DEMONSTRATION OF A SOLUBLE MEDIATOR THAT INDUCES EXUDATES RICH IN Ia-POSITIVE MACROPHAGES*

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Ia antigens play a fundamental role in the immunoregulatory function of macrophages and of other accessory cells, governing events such as antigen presentation to T cells and T cell proliferation in mixed leukocyte reactions. Recent studies have indicated that macrophages in the various tissues can be separated into two apparent sets, depending on the presence or absence of detectable surface-bound Ia molecules (1–6). Interestingly, the balance of Ia-positive and Ia-negative macrophages in peritoneal exudates or in spleen may be subject to environmental regulation (7). Intraperitoneal injection of inflammatory, nonimmune stimuli, such as mineral oil, resulted in exudates in which the great majority of macrophages had no detectable surface Ia and were thus similar to the resident population. However, exudates that developed during the response to immunogenic stimuli consisted predominantly of Ia-positive macrophages. The increase in Ia-bearing macrophages was particularly striking after infection with the intracellular bacterium *Listeria monocytogenes*. Experiments using adoptive transfer of immune cells indicated that the T cell, upon appropriate antigenic stimulation, was responsible for augmenting the level of Ia-bearing macrophages in the exudate. In this report, we have concerned ourselves with the mechanism by which T cells elicit this response. We present data indicating that a soluble mediator elaborated during interaction of antigen-stimulated T cells and macrophages in vitro is responsible for the induction of exudates rich in Ia-positive macrophages.

Materials and Methods

Mice. Most experiments were done with A/St mice of either sex, 8–12 wk of age, obtained from West Seneca Laboratories, Buffalo, N. Y. C57BL/6 (BL/6) mice were bred in our own facility at the Harvard Medical School, Boston, Mass.

Basic Experimental Protocol. Previously, we reported that intraperitoneal infection with *Listeria* produced a rapid increase in Ia-positive macrophages and that this cellular response was an immunological event transferable by immune T cells (7). The present experiments were designed to test whether a soluble mediator elaborated by the immune exudate cells might cause the increment in Ia-bearing macrophages: mice were infected intraperitoneally with live *L. monocytogenes* and their peritoneal exudate cells (PEC)[1] were harvested; the exudates were cultured with heat-killed *Listeria* organisms; the culture fluids were collected, filtered, and injected intraperitoneally into normal mice which were later killed and examined for the number of Ia-positive and Ia-negative macrophages in the PEC.

In most experiments, the PEC were harvested from three mice and pooled. Individual

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[1] *Abbreviations used in this paper:* BL/6, C57BL/6; MIRF, macrophage (Ia$^+$)-recruiting factor, PEC, peritoneal exudate cell(s); SDS, sodium dodecyl sulfate.

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experiments always employed mice of the same sex and age. Overall, results of experimental procedures were highly reproducible.

Macrophage surface Ia was detected by immunofluorescence using a hybridoma anti-I-A^k, clone 10-2.16, which originated in the laboratory of Dr. L. A. Herzenberg, Stanford University, Stanford, Calif. (8) or, occasionally, using an A.TH-anti-A.TL serum. Both antibodies gave identical results. Controls for specificity have been described previously (7). The PEC were cultured for 2 h in 15-mm-diameter wells that contained cover slips, fixed in paraformaldehyde, and then stained (7). The usual procedure consisted of exposing the cells on cover slips to the monoclonal anti-I-A antibody (5 μg/ml), followed by exposure to fluorescein isothiocyanate-conjugated $F(ab')_2$ fragments of rabbit-anti-mouse immunoglobulin (100 μg/ml). Each incubation was for 30 min on ice. For each protocol, we report the percentage of Ia-positive macrophages and the absolute number of Ia-positive and Ia-negative macrophages per mouse calculated from estimates of total exudate cells, the percentage of cells adherent to cover slips, and the percentage of Ia-positive macrophages. Ia-negative macrophages are those macrophages not exhibiting Ia immunofluorescence using the antibodies. Increasing the concentration of the anti-I-A antibody 100-fold did not change the results. We have confirmed the validity of these determinations by showing that the Ia-negative macrophages do not synthesize I-A and have no detectable I-A molecules using a sensitive assay with ^{125}I-labeled antibody; furthermore, similar results have been found scoring the cells in a fluorescence-activated cell sorter ([9]; and M. G. Scher, D. I. Beller, and E. R. Unanue. Unpublished observations.).

Culture Fluids. The culture fluids were generated by incubating the PEC for 24–48 h with heat-killed *Listeria* organisms. Usually, the exudates were from mice infected twice intraperitoneally with 2×10^4 live *Listeria* (mean lethal dose $[LD_{50}] = 2 \times 10^5$) 17 d and 10 d before and injected 3 d previously with 1.5 ml of 10% proteose peptone. The intraperitoneal injection of peptone results in a marked increase in *Listeria*-immune T cells in the peritoneal cavity. The entire peritoneal exudate, composed of ~70% macrophages and 30% small- and medium-sized lymphoid cells, was incubated at 4×10^6 cells/ml in RPMI-1640 medium with 0.5% fetal calf serum, 10 mM Hepes buffer, penicillin, streptomycin, and 10^6 heat-killed *Listeria* organisms/ml in 150-cm^2 tissue culture flasks (20 ml/flask). In a few experiments, the cells were cultured in 15-mm-diameter culture wells at 10^6 in 1 ml. Control fluids consisted of: (*a*) the same cells cultured without *Listeria*; in many instances, the fluids were obtained at the end of the culture period and *Listeria* was added to them; (*b*) media not conditioned by any cells, to which, in some instances, *Listeria* was added. All culture fluids were centrifuged at 1,000 rpm for 10 min, passed through a 0.45-μm filter (Millipore Corp., Bedford, Mass.), aliquoted, and injected immediately or frozen at −20°C until use.

We found that the ability of immune PEC to produce active culture fluids is lost between 24 and 48 h of culture. Thus, the medium was routinely harvested after 24 h. In some experiments, the cells were then resuspended in fresh medium with additonal *Listeria*. After another 24-h incubation, a second culture fluid was obtained. The culture fluids from the first and second day were then tested independently in vivo; and if both were found to be active, they were pooled for subsequent use.

In one experiment, the PEC were harvested from mice immunized to sheep erythrocytes or hemocyanin from giant keyhole limpets (*Megathura crenulata*). One group of mice was immunized twice intraperitoneally with 10^7 washed sheep erythrocytes at 7-d intervals, followed by 1.5 ml of 10% proteose peptone 7 d later. Another group was immunized subcutaneously with 50 μg of keyhole limpet hemocyanin in complete Freund's adjuvant in the hind foot pads and flanks, followed 7 d later by 50 μg in saline intraperitoneally; 1.5 ml proteose peptone was injected intraperitoneally 7 d later.

In some experiments, a lymphocyte preparation depleted of macrophages and enriched in functional T cells was obtained by culturing the PEC and eliminating the cells that adhered to the culture dish (20 ml of a suspension of PEC at 4×10^6/ml in a 150-cm^2 tissue culture dish for 60 min at 37°C). The adherence procedure was repeated three times. This produced a lymphocyte population that routinely contained 1–3% macrophages as determined by latex uptake and esterase staining.

Biosynthesis of I-A. PEC (10^6) were incubated in 1 ml of RPMI-1640 medium with 5% fetal calf serum and antibiotics in 15-mm tissue culture wells for 2 h. The wells were washed

extensively to yield an adherent population of >98% macrophages, as determined by criteria previously described (7), and pulsed with 1 μCi of [^{3}H]leucine (New England Nuclear, Boston, Mass.) in leucine-deficient RPMI-1640 medium for 6 h at 37°C. Wells were again washed extensively and the cells solubilized by the addition of 0.5 ml of 0.5% Triton X-100. A 200-μl aliquot of the soluble material was precipitated with 200 μl of anti-I-A^k, followed by binding to protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Stockholm, Sweden). Finally, material bound to protein A was released by sodium dodecyl sulphate (SDS), reduced by 2-mercapto-ethanol, and run in 10% polyacrylamide gel electrophoresis. As a control, the soluble material was incubated with 200 μl of normal IgG_{2b} protein and processed as above.

Miscellaneous. Fc receptors were quantitated by binding of sheep erythrocytes (30 min, room temperature) opsonized with IgG antibodies. C3 receptors were detected by binding of erythrocytes opsonized with IgM antibodies and C5-deficient immune serum as a complement source. Phagocytosis was determined after incubation with latex beads of 1-μm diameter for 2 h at 37°C.

Killing of T cells with anti-Thy-1.2 was done by using conventional methods with reagents previously standardized: AKR-anti-C3H thymocyte serum was employed at a 1:3 dilution for 30 min at 4°C, followed by 10% guinea pig serum for 45 minutes at 37°C as the complement source. Ia-positive macrophages were depleted by incubating PEC with the monoclonal anti-I-A antibody (10 μg/ml) for 30 minutes at 4°C, followed by incubation in a mixture of 10% rabbit and 10% guinea pig serum for 45 min at 37°C.

Treatment of culture fluid with insolubilized protease was done using Enzite Agarose Protease (lot 1449; Miles Laboratories, Inc., Elkhart, Ind.). The sample was incubated with 1 mg/ml of enzyme for 1 h on a rotary shaker (Bellco Glass, Inc., Vineland, N. J.).

Results

Production of Exudates Rich in Ia-positive Macrophages by a Soluble Molecule Elaborated in Culture. 24-h culture fluids from *Listeria*-immune PEC incubated with heat-killed *Listeria* were evaluated for their potential to substitute for intact immune T cells in the induction of peritoneal exudates highly enriched in Ia-positive macrophages. Fig. 1 summarizes the results of 10 different experiments performed during the course of this investigation in which we evaluated individual culture fluids. All the culture fluids were generated from *Listeria*-immune PEC cultured for 24 h, and the level of Ia-bearing macrophages in all exudates was evaluated after several injections of the culture fluid. The resident population in the peritoneum of uninfected mice contained an average of 1.45×10^5 Ia-positive macrophages representing 8.2% of the total macrophage population. This is based on an average yield of 28.4×10^5 PEC/mouse with a 62.3% adherent population. Injection of medium did not significantly change the composition of the exudate (1.9×10^5 Ia-positive macrophages/mouse representing 12.8% of the adherent population, based on an average yield of 23.6×10^5/mouse with 63% adherence). Injection of culture fluid from *Listeria*-immune cells not exposed to *Listeria* in vitro was also without substantial effect on the ratio of Ia-positive to Ia-negative macrophages, although the absolute number of macrophages sometimes significantly increased. Thus, whereas on the average 13.9% of the macrophages were Ia-positive, their number increased to 5.4×10^5, with a corresponding increase in Ia-negative macrophages (based on an average yield of 56.6×10^5 PEC/mouse with 68.6% adherence). The increase in macrophages in response to this control factor was not a consistent effect and varied considerably from experiment to experiment.

In contrast, fluids of *Listeria*-immune PEC cultured with *Listeria* generated exudates in which the absolute number of Ia-positive macrophages averaged 28.5×10^5, 5–20 times the level found in normal or in control-injected mice. The percentage of

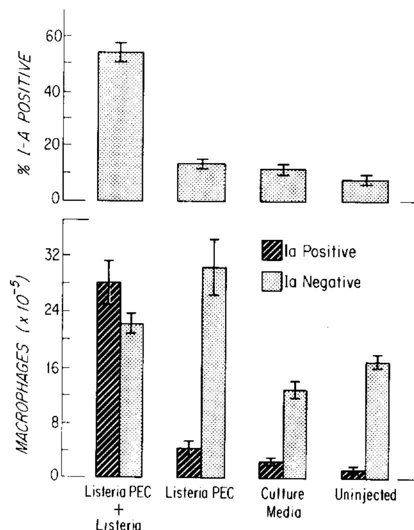

Fig. 1. Induction of exudates rich in I-A-positive macrophages by a soluble factor from immune PEC. This graph shows our first 10 experiments injecting 0.5 ml of culture fluid intraperitoneally every 6 or 12 h for 2 d. PEC were harvested either 6 or 12 h after the last injection. Indicated are the components of the 24-h culture. Listeria-PEC plus Listeria: immune PEC from *Listeria*-infected mice plus heat-killed *Listeria*. Listeria PEC: immune PEC not challenged with *Listeria*. Culture media: media not conditioned by any cells. Included in the last two groups are experiments in which *Listeria* organisms were added at the end of the 24-h incubation. The results were pooled because they are indistinguishable from those where *Listeria* was not added. Error bars: 1 SEM.

adherent cells that bore Ia ranged from 32 to 71%, with an average of 54%. This is based on an average yield of 71.0×10^5 PEC/mouse with 74.3% adherence.

At present, a total of 15 different culture fluids have been generated, all of which have been active, promoting an increase in both the number and percentage of Ia-positive macrophages relative to control culture fluids. However, there was virtually no change in the nonadherent populations, both in terms of the percentage they comprise of the total exudate elicited and in terms of the relative number of Thy-1.2- or immunoglobulin-positive cells. In a representative experiment, we evaluated the nonadherent populations for staining with either anti-Thy-1.2 antibody, followed by fluorescent $F(ab')_2$ fragments of rabbit anti-mouse immunoglobulin, or normal mouse serum, followed by fluorescent $F(ab')_2$ fragments of rabbit anti-mouse immunoglobulin. The latter procedure would stain Ig-bearing cells, whereas the former would stain both T cells and B cells. The distribution of Thy-1-bearing, Ig-bearing, and null (i.e., without T or B cell markers) lymphocytes in uninjected mice was 40.7, 42.3, and 17.0%, respectively; in mice that received a control culture fluid, we found 43.4, 38.1, and 18.5%, respectively; whereas mice that received the active *Listeria* culture fluid showed 44.1, 37.1, and 18.8%, respectively.

In several experiments, we quantitated the active principle by injecting the fluids diluted severalfold. Fig. 2 shows the results of a representative experiment. Routinely, dilution of the factor results in a corresponding reduction in the extent of the response, although comparison of the titration profile from five separate factors suggested a significant variability in their relative potency: in two experiments, culture fluids were diluted 20- to 50-fold with no significant loss of activity.

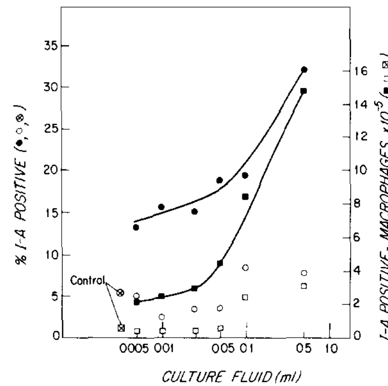

FIG. 2. Titration of active and control culture fluids: 0.5 ml of each dilution was injected intraperitoneally every 12 h for 2 d. PEC were harvested 12 h after the last injection. (●, ■) refer to culture fluids from *Listeria*-immune PEC cultured with heat-killed *Listeria*; (○, □), *Listeria*-immune PEC not challenged with *Listeria*; (⊗, ⊠), uninjected mice. Each point represents a pool of three mice.

It is noteworthy that our initial experiments employing a single intraperitoneal injection of undiluted culture fluid did not produce a significant change in either the percentage or absolute number of Ia-bearing macrophages in the peritoneal exudates. The results shown in Fig. 1 were obtained using a multiple-injection protocol. Representative experiments, evaluating various schedules, are shown in Table I. Experiments 1 and 2 show that the injection schedule, rather than the absolute amount of injected culture fluids, is the critical factor governing induction of exudates rich in Ia-positive macrophages and reveal the need for several injections in a 24–48-h period. Thus, in experiment 1, a total of 2 ml divided among four injections over a 2-d period resulted in a marked increase in the percentage as well as in the absolute number of Ia-positive macrophages. The same amount given in one or two injections produced little or no effect. Culture fluids from PEC not challenged with *Listeria* could augment the number of macrophages but had minimal effect on the percentage of Ia-positive macrophages.

We evaluated the kinetics of appearance of Ia-positive macrophages in the experiment shown in Fig. 3. An active culture fluid was injected one to four times, at 12-h intervals, and the PEC examined 12 h after each injection. The earliest increase in Ia-positive cells was found 12 h after the second injection, whereas the maximal level was found 12 h after the fourth injection. Although the absolute number of cells in the exudate dropped to approximately normal levels within another 12 h, the Ia-positive macrophage remained the dominant phenotype for at least 14 d, the last time point tested. In contrast, mice that were evaluated 24 h after the 3rd injection had an obvious reduction in Ia-positive macrophages (legend to Fig. 3). It is also clear in Fig. 3 that at no time tested was there an Ia-rich exudate generated using the control supernate. In subsequent experiments, therefore, we used the protocol of injecting 0.5 ml of culture fluid four times at 12-h intervals as the optimal schedule for inducing and maintaining high levels of Ia-positive macrophages. Thus, we have demonstrated that a soluble mediator produced during an in vitro immune reaction is capable of inducing exudates in vivo with a uniquely high representation of Ia-positive macrophages. We refer to this mediator as macrophage (Ia^{+})-recruiting factor (MIRF).

TABLE I

Demonstration of a Soluble Mediator Capable of Inducing Exudates Rich in Ia-positive Macrophages *

Culture fluid	Injection schedule	Percent Ia⁺	Ia⁺	Ia⁻
			$\times 10^{-5}$	$\times 10^{-5}$
Experiment 1				
No injection		5.8	0.6	9.3
Listeria-PEC + *Listeria*	2 ml × 1	13.8	2.0	12.5
	1 ml every 24 h × 2	26.8	5.4	14.6
	0.5 ml every 12 h × 4	48.2	35.1	37.8
Listeria-PEC	2 ml × 1	12.0	2.4	17.6
	1 ml every 24 h × 2	10.9	3.8	31.4
	0.5 ml every 12 h × 4	15.2	9.6	53.7
Experiment 2				
No injection		7.3	1.3	17.0
Listeria-PEC + *Listeria*	0.5 ml every 6 h × 4	37.3	9.3	15.7
	0.5 ml every 12 h × 4	56.3	25.3	19.7
	0.5 ml every 12 h × 2	13.8	3.7	23.3
	1.0 ml every 12 h × 4	45.2	18.2	22.1
	0.5 ml every 6 h × 8	58.7	29.4	20.7
Listeria-PEC	0.5 ml every 6 h × 8	17.0	12.0	58.7
Culture medium	0.5 ml every 6 h × 4	9.5	4.2	39.7
	0.5 ml every 12 h × 4	15.4	3.3	18.4
	0.5 ml every 24 h × 2	15.6	1.4	7.6
	1.0 ml every 12 h × 4	13.0	2.6	17.4

* PEC from all groups were harvested simultaneously 60 h after the first injection. Percent Ia-positive: percentage of macrophages with detectable surface Ia. The absolute number of Ia-positive or Ia-negative macrophages per mouse, indicated in the last two columns, were estimated from the total PEC recovered and the percentage of cells that adhered, usually 75% of the plated cells. *Listeria*-PEC, *Listeria*-immune PEC.

We more fully evaluated the control culture fluid generated from immune PEC cultured in the absence of additional antigen stimulation in experiments shown in Table II. We tested the possible activity of the heat-killed *Listeria* preparation (which is ineffective when injected by itself or with nonimmune T cells [7]), by reconstituting the control medium with this heat-killed *Listeria*. It is apparent from this experiment that the *Listeria* must exert its effect in culture during mediator production, rather than providing an independent stimulus in vivo. This interpretation is further substantiated by the results obtained in experiments 2 and 3 (Table II), in which we determined if immune PEC were required to produce the immunoregulatory activity in question. It is clear that peptone-elicited PEC from nonimmune mice, when cultured with *Listeria*—or for that matter, with other antigens like hemocyanin or sheep erythrocytes—did not produce an active culture fluid. It thus appears that, even when handled by macrophages in vitro before injection, heat-killed *Listeria* did not provide any direct stimulation in this system.

Evaluation of the MIRF-induced exudates indicated that the adherent cells were typical macrophages morphologically (Fig. 4), exhibited Fc receptor activity, and were capable of actively taking up latex particles. However, these macrophages were notably deficient in their representation of C3 receptors (Table III). The very few adherent cells lacking Fc receptors were small- to medium-sized round lymphoid elements.

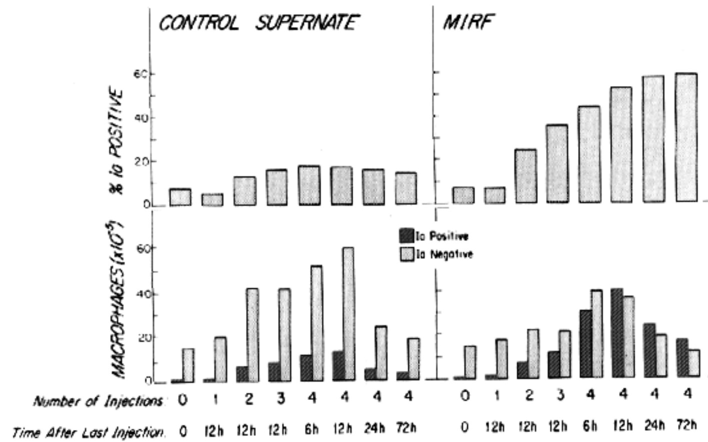

FIG. 3. Kinetics of development of exudates rich in Ia-positive macrophages. Active or control fluids were injected as described and the PEC harvested at the indicated times. Some of the data has not been included in this graph: (*a*) the PEC 24 h after the third injection of MIRF or control showed 21.7% (representing 8.5×10^5 Ia-positive macrophages/mouse) and 14.3% (4.7×10^5 Ia-positive macrophages/mouse), respectively: (*b*) the PEC 7 d after the fourth injection of MIRF or control contained 52.7% (21.4×10^5 Ia-positive macrophages/mouse), and 20.0% (4.4×10^5 Ia-positive macrophages/mouse), respectively; the PEC 14 d after the fourth injection of MIRF or control contained 40.5% (16.1×10^5 Ia-positive macrophages/mouse), and 12.6% (3.7×10^5 Ia-positive macrophages/mouse), respectivley.

We also evaluated the ability of the Ia-rich exudates induced by MIRF to synthesize Ia molecules. In the experiment shown in Fig. 5, the PEC induced by MIRF were cultured for 6 h in the presence of radioactive leucine, after which the cells were washed and treated with detergent. The detergent-solubilized material was immunoprecipitated with anti-I-A^k and examined in SDS polyacrylamide gel electrophoresis. The typical pattern of I-A molecules could be identified with the β-chain of 28,000 mol wt and the α-chain of 32,000 mol wt.

Antigen Specificity of MIRF Production. We have evaluated the specificity of the antigen requirement for MIRF production. PEC from mice infected with *Listeria* or immunized with sheep erythrocytes or with keyhole limpet hemocyanin were cultured with each of the three antigens for 24 h and their culture fluids tested in vivo. The data in Fig. 6al that MIRF was derived only from PEC cultured with the antigen used for immunization. It is apparent that the fluids from the *Listeria* exudates were much more potent than those from exudates induced by sheep erythrocytes or hemocyanin. This finding is in agreement with our previous studies in which *Listeria* infection was shown to be more effective than keyhole limpet hemocyanin in producing exudates rich in Ia-positive macrophages (7).

Cellular Requirements for MIRF Production. We know from our previous studies that *Listeria*-immune PEC contain high numbers of both Ia-positive macrophages and highly active immune T cells. We continued our evaluation of MIRF by attempting to determine if either of these cells, alone, elaborated MIRF. In experiment 1 of Table IV, the two cell types were separated and cultured with or without addition of heat-killed *Listeria*. Isolated lymphocytes in the presence of heat-killed *Listeria* were inactive. Likewise, treatment of the entire PEC or the nonadherent lymphocyte fraction with anti-Thy-1.2 and complement resulted in marked loss of MIRF production (Table

TABLE II

*MIRF Production Requires Immune PEC**

Culture fluid	*Listeria* injection (i.p.)‡	Percent Ia^+	Ia^+	Ia^-
			$\times 10^{-5}$	$\times 10^{-5}$
Experiment 1				
No injection	−	8.3	1.9	20.6
Media	−	11.7	2.3	17.4
Media	+	13.7	5.0	31.4
Listeria-PEC + *Listeria*	−	71.1	60.3	25.4
Listeria-PEC + *Listeria*	+	57.3	41.3	43.6
Listeria PEC	−	17.0	8.9	43.6
Listeria PEC	+	15.4	10.7	58.6
Experiment 2				
No injection		6.1	1.2	18.8
Listeria-PEC + *Listeria*		60.0	23.2	15.5
Peptone PEC + *Listeria*		14.5	5.7	33.5
Peptone PEC		10.8	2.4	19.6
Experiment 3				
No injection		4.4	0.7	15.5
Listeria PEC		9.2	0.9	8.9
Listeria-PEC + *Listeria*		43.2	30.9	40.6
Peptone PEC		10.5	3.1	26.2
Peptone PEC + *Listeria*		11.3	1.7	13.5
Peptone PEC + SRBC§		6.5	1.9	26.7
Peptone PEC + KLH‖		12.9	2.2	14.5

* Culture fluids were generated by the standard protocol for experiments 1 and 2. For experiment 3, culture fluids were generated in 15-mm-diameter culture wells. Experiment 1: 0.5 ml injected intraperitoneally every 6 h for 2 d. PEC harvested 6 h after last injection. Experiments 2 and 3: 0.5 ml injected intraperitoneally every 12 h for 2 d. PEC harvested 12 h after last injection.

‡ Heat-killed *Listeria* organisms (10^7) given with the first intraperitoneal (i.p.) injection of culture fluid.

§ SRBC, sheep erythrocytes.

‖ KLH, keyhole limpet hemocyanin.

IV; experiments 2 and 3). Although the conditioned medium from adherent PEC plus heat-killed *Listeria* had a small effect on the percentage of Ia-positive macrophages in experiment 1 (Table IV) (25.5%), there was virtually no effect on the absolute number of Ia-bearing macrophages (3.4×10^5). The overall ineffectiveness of isolated macrophages in the absence of T cells is further demonstrated in experiments 2 and 3 of Table IV. It thus appears that both macrophages and immune T cells are needed to produce MIRF.

These cellular requirements, when viewed in the context both of the antigen dependence of MIRF production and the well-documented requirement for Ia-positive macrophages in antigen presentation, suggested that Ia-positive macrophages might be necessary to generate MIRF. Using *Listeria*-induced PEC, we found that anti-I-A-plus-complement treatment of the plated macrophages completely abrogated MIRF production. We have also evaluated the response of peptone-elicited exudates, which are ~75–90% Ia-negative. In experiment 4 of Table IV, PEC induced by peptone were used as the source of macrophages and were left untreated or were exposed to anti-I-A or nonimmune IgG_{2b} and complement while in suspension. Residual PEC

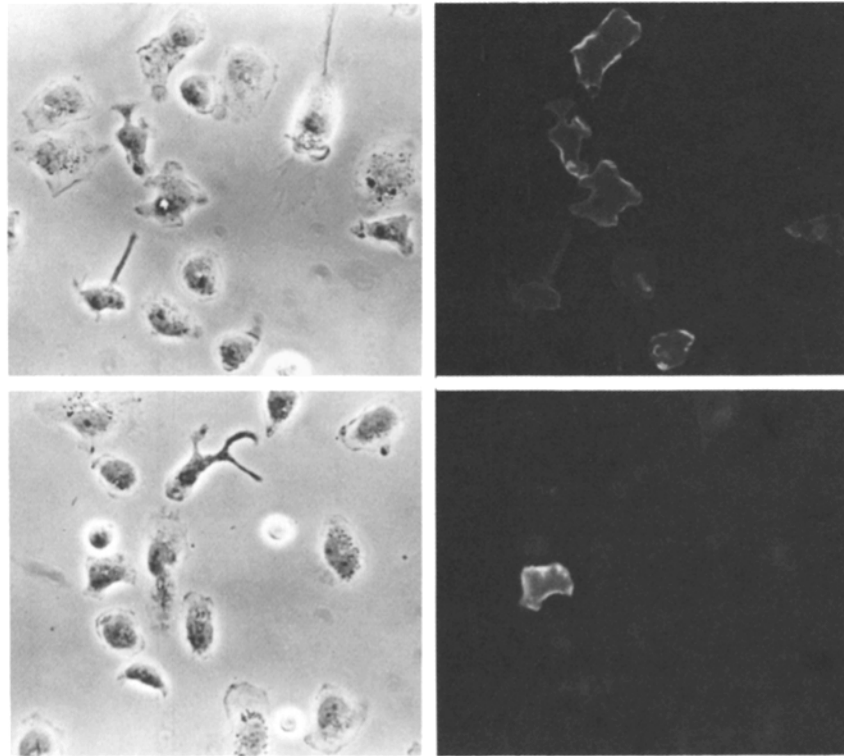

FIG. 4. Demonstration of I-A antigens on MIRF- and control-factor-induced peritoneal exudate macrophages. Active or control culture fluids were injected as described, and adherent cells from the resulting exudates were fixed and stained for I-A. On top are photographs of MIRF-induced macrophages, taken with phase (left) and fluorescent (right) optics. On the bottom is a similar set of photographs taken from macrophages from an exudate induced by a control culture fluid.

TABLE III
Evaluation of MIRF-induced Macrophages

Culture fluid	Percent Ia^+	Ia^+	Ia^-	Percent Fc receptor^{+*}	Percent C3 receptor^{+*}	Percent latex$^{+‡}$
		$\times 10^{-5}$	$\times 10^{-5}$			
No injection	5.8	0.6	9.3	99.1	95.2	94.2
Listeria-PEC + *Listeria*	48.2	35.1	37.8	98.2	12.1	87.9
Listeria-PEC	15.2	9.6	53.7	99.2	28.4	91.0
Peptone§	14.1	4.9	29.5	98.6	97.9	93.3

* Positive denotes binding of five or more opsonized SRBC.
‡ Positive denotes uptake of five or more latex particles.
§ 1.5 ml of 10% peptone injected in place of culture fluid.

from each of the three groups were then plated at several densities and *Listeria*-immune lymphocytes and antigen added to these cultures. MIRF was then assayed by the standard procedure. Significant although not complete reduction of MIRF production was found after depletion of Ia-positive macrophages in culture. This residual activity might be because the treatment with anti-I-A still left 2% of weakly stained Ia-positive macrophages, in contrast to the control which had 24%. The results

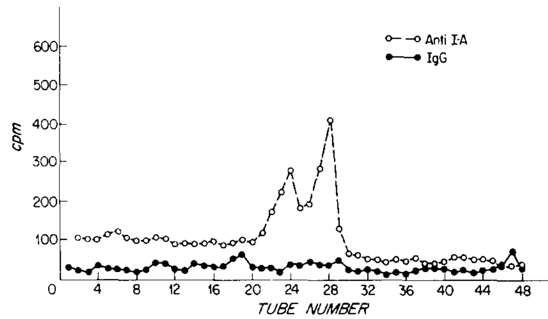

FIG. 5. SDS-polyacrylamide gel of I-A biosynthesis by macrophages induced by MIRF. The molecular weights of the two peaks have been calculated to be 32,000 and 28,000. IgG refers to supernates incubated with normal IgG_{2b} protein.

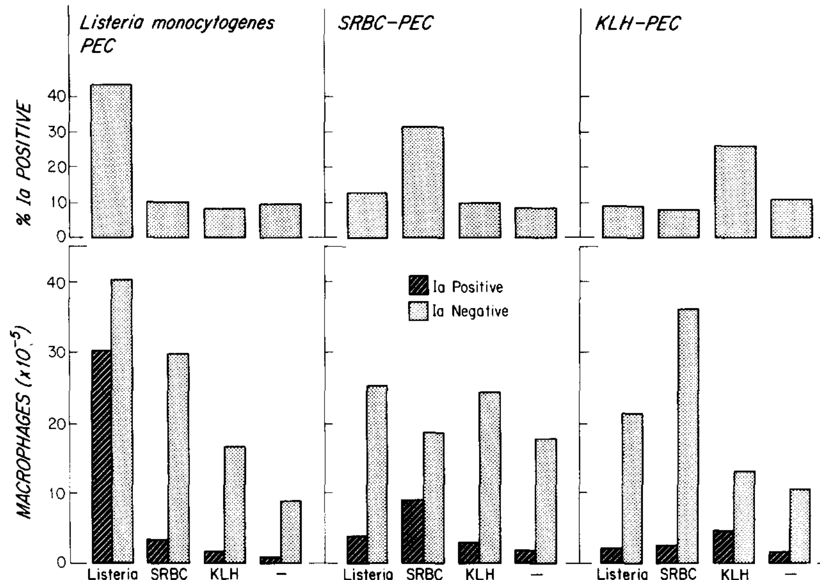

FIG. 6. MIRF production by PEC from mice immunized with different antigens. Culture fluids were generated from 10^6 PEC with 10^6 *Listeria* organisms, or 2×10^6 sheep erythrocytes (SRBC) or 10 μg of keyhole limpet hemocyanin (KLH) in 1 ml in 15-mm-diameter culture wells.

of experiment 4 of Table IV also suggest that the ratio of macrophages is important in the generation of MIRF and that some inhibition can be found with high number of macrophages; compare, for example, results of MIRF production using 5×10^5 and 1.5×10^6 macrophages.

Some Characteristics of MIRF. MIRF, once generated, is not restricted in its action by the H-2 gene complex. We tested this by generating *Listeria*-immune exudates in BL/6 and A/St mice, culturing the PEC with *Listeria* and testing the fluids in vivo in each strain. Table V shows that culture fluids from A/St and BL/6 immune exudates were each active in both A/St and BL/6 recipients. Moreover, the MIRF generated in A/St PEC induces an Ia-rich exudate in BL/6 that is not detected by the hybridoma anti-I-A (specificity 17) but that can be detected with an antiserum like A.TH-anti-A.TL that reacts with Ia specificity 3 of the BL/6. The failure of the anti-

TABLE IV

Cell Requirements for MIRF Production

Culture fluids*				Percent Ia^+	Ia^+	Ia^-
Entire PEC	Macrophages	Lymphocytes	*Listeria*			
					$\times 10^{-5}$	$\times 10^{-5}$
Experiment 1‡						
No injection				6.1	1.2	18.8
+	–	–	–	10.4	1.5	12.9
+	–	–	+	52.2	27.7	25.4
–	+	–	–	10.3	2.0	17.2
–	+	–	+	25.5	3.4	9.9
–	–	+	–	16.2	0.9	4.9
–	–	+	+	11.8	1.4	10.6
Experiment 2§						
No injection				6.6	1.8	24.9
Untreated	–	–	–	12.5	4.4	31.0
Untreated	–	–	+	52.5	18.8	17.0
Anti-Thy-1.2 + C	–	–	–	16.3	2.9	14.6
Anti-Thy-1.2 + C	–	–	+	18.9	4.5	19.3
NMS + C	–	–	–	18.3	2.8	12.4
NMS + C	–	–	+	57.7	12.3	9.0
Experiment 3‖						
No injection				6.6	1.8	24.9
–	+	Untreated	–	11.5	4.4	34.1
–	+	Untreated	+	58.7	28.6	20.1
–	+	Anti-Thy-1.2 + C	–	18.9	2.0	8.5
–	+	Anti-Thy-1.2 + C	+	19.7	4.2	17.1
–	+	NMS + C	–	18.2	9.1	40.9
–	+	NMS + C	+	50.2	17.9	17.7
Experiment 4¶						
No injection				6.1	1.2	18.5
–	Untreated (10^6)	+	–	19.7	4.4	36.8
–	Untreated (10^6)	+	+	31.6	21.1	45.6
–	Anti-I-A^k + C (1.5 $\times 10^6$)	+	+	19.8	3.2	13.1
–	Anti-I-A^k + C (10^6)	+	+	19.7	4.7	19.3
–	Anti-I-A^k + C (5 $\times 10^5$)	+	+	19.6	7.1	28.9
–	IgG_{2b} + C (1.5 $\times 10^6$)	+	+	29.5	12.2	29.1
–	IgG_{2b} + C (10^6)	+	+	32.8	16.7	34.3
–	IgG_{2b} + C (5 $\times 10^5$)	+	+	35.3	27.2	49.9
–	–	+	+	7.1	4.7	61.9

* Culture fluids were generated in a volume of 1 ml/15-mm culture well.

‡ Experiment 1: 10^6 *Listeria*-immune PEC were incubated for 2 h after which either entire PEC were left intact or the nonadherent cells removed before the addition of *Listeria*. The lymphocyte populations, primarily T cells, were *Listeria*-immune PEC depleted of macrophages by three cycles of adherence and were added at 2.5 $\times 10^5$/well.

§ Experiment 2: *Listeria*-immune PEC were left untreated or incubated with either anti-Thy-1.2 or normal mouse serum (NMS) plus complement (C) in suspension. Residual cells (10^6) were then plated with or without *Listeria*.

‖ Experiment 3: *Listeria*-immune PEC (10^6) were adhered for 2 h, after which the nonadherent cells were removed. *Listeria*-immune lymphocytes were left untreated or incubated with either anti-Thy-1.2 or normal mouse serum (NMS) plus complement (C) and added at 5 $\times 10^5$/well.

¶ Experiment 4: Peptone-elicited PEC were left untreated or incubated with either anti-I-A^k or IgG_{2b} plus complement (C). Numbers in parentheses refer to the numbers of those PEC which were plated for 2 h; the nonadherent cells were then removed. *Listeria*-immune lymphocytes (5 $\times 10^5$) were then added to each well.

TABLE V

MIRF Is Active in Allogeneic Mice

Strain generating MIRF	Recipient strain	Anti-I-A^k			A.TH-anti-A.TL		
		Percent Ia^+	Ia^+	Ia^-	Percent Ia^+	Ia^+	Ia^-
			$\times 10^{-5}$	$\times 10^{-5}$		$\times 10^{-5}$	$\times 10^{-5}$
A/St	A	45.9	20.8	24.5	ND*	ND	ND
A/St	BL/6	1.3	—	—	26.3	20.1	56.4
BL/6	A	48.1	34.9	37.6	52.1	37.8	34.7
BL/6	BL/6	0	—	—	28.4	14.7	37.0
BL/6 control fluid	A	12.9	4.4	29.9	17.2	5.9	28.5
A/St control fluid	BL/6	0.5	—	—	12.6	6.1	42.1
—	A	6.7	1.6	22.5	—	—	—
—	BL/6	0	—	—	11.5	8.2	64.5

* ND, not determined. There is no significant difference in the percent of A/St macrophages reacting with the hybridoma anti-I-A^k versus A.TH-anti-A.TL (7). A.TH-anti-A.TL recognizes Ia specificity 3 in the BL/6 mouse. Anti-I-A^k recognizes specificity 17 and does not react with the H-2^b haplotype.

TABLE VI

Preliminary Characterization of MIRF

Culture fluid	Treatment	Percent Ia^+	Ia^+	Ia^-
			$\times 10^{-5}$	$\times 10^{-5}$
No injection		7.2	0.9	12.2
Listeria-PEC + *Listeria*	None	61.7	28.1	17.4
Listeria-PEC + *Listeria*	Dialyzed	57.4	25.7	19.0
Listeria-PEC + *Listeria*	56°C, 1 h	51.4	13.3	12.6
Listeria-PEC + *Listeria*	37°C, overnight	49.0	14.9	15.1
Listeria-PEC + *Listeria*	Insoluble protease	14.7	2.7	15.5

I-A^k hybridoma to detect the Ia specificity displayed by the MIRF-induced BL/6 macrophage indicates that the Ia antigens must derive from the host and not from MIRF. It is also apparent that there is a marked difference in the potency of the two culture fluids that seems to be dependent more on the recipient strain than on the strain of origin of the exudates generating the MIRF. Thus, the BL/6 mice injected with either A/St- or BL/6-generated factor had, in each case, a 1.5- to twofold increase in Ia-positive macrophages. The A/St mice had, in both cases, an increase of about five- to eightfold.

Our initial evaluation of some of the properties of MIRF appear in Table VI. The retention of the activity during dialysis indicates a molecular weight >10,000. The molecule displays minimal heat lability and appears to be a protein because of its sensitivity to insoluble protease.

Discussion

When T lymphocytes are stimulated by strongly immunogenic antigens, e.g., *Listeria*, they elicit a response characterized by a dramatic and acute augmentation of the local Ia-bearing macrophage population (7). In the present study, we have described the production and activity of a soluble mediator that serves the same function. The production of this mediator, which appears to be a protein, requires Ia-

positive macrophages, immune T cells, and challenge with specific antigen. In addition to describing the basic phenomenon of factor-mediated regulation of Ia-positive macrophages, we have made a preliminary appraisal of the mode of action of MIRF. This factor must be repeatedly injected to generate and to maintain an exudate displaying high levels of Ia-positive cells. The most likely explanation for this requirement is that the factor is labile in vivo and that multiple injections are required to maintain a threshold concentration during the induction process. MIRF is not restricted in its mode of action by the H-2 gene complex and, in this sense, is similar to most previously described soluble mediators.

In a previous study (7), we discussed the issue of two different stimuli which induced exudates that bore significantly different proportions of Ia-positive and Ia-negative macrophages. Exudates induced by inflammatory stimuli (e.g., oil) were enriched for Ia-negative cells, whereas Ia-positive macrophages predominated in the exudates induced during an immune reaction. In experiments to be published, we have found that both stimuli require a radiosensitive bone marrow precursor. Furthermore, we have found that, like these stimuli, MIRF is inactive when injected into x-irradiated mice and that, in culture, it does not induce Ia-negative macrophages to synthesize Ia. It would appear that the most likely functions of MIRF would be either to control the differentiation of the mononuclear phagocyte lineage at the level of the bone marrow, thereby enhancing production of Ia-positive phagocytes, or to regulate the selective migration of recently divided Ia-positive phagocytes into the tissues. These functions are not mutually exclusive and both could be operant. That nonimmune stimuli recruit predominantly Ia-negative macrophages might indicate that exudates that develop in response to inflammatory stimuli are regulated by a mechanism quite distinct from that which governs the induction of Ia-rich exudates by immunogenic stimuli. Alternatively, both stimuli—nonimmune and immune—might induce monocytosis by a common mechanism, which, during an immune response, might be further modulated to result in the preferential production and/or export of Ia-positive phagocytes. It has been shown that inflammatory stimuli can induce monocytosis by way of a soluble factor (10). Such a factor could be part of the sequence by which exudates enriched for Ia-positive macrophages are induced. In this light, it is interesting to note that, whereas MIRF is produced only by immune PEC in the presence of antigen, the antigen is not required to produce culture fluids that increase PEC number nonspecifically.

The significance to the phagocyte lineage of two sets of macrophages which have different surface phenotypes is not known, although it is tempting to speculate that Ia-negative as well as Ia-positive macrophages are suited for a distinct biological role. Regardless of whether the lineage splits into two sublines in the bone marrow, or whether it represents a single line with expression of Ia at different maturational stages, our results strongly argue for differential modes of regulation of the two populations. The discovery of MIRF offers the possibility of isolating and characterizing biochemically and biologically a specific effector that regulates at least one stage of the recruitment event previously studied with antigen and immune T cells.

The relationship of MIRF to other biologically active molecules elaborated by immune cells in culture remains to be determined. Productive comparison with past studies can be made only after we have more information on the biochemistry and biology of MIRF. We do know that MIRF is generated in the same system that has

previously been shown to produce a number of lymphostimulatory molecules including a thymocyte mitogen (11) and a T cell-differentiating molecule (12). Our cultures also contain a macrophage-activating factor characterized by the induction of cytocidal activity in macrophages (13), small amounts of interferon (12), and of granulocyte-stimulating factor (E. G. Calamai. Unpublished observations). Purified preparations of these different molecules are now being evaluated in our assay, concurrent with our attempts to characterize MIRF.

Our final point is a speculation on the possible function of MIRF in the T cell regulation of the development and distribution of Ia-positive macrophages. Because the elaboration of this factor itself requires Ia-positive macrophages, MIRF production would appear to be a regulatory mechanism distinct from the processes that give rise to the basal level of Ia-positive macrophages during ontogeny (14, 15). Thus, there may be two or more distinct mechanisms regulating Ia expression, only one of which may be immunologically induced. It would seem that, if an individual requires Ia-bearing accessory cells to develop immune responsiveness, then it must be able to produce this population independently of, and before, immune challenge. The subsequent implementation of a second immunological mechanism to regulate macrophage Ia expression would then allow precisely those cells that require the collaboration of Ia-positive macrophages—the antigen-specific T cells—to regulate the size and perhaps the distribution of the Ia-bearing macrophage subpopulation. Studies in germ-free and in athymic mice showing normal basal levels of Ia-positive macrophages support our speculation (C. Y. Lu and D. I. Beller. Unpublished results.).

Summary

Previous studies have shown that *Listeria monocytogenes*-immune T cells, adoptively transferred into normal mice with killed *Listeria* organisms, induced peritoneal exudates rich in Ia-positive macrophages. We show now that culture fluids generated by *Listeria*-immune exudate cells and *Listeria* contain an activity that elicits Ia-rich exudates when injected intraperitoneally. The factor that recruits Ia-positive macrophages must be injected several times during a 2-d period for optimal demonstration of its activity. The induction of the factor is immunologically specific and requires Ia-positive macrophages, primed T lymphocytes, and antigen challenge. The factor is a nondialyzable protein and is not genetically restricted in its activity. The macrophages in the exudates induced by the factor bear Fc receptors, take up latex, synthesize I-A, but bear few C3 receptors. We have thus identified an immune mediator capable of controlling the Ia phenotype of the exudate macrophages.

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