Induction of a Macrophage Migration Enhancement Factor after Desensitization of Tuberculin-Positive Rabbits with Purified Protein Derivative

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The production of a macrophage migration enhancement factor (MEF) has been achieved as a consequence of administering a desensitizing dose of purified protein derivative (PPD) to *Mycobacterium bovis* BCG-sensitized rabbits. The migration-enhancing effect was first demonstrated when alveolar macrophages (AM) harvested from desensitized rabbits exhibited marked migration stimulation; whereas maximum migration enhancement was observed 8 days after the administration of PPD, migration enhancement of the AM from these rabbits persisted for up to 12 days. Sera from BCG-sensitized, PPD-desensitized animals exhibited a peak of MEF activity 4 days after desensitization. Maximal MEF activity was demonstrated in culture supernatants of nonadherent spleen cells harvested 8 days after the intravenous desensitizing dose of PPD was given. Control spleen cell culture supernatants did not produce detectable MEF. The route of desensitization with PPD was critical. When PPD was administrated intratracheally, MEF activity was not induced. The intravenous administration of BCG after PPD desensitization reversed migration enhancement to strong migration inhibition. Ammonium sulfate fractionation indicated that two fractions contained MEF activity. MEF activity was retained by dialysis membranes with a 15,000-molecular-weight cutoff but passed through dialysis membranes with a 25,000-molecular-weight cutoff. The mixture of migration inhibition factor with MEF-containing supernatants resulted in the mutual cancellation of both activities. These observations suggest that MEF may be a modulator of macrophage effector responses mediated by migration inhibition factor.

Assays for migration inhibition factor (MIF) have been plagued in many systems by wide variations in the degree of inhibition. It is of special interest that several investigators have observed stimulation of migration in some MIF assays. For example, migration stimulation has been observed when fetal bovine serum of lymph node supernatants were added to the migration assay system (1, 2). Weisbart et al. (16) were the first investigators to propose that the migration enhancement factor they studied was a lymphokine.

Studies on the interactions of MIF and migration enhancement factor(s) (MEF) are of special importance. Weisbart et al. (16) reported that their MEF cancelled the effects of MIF. In addition, Fox et al. (3) reported that migration stimulation factor and MIF neutralized each other in mixing experiments.

We have repeatedly noted that, when *Mycobacterium bovis* BCG-immunized rabbits are challenged intravenously (i.v.) or intratracheally (i.t.) with heat-killed BCG, they develop an extensive pulmonary granulomatous response after challenge (4, 8–11). In this case, the alveolar macrophages (AM) invariably exhibit migration inhibition when harvested and tested in the capillary tube migration test (no antigen added). We have referred to this phenomenon as autoinhibition (9). In this case, the state of migration inhibition in vivo may be the result of MIF production by immune lymphocytes triggered by the antigens of BCG administered i.v. or i.t.

In contrast, when 1 mg of PPD is injected i.v. in a BCG-immunized rabbit, the AM harvested 8 days after injection exhibit marked enhancement of migration. We have observed also that AM from neonatal rabbits are in a state of migration enhancement compared with adult rabbit AM (14). Enhanced AM migration was highest in 42- to 46-day-old animals but declined rapidly thereafter to normal adult levels. In addition, we have found that, when spleen cells from 42-day-old rabbits are cultured for 4 days, they spontaneously produce MEF. Sera from these young animals also stimulate the migration of adult AM. This communication will describe the induction, the cell source, and the partial characterization of an MEF that was induced by purified protein derivative (PPD) desensitization of adult rabbits.

MATERIALS AND METHODS

Animals. New Zealand White rabbits of either sex, weighing between 2 and 3 kg, were used in these experiments. They were sacrificed with a lethal dose (60 mg/kg) of pentobarbital (Barber Veterinary Supply, Fayetteville, N.C.).

BCG preparation. A BCG strain of *M. bovis* was grown on Proskauer-Beck broth. When the bacterial growth covered the entire surface of the medium, the cultures were autoclaved. The organisms were then harvested by filtration, washed three times with distilled water, suspended in a small volume of distilled water, lyophilized, and stored at −20°C. For use, lyophilized BCG was ground with a sterile mortar and pestle with mineral oil or saline. PPD of *Mycobacterium tuberculosis* was obtained from Connaught Laboratories, Willowdale, Ontario, Canada.

Immunization procedures. Rabbits were sensitized subcutaneously (s.c.) by injecting 200 µg of BCG in 0.2 ml of light mineral oil. The dose was divided into two sites, one at the base of each ear. After 21 days, the animals were skin tested

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by injecting 25 μg of PPD intradermally into the shaved flank of each rabbit. The reaction was quantitated by comparing the thickness of the skin at the reaction site with normal skin thickness at 24 and 48 h after injection by using a Schnelltaster skin caliper (Kroplin, Schluchtern, Federal Republic of Germany). Desensitization was accomplished by administering 1 mg of PPD i.v. Some animals received a BCG challenge which consisted of an injection of 3 mg of BCG in saline given either i.v. or i.t.

Standard medium. Unless otherwise specified, the medium used was RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 0.1 M L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% pooled normal rabbit serum (NRS; Hazleton Research Animals, Denver, Pa.) and buffered with 40 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.2; Research Organics, Cleveland, Ohio).

Cell procurement. Alveolar macrophages were obtained by pulmonary lavage by an aseptic technique previously described (6). Spleen cells were procured by gently teasing spleen tissue into RPMI 1640 medium with an 18 gauge hypodermic needle. Cell aggregates and connective tissue were removed by filtration through a 200 mesh screen. Centrifugation of cells was carried out at a low speed (55 × g) to preserve cell viability. Viability was assessed by trypan blue exclusion (5, 15). Differential cell counts were performed by the staining procedures of Giemsa and Wright. Normal AM preparations contained 95 to 99% macrophages. Lavaged AM preparations from BCG-sensitized, PPD-desensitized animals had 80 to 85% macrophages, 10 to 15% lymphocytes, and 5 to 10% neutrophils.

Migration assay. The effects of MEF and MIF were quantitated by direct and indirect versions of the standard capillary tube migration assay (12). In the direct assay, rabbit AM, obtained from age- and weight-matched untreated animals and animals receiving the appropriate immunization schedule, were washed twice and suspended in a volume of medium equal to the volume of the packed cell pellet. The cells were then loaded into capillary tubes, one end of each tube was plugged, and the tubes containing the cells were centrifuged at 55 × g for 10 min. The capillary tubes containing the packed AM were then broken into 5-mm lengths and mounted with silicon stopcock grease into migration chambers. Medium was added to the chambers, and the chambers were sealed and incubated at 37°C for 24 h. The resulting migrations were projected at ×10 magnification and traced, and the area was measured with a planimeter. In the indirect assay, tissue culture supernatants, sera, or lavage fluids were assayed for MIF or MEF activities with AM from untreated adult rabbits as indicator cells. The fluid to be tested was added to the culture medium in the migration chamber. Six replicate assays were done for each sample.

Cell culture. Spleen cells, obtained from animals of specified protocols, were cultured at 2×10^7 cells per ml in RPMI 1640 medium at 37°C in tissue culture flasks for 4 days. The supernatants were harvested by centrifugation at $200 \times g$ for 10 min.

Nylon wool adherence. Scrubbed nylon fibers (Associated Biomedic Systems Inc., Buffalo, N.Y.) were placed loosely into the barrel of a hypodermic syringe (2 g of fibers per 30-ml syringe) to form a continuous nylon wool column. The column was treated with 50% normal rabbit serum (NRS) in RPMI 1640 at 37°C for 1 h and washed with RPMI 1640–10% NRS. Cells were then applied and allowed to settle into the nylon wool and adhere for 1 h at 37°C in 5% CO_2 in air.

Nonadherent cells were eluted by gently washing the column with RPMI 1640–10% NRS at 37°C. Adherent cells were removed by the addition of cold medium (15°C) in conjunction with mild mechanical shock (tapping and drawing cold medium in and out with the plunger or by teasing the wool in cold medium with a hypodermic needle).

PNA fractionation. Spleen cells were treated with 0.5 mg of peanut agglutinin (PNA) per ml in RPMI 1640 with no serum (10^7 cells per ml) for 10 min at 37°C. Five times the volume of RPMI 1640–10% NRS was added with vortexing, and the cells were allowed to settle and incubate for an additional 90 min at 37°C. The sedimented (PNA$^+$) and suspended (PNA$^-$) cells were separated and washed three times with 0.3 M D-galactose. The suspended cell fraction was additionally absorbed with PNA-agarose to remove any PNA$^+$ cells that may have remained unagglutinated. Cell separation was monitored by visualization of fluorescein isothiocyanate-labeled PNA on the cells with a fluorescent microscope (Leitz Inc., Rockleigh, N.J.).

Dialysis. Lengths of membrane dialysis tubing (Spectrapore type 6; Spectrum Medical Industries; Los Angeles, Calif.) with molecular weight cutoff values of 6,000, 10,000, 25,000, and 50,000 were washed and soaked in three changes of sterile distilled water over a 24-h period. The membranes were autoclaved in water. The samples were added and dialysed for three successive 24-h periods against volumes of sterile distilled water or phosphate-buffered saline (PBS; pH 7.2) equivalent to 100 times the volume of the sample.

Ammonium sulfate fractionation. Precipitation of proteins in spleen cell supernatants was accomplished by the stepwise addition of $(NH_4)_2SO_4$ crystals (Sigma Chemical Co. St. Louis, Mo.) to achieve 30, 40, 50, 60, 70, 85, and 100% saturation. Precipitation was allowed to occur for 30 min at room temperature at each concentration; the precipitates were removed by centrifugation at $30,000 \times g$ for 30 min. $(NH_4)_2SO_4$ was removed by extensive dialysis (three 100-volume changes) against distilled H_2O.

Statistics. The means and standard deviations were calculated on all data. Each test result was expressed as a percentage of control migration and was calculated by the following formula: percentage of control migration = (area of test migration/area of control migration) × 100. Unless otherwise indicated, the standard deviation is shown in the figures. In addition, by using the Student t test, we have determined that a deviation of greater than 20% from the control was significant at the 95% confidence level. Our interpretations of the data presented in this communication were in accordance with this statistical parameter.

RESULTS

In the course of attempting total desensitization of BCG-immunized rabbits, we noted that dermal anergy was readily accomplished by injecting 1 mg of PPD by the i.t. route. However, we never accomplished desensitization of the harvested alveolar or interstitial cell populations based on MIF production. Our observations suggest that circulating immune T cells enter tissues as a result of the desensitizing dose of PPD. Presumably, dermal anergy resulted from a depletion of circulating immune T cells that moved to extravascular sites in the lung and possibly other organs. The following line of investigation was followed because of the chance observation that, when PPD was given i.v., MEF was produced.

Demonstration of a macrophage MEF. The migration potentials of AM from s.c. BCG-sensitized, i.v. PPD-desensitized animals were compared with AM from s.c.

BCG sensitized, i.v. BCG challenged animals. AM from BCG-sensitized, BCG-challenged animals exhibited marked migration inhibition (50 to 70%) (Fig. 1, fourth bar). In contrast, AM from BCG-sensitized, PPD-desensitized animals showed a large enhancement in migration (2 to 2.5 times) compared with control AM (Fig. 1, second bar). It is of special interest that the stimulation produced by the PPD desensitization was reversed by a subsequent challenge with BCG (Fig. 1, third bar). Photographs of representative migrations are presented in Fig. 2.

Time course of enhanced migration of AM from BCG-sensitized, PPD-desensitized rabbits. A series of experiments was carried out to determine the optimal interval after PPD was given which produced maximal migration stimulation of AM. Rabbits were sensitized s.c. with BCG and given 1 mg of PPD i.v. 3 weeks later. Alveolar cells were harvested 2, 4, 8, and 12 days after administration of PPD. Maximal migration enhancement occurred 8 days after PPD challenge (Fig. 3). It can be noted that sensitized controls (Fig. 3, sixth bar) that did not receive PPD showed control migration patterns. In addition, AM from nonsensitized animals that received PPD also exhibited normal migration patterns (Fig. 3, seventh bar).

Demonstration of an MEF in the sera of BCG-sensitized, PPD-desensitized rabbits. A time course experiment was done to determine the interval after the administration of PPD which resulted in maximal MEF activity of the sera. Six animals were bled serially, and their sera were tested in the

FIG. 2. Dark-field photographs of migration of AM from a normal adult rabbit (A), migration of AM from a BCG-sensitized, PPD-desensitized rabbit (B), and migration of AM from a BCG-sensitized, BCG-challenged rabbit (C).

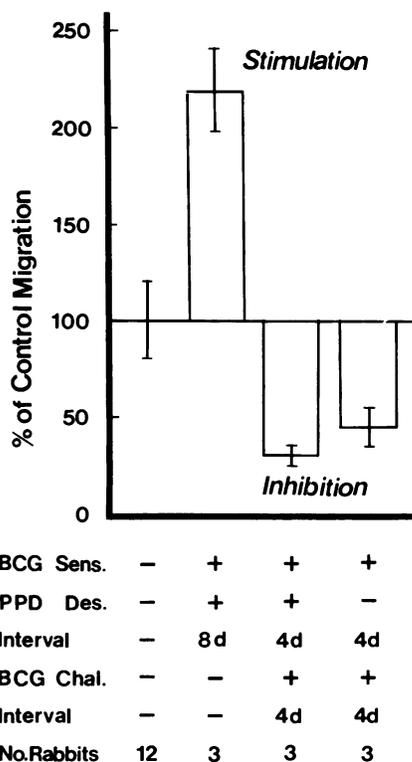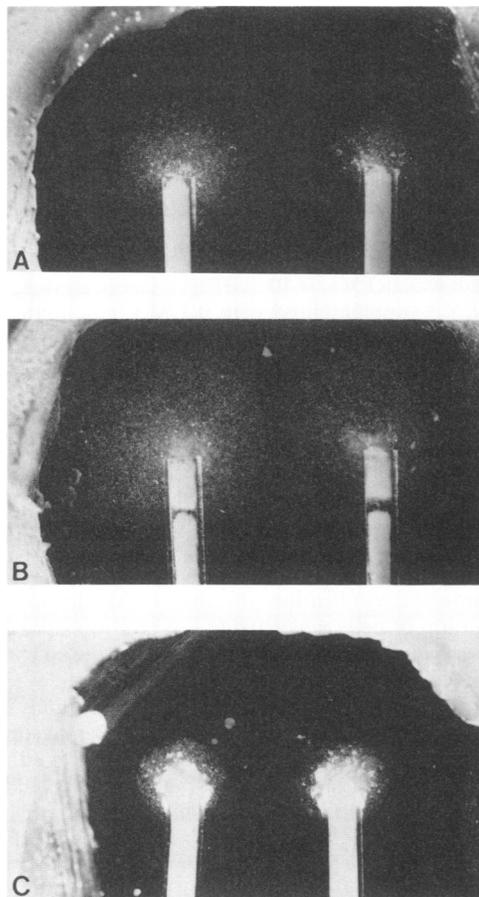|			
BCG Sens.	−	+	+	+
PPD Des.	−	+	+	−
Interval	−	8d	4d	4d
BCG Chal.	−	−	+	+
Interval	−	−	4d	4d
No.Rabbits	12	3	3	3

FIG. 1. Effects of PPD desensitization and BCG challenge of BCG-sensitized (BCG sens) rabbits on the migration of their AM. PPD des, PPD-desensitized rabbits; BCG chal, BCG-challenged rabbits. Control animals were given sham injections (saline only). The total interval after desensitization was 8 days, with the BCG challenge being given on day 4 after desensitization (see Materials and Methods).

standard migration assay. The results summarized in Fig. 4 were obtained from one animal and are representative of the experiment. It is noteworthy that peak MEF activity occurred 4 days after PPD desensitization. It can be recognized that peak MEF activity in the sera preceded peak resident AM migration enhancement by about 4 days.

Effect of route of PPD administration on the migration enhancement of AM. Three weeks after BCG sensitization, rabbits were given 1 mg of PPD either i.t. or i.v. Alveolar cells were harvested either 2 or 4 days after administration of PPD. The results reveal that an i.t. injection of PPD resulted in migration inhibition, whereas an i.v. injection of PPD resulted in marked stimulation of migration. (Fig. 5).

Studies on spleen cells as a possible source of MEF. Since PPD desensitization by the i.v. route was required to induce MEF, spleen cells were implicated as a possible source of MEF. Accordingly, spleen cells were harvested from BCG-sensitized rabbits 4 days after i.v. administration of PPD. The spleen cell cultures were incubated for 4 days, and the supernatants were harvested and tested for MEF activity with adult normal AM.

The results indicate that spleen cell supernatants from BCG-sensitized, PPD-desensitized rabbits markedly stimulated the migration of control AM (Fig. 6). It is important to note that comparable spleen cell culture supernatants prepared from control rabbits had no detectable MEF activity.
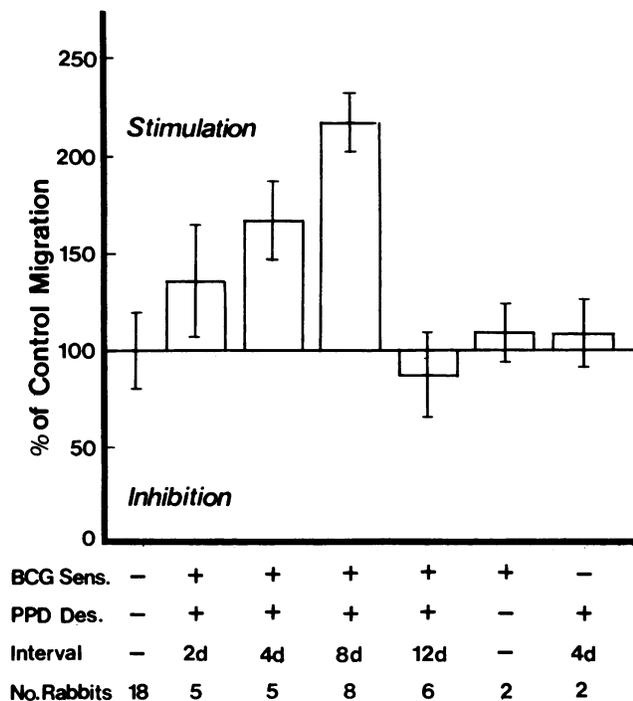
FIG. 3. Effect of varying the interval after desensitization on the migration of AM (see Materials and Methods for experimental protocols). Abbreviations are defined in the legend to Fig. 1.

In particular, spleen cell supernatants from nonsensitized rabbits that received PPD were inactive.

Lavage fluids (10%) tested for MEF activity in representative experiments did not have any migration stimulatory effects. In this regard, MEF activity was not detected in the lavage fluids obtained from BCG-sensitized animals given 1 mg of PPD either i.v. or i.t. at intervals between 4 and 12 days after administration of PPD (data not shown).

Titration of MEF activity in spleen cell culture supernatants. To quantify the amount of MEF activity in spleen cell supernatants, twofold serial dilutions were assayed. In these experiments, spleen cells from BCG-sensitized, PPD-desensitized adult rabbits were cultured as described in Materials and Methods, and the supernatants were col-
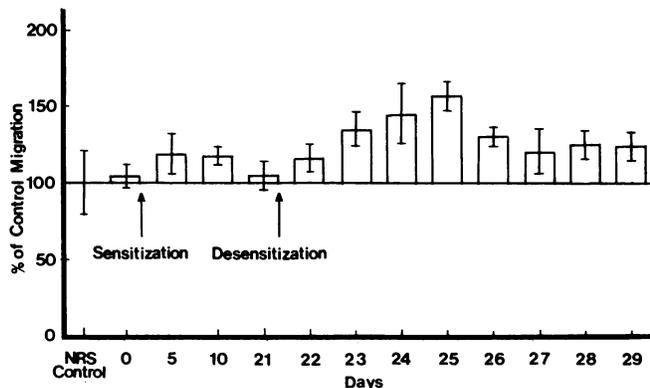

FIG. 4. Effect of serial serum samples from a single representative BCG-sensitized, PPD-desensitized rabbit on the migration of untreated adult AM (see Materials and Methods for experimental protocols).
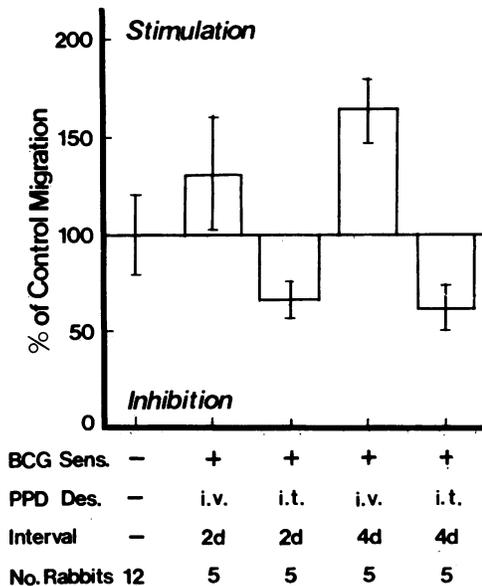

FIG. 5. Effect of the route of PPD desensitization on the migration of AM (see Materials and Methods for experimental protocols).

lected. Dilutions were made with fresh medium, and each dilution was tested for its effect on the migration of adult normal rabbit AM. MEF activity increased with increasing dilution, peaking at dilutions of 1:16 or 1:32 (Table 1).

Partial characterization of cells which produce MEF. Previous studies in our laboratory revealed that an MEF was produced by neonatal rabbit spleen cells that were nonadherent PNA-agglutinable lymphocytes. We attempted to determine whether the cells which produced MEF in the desensitized adult rabbit model had similar characteristics. Spleen cells were harvested from BCG-sensitized, PPD-desensitized rabbits. Portions of the spleen cell population were immediately cultured, and the remaining cells were
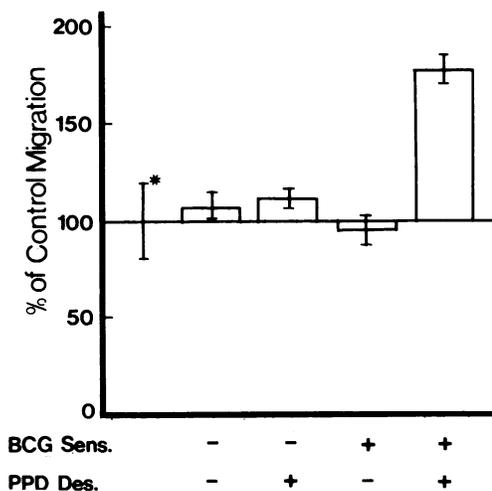

FIG. 6. Effect of spleen cell culture supernatants on the migration of untreated adult rabbit AM (see Materials and Methods for experimental protocols). These results are representative of 10 experiments. Control (*) was standard tissue culture medium (RPMI 1640–10% NRS). Abbreviations are defined in the legend to Fig. 1.

TABLE 1. Effects of diluting BCG-sensitized, PPD-desensitized rabbit spleen cell culture supernatants on their MEF activity

Dilution	% of control migration[a]		
	Expt 1	Expt 2	Expt 3
Undiluted	104.4	78.1	ND[b]
1:2	146.2	ND	132.1
1:4	188.0	130.7	143.1
1:8	222.8	131.4	148.9
1:16	**229.7**	135.0	157.7
1:32	162.0	**144.5**	**173.7**
1:64	165.2	107.3	150.4
1:128	146.2	113.1	123.4
1:256	122.8	114.6	129.9
1:512	ND	108.8	119.0
1:1,024	ND	127.0	102.9

[a] Peak stimulation in each experiment is in boldface.
[b] ND, Not determined.

separated into adherent and nonadherent populations by using a nylon wool column. Half of each population was cultured, and the remaining cells were separated into PNA-agglutinable and PNA-nonagglutinable fractions and cultured. Each fraction was cultured for 4 days, and the supernatants were collected and tested with adult AM for MEF activity (Fig. 7). Additionally, each cell fraction was monitored for viability, stained with fluorescein isothiocyanate anti-rabbit immunoglobulin or Wright stain, and visualized by conventional and fluorescent microscopy. The data in Fig. 7, in conjunction with the microscopic appearance of the cells in each fraction, clearly show that the MEF-producing cells are nonadherent lymphocytes. Experiments to determine preferential MEF production by PNA[+] or PNA^{-} cells were inconclusive (results not shown).

Effect of mixing MIF-containing supernatants with MEF-containing supernatants on the migration of normal AM. Since Fox et al. (3) reported that MIF-containing supernatants mixed with MEF-containing supernatants resulted in a mutual cancellation of both activities, it became important to test this principle in our system.

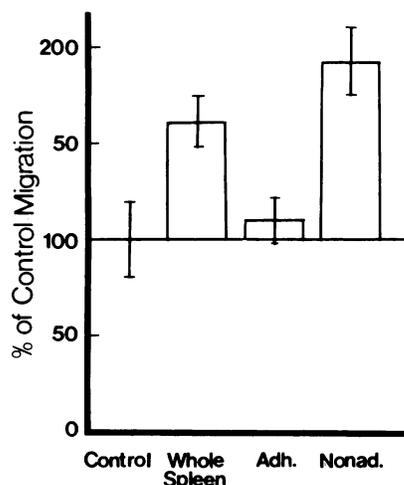MIF-containing supernatants were prepared by incubating

FIG. 7. Comparative abilities of adherent and nonadherent BCG-sensitized, PPD-desensitized rabbit spleen cells to produce MEF (see Materials and Methods). These results represent the mean ± standard error of the mean of four experiments.
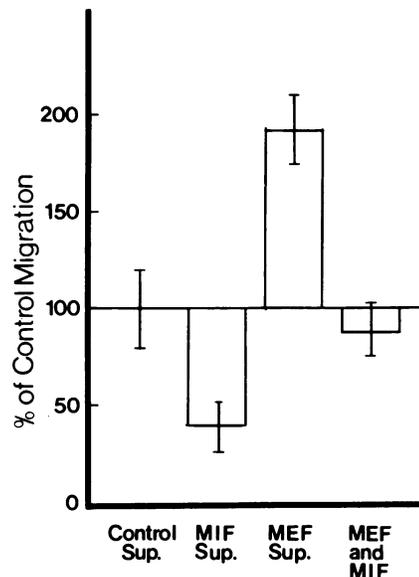

FIG. 8. Effect of mixing MIF-containing supernatants (MIF Sup.) with MEF-containing supernatants (MEF Sup.) on the migration of normal adult rabbit AM (see Results). Data represent means of 30 experiments.

2×10^7 spleen cells per ml (from BCG-sensitized rabbits) in the presence of 10 μg of BCG per ml for 24 h. The supernatants were harvested by centrifugation at $200 \times g$ for 30 min. Equal volumes of MEF-containing spleen cell supernatants and MIF-containing spleen cell supernatants in RPMI 1640–10% NRS were tested singly and together in the migration assay. The data (Fig. 8) indicate that the effects of MEF and MIF activities on the migration of normal AM cancelled each other. In addition, when MIF- and MEF-containing supernatants were mixed at ratios other than 1:1, the percentage of control migration observed (Inhibition or stimulation) was dependent on the relative concentration ratios of MIF to MEF. (Data not shown).

Ammonium sulfate fractionation of MEF from spleen cell culture supernatants. Spleen cell supernatants known to contain MEF activity were subjected to ammonium sulfate precipitation. The collected fractions were dialyzed to remove the ammonium sulfate. The dialyzed fractions were reconstituted in RPMI 1640 and tested in the migration assay. The data revealed MEF activity in two fractions (50 to 60% and 70 to 85%). Most of the MEF activity resided in the 50 to 60% fraction.

Estimation of molecular weight of MEF by using dialysis membranes with various molecular weight cutoffs. Spleen cell supernatants known to contain MEF activity were dialyzed against distilled water by using membranes with molecular weight cutoffs of 6,000, 10,000, 15,000, 25,000, and 50,000. MEF activity was retained by membranes with a molecular weight cutoff of 15,000 but not by membranes with a cutoff of 25,000. This indicates that MEF has a molecular weight between 15,000 and 25,000. Because dialysis is largely dependent on molecular shape and size, the molecular weight cutoff can only be a rough approximation.

DISCUSSION

The data obtained in this study clearly establish that the desensitization protocol with PPD administered i.v. leads to the generation of a macrophage MEF. To our knowledge,

this is the first demonstration of MEF induction as result of an in vivo immunologic protocol. It is of particular importance to note that MEF appeared in the sera of desensitized rabbits a few days before the peak migration stimulation exhibited by the resident AM. This would suggest that spleen cells are mobilized or induced or both to make MEF after the i.v. dose of PPD. Accordingly, MEF is most likely transported to the AM in the lung by way of the circulation.

The requirement that the desensitizing dose of PPD must be given i.v. strongly supports the idea that MEF is made by cells in the spleen. For example, when PPD was administered i.t., no macrophage migration enhancement was expressed by resident AM. In some instances, migration inhibition was noted after the administration of PPD by the i.t. route. These data support the idea that cells in the lung do not make MEF under the conditions of our experiments.

It is of special interest that a suspension of BCG given i.v. to BCG-sensitized rabbits produced migration inhibition instead of migration enhancement. It appears that the induction of MEF requires a soluble form of the antigen. In this regard, we have demonstrated that soluble PPD does not elicit granulomas, whereas PPD made insoluble by cross-linking with glutaraldehyde elicits typical tubercle-type granulomas (7). The mechanisms involved which require soluble antigens to produce MEF are not known.

The spleen cells responsible for the synthesis of MEF appear to be nonadherent lymphoid cells. The difficulties in determining whether the cells were PNA$^+$ or PNA$^-$ could be due to the large numbers of PNA$^-$ cells present in the adult spleen. Nevertheless, the cells responsible for MEF production could be similar to the nonadherent PNA-agglutinable neonatal spleen cells which synthesize MEF spontaneously in culture (14).

The molecular weight of MEF remains uncertain, based on the data obtained in our study. The results of dialysis membrane cutoff experiments suggest a molecular weight of between 15,000 and 25,000. The ammonium sulfate fractionation studies suggest a higher molecular weight. Experiments in progress are concerned with the resolution of this ambiguity.

The major point of interest in this work stems from the observation that a desensitizing dose of PPD can induce production of a macrophage MEF which appears to be an antagonist of MIF. Our studies, as well as others, indicate that the effects of MIF and MEF, which appear to be modulators of macrophage migration, cancel each other when they are mixed together. Additionally, the rise in MEF activity with increasing dilution of spleen cell supernatants suggests the presence of an antagonist in cultured spleen cell supernatants which could possibly be MIF. The observation that a BCG challenge superimposed on PPD desensitization can reverse migration enhancement supports the idea that modulation of a cell-mediated immune response can occur in vivo. In this particular case it was noted that the marked migration enhancement that was induced by PPD desensitization was reversed to an intense migration inhibition by the BCG challenge dose.

The possible role of MEF in mediating anergy is still uncertain. Desensitization produces many events, and it is unlikely that anergy results only from the production of MEF. For example, desensitization with PPD by the i.v. route seems to deplete the circulation of immune T cells. Furthermore, there is some evidence that desensitization will drive circulating T cells into the tissue. Accordingly, dermal anergy could be the result of depletion of circulating immune T cells. Nevertheless, the possible role of MEF in

preventing or attenuating macrophage participation in a cell-mediated immune response warrants further study.

It is of special interest that the enhanced migration of resident AM in desensitized rabbits persists for about the same time as anergy is sustained. For example, stimulated migration of AM from BCG-sensitized, PPD-desensitized animals persisted for up to 12 days after administration of PPD. It was also noted that dermal responsiveness returned shortly after that interval. The persistence of the enhanced migration of AM in desensitized rabbits even after MEF was not detectable in the serum was unexpected; this suggests that the migration stimulatory effects persist because MEF is bound but not rapidly metabolized by AM or that the AM Can retain enhanced migration even when the availability of MEF is curtailed.

Collectively, the data from our laboratory support the concept that MEF is a lymphokine that could be an important modulator of cell-mediated immune responses. In view of our observation that immature PNA$^+$ lymphoid cells from the spleen of neonatal rabbits produce MEF spontaneously, we postulate that MEF could be a lymphokine made by nonspecific (natural) suppressor cells (13, 14), which are prevalent in the neonatal spleen. This hypothesis is being investigated in experiments currently in progress.

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