature. The SRBC were then washed four times with 0.5% ClNa solution and taken to a 7% concentration in MEM. Direct and indirect PFC were assayed by conventional procedures. A polyvalent rabbit antimouse immunoglobulin (Ig) was used as a facilitating antisera. In control experiments it was determined that anti-F PFC were readily blocked by addition of 10^{-6} M to 10^{-8} M free F into the medium.

All experiments employed spleen cells from A/St mice immunized with F-keyhole limpet hemocyanin (KLH) in alum. Two groups of mice were used: (a) mice primed with KLH in alum (i.p. with 50 to 100 μg) once to twice about 3–4 mo previously; these mice usually responded with about 300 to 400 direct and indirect PFC with background number of PFC never exceeding 150; and (b) mice primed as above but boosted 1–4 wk before the experiment with 50 μg of F-KLH in alum; these cells responded with about 1,000 to 3,000 direct and indirect PFC.

Fluorescein was conjugated to KLH or rabbit gamma globulin (RGG) by standard procedures. KLH was prepared by ultracentrifugation of the hemolymph of giant keyhole limpets. Rabbit IgG was obtained from Pentex Biochemical, Kankakee, Ill. Ratio of F:P were 9.0 for 100,000 mol wt of KLH and 2.5 for RGG.

Physicochemical Analysis. PEC from CD-1 mice injected with peptone were placed in culture with 1640 medium containing lactalbumin hydrolyzate for 24 h at which time the fluids were harvested and concentrated about 10-fold by ultrafiltration. 2 ml of the concentrated fluid were passed through a Sephadex G200 column (Pharmacia Fine Chemicals, Piscataway, N.J.), 39 cm long and 3 cm in diameter and eluted with 0.15 M phosphate-buffered saline, pH 7.2, at a flow rate of 4.5 ml per hour. Eluted fractions, each of 32 ml, were dialyzed 48 h against distilled water, lyophilized, and resuspended to one-tenth of the original volume with 1640 medium and tested. The fractions containing the stimulatory activity were pooled, passed through a Sephadex G100 column under the same conditions as described above, and the eluted fractions tested. Each of the columns had been calibrated with known protein markers. The molecular weight of the stimulatory molecule was calculated from a chart where the Kav values for markers were plotted against the logarithm of the molecular weight.

In one experiment culture fluids were passed through a Sepharose 4B column which had anti-*H-2* antibodies bound covalently. Anti-*H-2*ᵃ antibodies were prepared in C57BL/6 mice by repeated immunization with A/St spleen cells. The antiserum had been standardized and found to be cytolytic to A/St spleen cells at dilutions of 1×160. A globulin-rich fraction of the anti-*H-2* serum and of normal mouse serum was obtained by ammonium sulphate precipitation (at 40% concentration); 10 mg of the globulin was bound to 20 ml of Sepharose 4B by cyanogen bromide as per details given by March, et al. (19). Control experiments with radioiodinated globulin confirmed that 32% of it was bound to the column. 8 ml of macrophage culture fluid was passed through each column.

Miscellaneous Manipulations. In one experiment PEC fluids were incubated with a known batch of diisopropylfluorophosphate (DFP) (from Aldrich Chemical Co., Milwaukee, Wis.) 10^{-4} or 10^{-5} M, for 1 h at 37°C, then dialyzed and tested.

An active fraction eluted from G100 column was treated with various enzymes: trypsin (up to 2 mg per ml), papain (70 μg per ml), pronase (50 μg per ml), carboxypeptidase A (70 μg per ml), ribonuclease (50 μg per ml), chymotrypsin (2 U per ml), pepsin (82 μg per ml) and neuraminidase 50 μg per ml), for 4 h at 37°C under appropriate conditions. All proteolytic enzymes, obtained from Worth-

ington Biochemical Company, Freehold, N. J., were tested on a hemoglobin substrate and found to be active.

Results

Effect of Macrophage Culture Fluids on Thymocyte and Spleen Cell Proliferation. Most of the experiments were made with culture fluids obtained from PEC of A/St mice harvested 3 days after injection of 1.5 ml of 10% proteose peptone (Difco Laboratories). In Table I we present the result of 19 different

TABLE I

Thymocyte Response to A/St PEC Culture Fluids

Culture fluid no.	Background	Amount of PEC supernate		
		10%	25%	50%
	cpm			
147	678 ± 115	4,957 ± 608	8,294 ± 699	9,719 ± 1,008
154	314 ± 41	13,138 ± 819	14,580 ± 1,564	—
157	1,082 ± 50	22,628 ± 5,032	12,255 ± 1,753	18,922 ± 2,265
158	1,199 ± 136	1,624 ± 109	—	2,717 ± 175
160	445 ± 86	2,591 ± 202	—	9,890 ± 669
161	586 ± 77	910 ± 124	—	876 ± 164
171	571 ± 10	761 ± 86	—	1,801 ± 262
172	468 ± 39	3,123 ± 1,314	—	7,716 ± 221
174	785 ± 128	10,522 ± 1,648	20,827 ± 1,057	27,747 ± 1,919
175	615 ± 77	7,060 ± 935	17,040 ± 1,309	31,057 ± 1,827
176	801 ± 58	3,331 ± 157	7,448 ± 206	15,911 ± 1,130
176-A	736 ± 48	1,419 ± 160	2,840 ± 235	5,112 ± 371
189	425 ± 22	647 ± 54	621 ± 48	629 ± 48
190	425 ± 22	628 ± 59	774 ± 65	1,120 ± 130
191	961 ± 47	—	3,400 ± 450	6,115 ± 625
192	961 ± 47	—	1,493 ± 272	2,216 ± 186
196	425 ± 22	3,870 ± 200	6,571 ± 256	—
197	836 ± 176	—	8,629 ± 251	12,912 ± 1,623
200	402 ± 82	644 ± 120	998 ± 109	2,124 ± 135

Thymic cells were cultured for 72 hs with different amounts of macrophage culture fluid or in regular medium. Values represent cpm of [^{3}H]thymidine in trichloracetic-insoluble material.

culture supernates tested on thymocytes. Of the 19 supernates, 15 stimulated significantly the incorporation of thymidine into DNA, while four had minimal, if any, effect. The degree of stimulation varied among the 15 stimulatory fluids—indeed, six of these produced stimulation of over 10,000 cpm over background incorporation usually not exceeding about 600 cpm, while the remaining produced stimulations ranging from about 2,000 cpm to about 9,000 cpm.

Several experiments clearly indicated that the stimulatory activity of PEC fluid was not restricted in its action to thymocytes of the same strain. For example, PEC fluids generated in cells of A/St (an H-2^a) mice stimulated proliferation of syngeneic thymocytes or thymocytes from BALB/c (H-2^d) or A.By (H-2^b) to the same extent.

The response of spleen cells to macrophage culture fluid was of small magnitude, not exceeding in nine different culture fluids tested an E/C value of three (see also Reference 16). Culture fluids that produced good stimulation of thymocytes only induced meager stimulation of spleen cells. One such culture fluid, for example, resulted in an E/C value on thymocytes of 30 while only 1.9 on spleen cells.

The In Vitro *Antibody Response to F in the Presence of Macrophage Culture Fluids.* We set up cultures of spleen cells from mice immune to F-KLH without the addition of antigen, adding F-KLH or F-RGG, and with or without different concentrations of macrophage culture fluids. In most experiments the macrophage culture fluids were obtained from peptone-stimulated mice—of nine such culture fluids tested, seven displayed good stimulatory activity. In the experiment shown in Fig. 1, spleen cells were cultured at three concentrations of the two antigens as well as in three different concentrations of macrophage supernates (10, 25, and 50% vol/vol). The spleen cells were obtained from mice primed 3 mo previously with F-KLH in alum. At a concentration of 0.1 μg of F-KLH the PFC response was markedly augmented proportional to the dose of macrophage supernate added to the culture. Of interest are the results using a heterologous carrier, F-RGG. PFC to F-RGG in the absence of macrophage supernate were not over the background level. In the presence of macrophage supernates, however, the cells responded with 530 direct PFC and 840 indirect PFC. That is to say the macrophage supernate had stimulated the cells for a
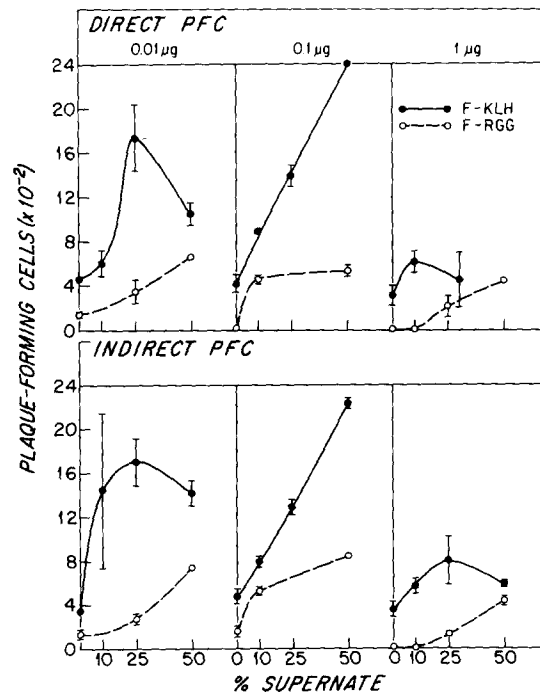

Fig. 1. The PFC response of F-KLH-primed spleen cells to the F-KLH or F-RGG at three concentrations of each in the presence of different doses of macrophage culture fluids. Bars represent value of one standard error of the mean.

response similar to that present under conditions of complete stimulation with F-KLH. (Cell viability was not determined. However, in other experiments, after 4 days of culture, viability was about 30% regardless of whether the cultures contained macrophage supernatants and/or antigens.)

In the experiment of Fig. 1 we did not include the effects of macrophage cultures in the absence of antigen. This point was studied in the experiment of Fig. 2 which also evaluated macrophage supernates obtained from various strains
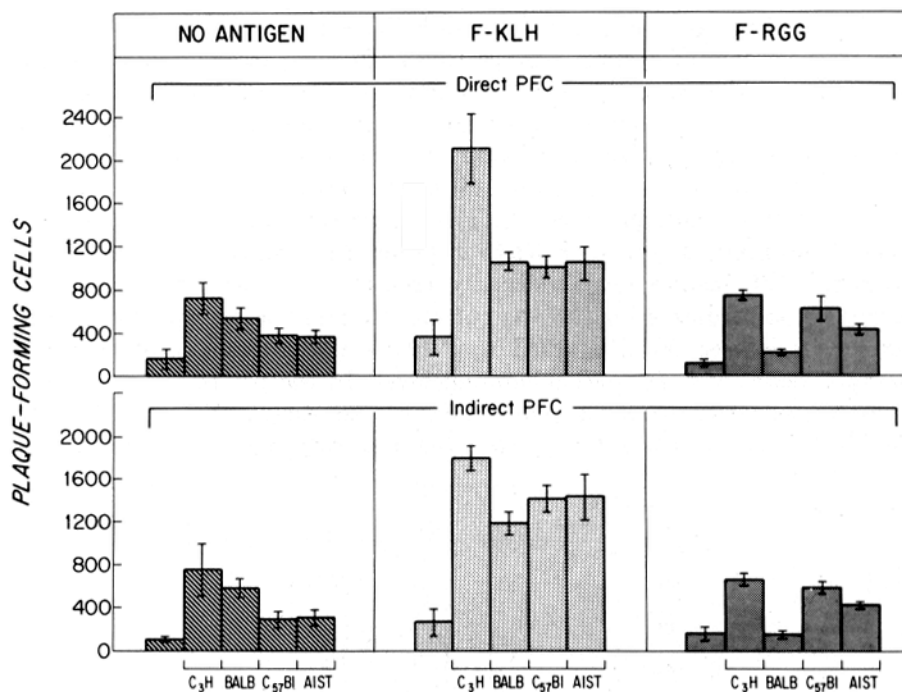

FIG. 2.   The PFC response to F induced by macrophage supernatants from various strains of mice differing in their H-2 haplotype. F-KLH-primed A/St spleen cells were incubated 4 days in the presence or absence of hapten conjugates on homologous (KLH) or heterologous carrier (RGG).

of mice differing in their H-2 haplotype from the A/St spleen cells used in cultures. Despite some variations, all the macrophage culture fluids irrespective of the donor strain increased the response to F. In this experiment of Fig. 2 the response to F-KLH was of the order of 367 direct and 287 indirect PFC; addition of F-KLH and the macrophage supernate—for example, from C3H mice at a 50% concentration—increased it to 2,133 direct and 1,817 indirect PFC; the culture of spleen cells with the macrophage culture fluids in the absence of any antigen increased the response to 730 direct and 773 indirect PFC, which was of the same order of magnitude as cultures with F-RGG plus the macrophage supernate (760 direct and 660 indirect PFC). In other words, the culture fluids from macrophages had produced a stimulation of the B cells without the need for addition of antigen to the culture. Likewise, as in the experiment of Fig. 1, the stimulation of carrier T-cell function was quite evident.

The Use of Anti-θ-Treated Spleen Cells.   In the experiment shown in Fig. 3 we examined the PFC response of spleen cells depleted of T cells by treatment with anti-θ and complement. Untreated spleen cells made a modest number of PFC upon challenging with F-KLH; similar results were obtained by culturing the cells in the presence of macrophage culture fluids without addition of F-KLH; as before, a marked enhancement was observed in cells cultured in the presence of
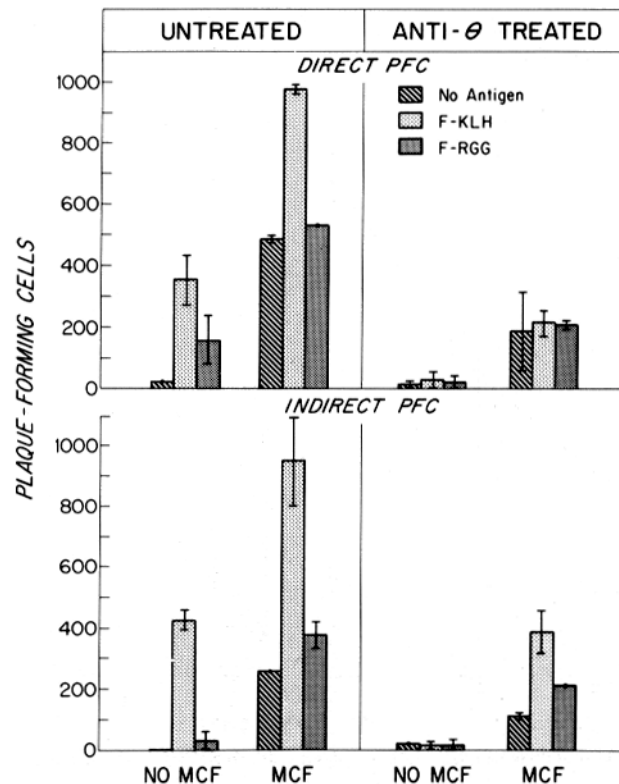

FIG. 3.   The PFC response of spleen cells treated with anti-θ and complement to macrophage culture fluids (MCF). Normal or treated F-KLH-primed spleen lymphocytes were incubated with or without antigen as in the previous figure in the presence or absence of macrophage supernatants.

both macrophage culture fluids and F-KLH. Anti-θ-treated spleen cells did not respond as expected. Culture of such cells in macrophage fluids, however, produced a response to about 200 direct and indirect PFC. Not shown in Fig. 3 is the additional control that brings out an interesting relationship. We added to the anti-θ-treated spleen cells (10^7) an equal number of spleen cells from a KLH-immune mouse given 750 R of whole-body X-irradiation just before harvesting. Such cells provided helper T-cell activity producing now strong responses upon challenged with F-KLH of 3,010 direct and 4,560 indirect PFC, respectively. This strong anti-F response was not enhanced any further by culturing the cells with macrophage culture fluids and F-KLH (i.e., 2,740 direct and 4,500 indirect PFC). The experiment suggested that in order to see the effects

of the macrophage culture fluids in enhancing helper activity it was best to use culture conditions of limited T-cell function.

Effects of Macrophage Supernates on Cultures of Nude Mice Spleen Cells. Spleen cells from nude athymic mice developed no indirect PFC in culture and a small direct PFC response (Fig. 4). The addition of culture supernates from macrophages plus SRBC increased the direct PFC response and allowed for an indirect PFC response. Note that the increase in PFC necessitates the addition of both SRBC plus macrophage supernate; the latter by itself only produced a marginal increase in PFC.
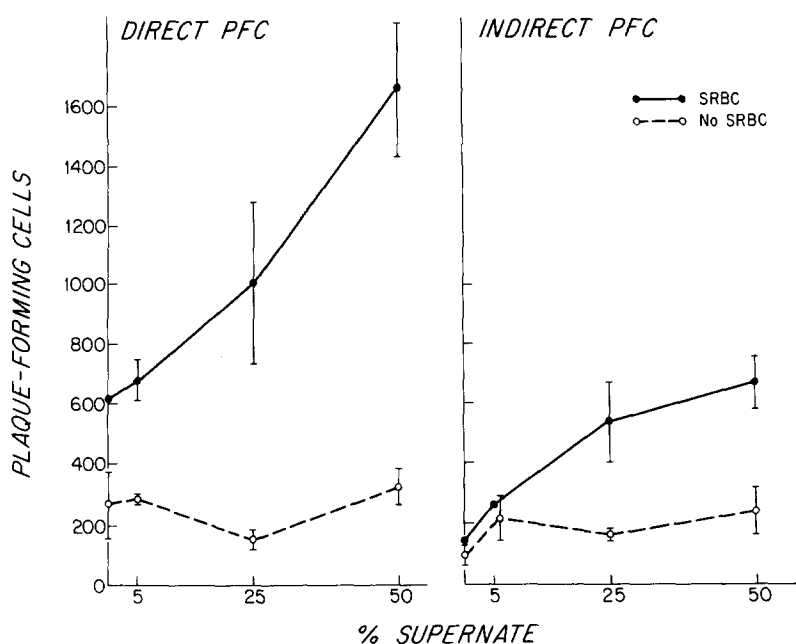

Fig. 4. The PFC response of nude mouse spleen cells to SRBC in the presence of macrophage supernates.

The Cell Source of the Stimulatory Activity. It is possible that other cells in the PEC besides the macrophages could be the source of the stimulatory material. The best candidate could be a T lymphocyte adherent to the dish and not eliminated by the handling and washing procedure. To eliminate such putative T cells, the PEC were treated with anti-θ antibodies in the presence of fresh guinea pig serum. Culture fluids were generated from these anti-θ-treated PEC, from cells treated with normal mouse serum and complement, and from untreated cells. It was observed that PEC treated with anti-θ produced the same degree of stimulation as PEC treated with normal mouse serum. This was tested on thymocyte proliferation and on the PFC response to F with identical results. In agreement with this observation, it was found that PEC obtained from X-irradiated mice generated in culture good stimulatory activity. Such PEC were made entirely of macrophages. Culture fluids from 3T3 cells, mouse embryo cells, or unstimulated spleen lymphocytes did not contain stimulatory activity.

The experiment in Fig. 5 shows similar increases of PFC without the need of addition of antigen in the presence of PEC fluids generated from either untreated PEC or anti-θ-treated PEC. The experiment of Fig. 5 also stresses the important relationship between the stimulatory activity from macrophages and the T-cell cooperative activity. The spleen cells used for this experiment were harvested from recently boosted mice. These cells, upon challenge with F-KLH, exhibited
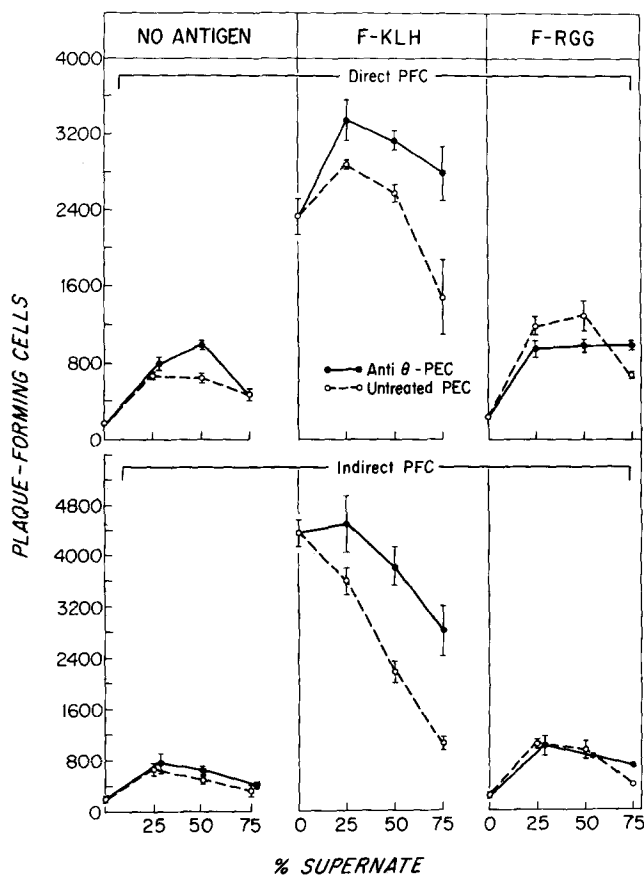
Fig. 5.   Assessment of PFC on spleen cells incubated with supernates from peritoneal exudate cells treated with anti-θ or normal serum and complement.

a much larger number of PFC than those shown in the previous series depicted in Figs. 1-3. In the presence of such larger responses, the addition of macrophage supernates results only in a slight enhancement of the F-KLH response (central panels); furthermore, at higher concentrations, the supernates clearly had an inhibitory effect, the response being lowered up to one-half or one-third of its original level.

Attempts to Establish the Nature of the Stimulatory Molecule.   The nature of the stimulatory factor is not known. The factor can be generated in cultures free of fetal calf serum, is resistant to freezing and thawing, and sensitive to boiling.

Because macrophages can secrete a plasminogen activator-like material (20),

we examined whether the stimulatory material was sensitive to DFP. There was no loss of activity by treatment with DFP (i.e., 25 to 50% concentrations of untreated supernates stimulated thymocyte incorporation of thymidine to 2,910 cpm and 12,722 cpm, respectively; figures for DFP-treated supernates [at 10⁻⁴ M] were 3,367 cpm and 10,341 cpm; background incorporation was 642 cpm).

Culture fluids generated from A/St PEC were found not to lose any of their thymocyte-stimulating activity by passing through an anti-H-2^a Sepharose 4B column (i.e., untreated PEC fluid: stimulation of 3,917 cpm ± 423; PEC fluid after passing through normal mouse serum column: 4,923 ± 392; PEC fluid passed through anti-H-2^a column: 6,087 ± 698; all fluids were tested at three concentrations, but the indicated results are at 50%.

The size of the active fraction was investigated using gel filtration (Fig. 6). The active moiety eluted from a G100 column in a position lighter than trypsin and heavier than ribonuclease. Activity stimulating thymocyte proliferation was only found in fractions ranging from 15,000 to 21,000 daltons (Fig. 6), in general agreement with data obtained by Gery and Handschumacher (21). Limited studies have been done, so far, assaying on PFC responses. It is clear, however, that a pool of fraction active in thymocyte proliferation likewise resulted in PFC cell stimulation. The active fraction eluted from a G100 was treated with several enzymes and analyzed for stimulation of thymocyte proliferation. The stimulatory material was resistant to trypsin, ribonuclease, carboxypeptidase A, papain, and neuraminidase. It was reduced by 41% by treatment with pronase and totally destroyed by chymotrypsin and pepsin.
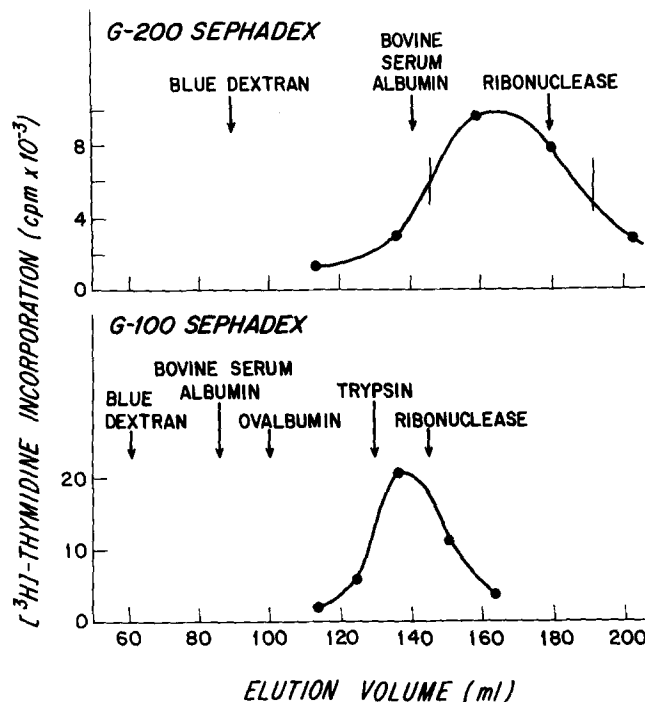

Fig. 6. The graph shows the fractionation of macrophage culture fluids in Sephadex G200 or G100. The figure shows only those fractions with cpm over background.

Discussion

Cultures of peritoneal cells rich in macrophages contained activities that stimulated DNA synthesis in thymocytes and, to lesser extent, in spleen lymphocytes. The stimulation of thymocytes did not require the addition of lectins to the culture, as was first reported in the original papers of Gery, et al. (10, 11). In agreement with them, a marked potentiation of proliferation was observed by addition of PHA (16). The culture fluids also contained an activity that influenced B-cell differentiation. Whether both activities—thymocyte proliferation and B-cell stimulation—reside in the same molecule, as suggested by the early fractionation experiments, needs to be verified using more purified materials. (In the context of this discussion, both activities are considered as a single entity.) An important requirement for detecting the stimulatory molecule was to eliminate by dialysis a small molecular weight inhibitor of DNA and protein synthesis (17). This inhibitor of less than 1,000 daltons stopped cell division and protein synthesis of a number of cells in a reversible manner. As with the stimulatory molecule, it was found associated with macrophages in cultures. The exact relationship of these contrasting molecules clearly needs to be established.

Several points concerning the stimulatory molecule have been reasonably well documented. All evidence points to the macrophage as the cell source. Indeed, cultures of macrophages free of lymphocytes, in particular those of the T class, generated active fluids. In contrast, cultures of unstimulated lymphocytes or of other cell lines failed to produce the activity. A second point of interest is the relationship between the biosynthesis and/or secretion with the activity of the macrophage. Indeed, in experiments to be reported, we have found that phagocytosis enhanced the amount of active material in culture by several fold. Further studies are in progress attempting to dissect the relationship between membrane binding, interiorization, and/or cellular activation by various materials with the actual biosynthesis and secretion of the stimulatory molecule.

The stimulatory molecule has a size ranging between 15,000 to 21,000 daltons and lacks H-2 determinants. Hence, by both these criteria, this molecule can be separated by the thymic factors which are of larger size and which contain some antigens coded by H-2 complex, therefore, binding to anti-H-2 antisera (22–29). It is possible, however, that crude, unfractionated preparations of thymic factors could contain this macrophage molecule, since, under the culture condition where they are generated, macrophages have not been excluded. The stimulatory molecule was heat labile, resistant to trypsin treatment, and partially affected by pronase. This suggests that it is a protein with a low content of basic residues not available to tryptic digestion. The fact that it is resistant to DFP rules out that it is a serine esterase such as the plasminogen activator-like molecule secreted also by macrophages.

In order to obtain a better perspective of the functional significance of this stimulatory molecule from macrophages, certain points need to be clearly evaluated: (a) The stimulatory molecule clearly exerted an effect on thymocytes and on T helper cells; the observation that anti-θ-treated spleen cells or nude mice spleen cells responded can be interpreted to mean that the stimulatory molecule also acted directly on B cells. Alternatively, the above results may be

explained by a primary effect on some low level of residual T cell or of stem cells. Hence, it is possible that the macrophage stimulatory molecule may act directly on B cells or via T-cell stimulation or under both conditions. (*b*) The stimulation of hapten-primed cells without antigens raises the possibility that macrophage stimulatory molecule by itself—directly or indirectly—could allow for expansion and differentiation of any B-cell clone. However, the need for antigen in the experiments with nude mice spleen cells contrasts with the apparent lack of requirement in the F-KLH system. Either lymphocytes differ in the response to the molecule—the primed population having a lower threshold for response—or some sequestered antigen, present in the F-KLH-primed cells, is being exposed as a result of disruption of the spleen and serves as an added stimulus to the macophage molecule. (*c*) The fact that macrophages are made to secrete more of the stimulus as a result of uptake and phagocytosis of particles clearly indicates that the process of synthesis and/or secretion is under some form of modulation by extracellular materials that interact with the macrophage. Investigation of the factors that modulate the synthesis and secretion of this molecule may give us some clue as to its possible intervention in immune process—indeed, we are thinking along the lines that lymphocyte products or a number of adjuvants (most of which are known to be taken up by phagocyte) could have some of their effects via the stimulation of the macrophage. And (*d*) the relationship between the stimulation of macrophages and the T-helper function needs to be closely evaluated—the results suggest a clear synergism of both when T-cell helper function is less apparent (Figs. 1 and 2) and an antagonistic effect under optimal T-cell helper function (Fig. 5). Perhaps the B cell is turned off after a certain level of stimulation or perhaps the antagonism reflects competition for a membrane receptor of two activities.

It is our hypothesis that the macrophage plays a very early regulatory role in immune induction. Clearly, the regulatory role of phagocytes could be accomplished not only as a result of antigen uptake, degradation, and focusing, but also as a result of secretion of the kind of molecule described herein.

Summary

Culture fluids of peritoneal exudate cells rich in macrophages stimulated DNA synthesis of thymocytes and, to lesser extent, of spleen cells. We also investigated the effects of culture fluids from macrophages on the in vitro response to a hapten-carrier protein (fluorescein-hemocyanin) using spleen cells from immune mice. Macrophage culture fluids contained an activity that increased the plaque-forming cell response of both IgG and IgM class. This increase was observed in the absence of any added hapten protein to the culture. The helper function of T lymphocytes (as evidenced by challenging with the hapten on the homologous carrier) was also increased by the macrophage culture fluid. However, this enhancement was best observed in conditions of relatively low T-cell activity. Also, the macrophage fluid allowed spleen cells of nude athymic mice to make a plaque-forming cell response to sheep red blood cells of both the IgM and IgG class. The macrophage was the cell source of the stimulatory molecule since it was generated only in cultures of macrophages devoid of

significant number of lymphocytes. Stimulatory activity was not found in cultures of lymphocytes, mouse embryo cells, or 3T3 cells. The thymocyte stimulatory molecule did not contain *H-2* antigens, was resistant to diisopropylfluorophosphate treatment, eluted from Sephadex with a size ranging from 15,000 to 21,000 daltons, and was sensitive to chymotrypsin and pepsin.

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