Macrophage Migration Inhibition Studies with Cells from Mice Vaccinated with Cell Walls of 
*Mycobacterium bovis* BCG: Characterization of the Experimental System 

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The macrophage migration inhibition test was applied to the study of delayed hypersensitivity in mice vaccinated intravenously with oil-treated cell walls of *Mycobacterium bovis* BCG. Migration inhibition of peritoneal exudate cells from sensitized mice was demonstrated directly upon incubation of the cells with purified protein derivative, but indicator cells such as normal peritoneal cells had to be included to demonstrate migration and migration inhibition with sensitized lung cells. Inhibition of migration induced by mouse cells was greatest 3 to 4 weeks after sensitization but was still considerable after 11 weeks. The migration inhibitory factor (MIF) was not detected in cells freshly isolated from sensitized mice but was released into the supernatant fluid when cells were incubated with purified protein derivative for 24 hr at 37 C in a tissue culture system. Production of MIF was inhibited by actinomycin D and puromycin. MIF was nondialyzable, resistant to heating at 56 C for 1 hr, and of a lower molecular weight than mouse gamma globulin. All data indicated that migration inhibition induced by cells from cell wall-vaccinated mice was very similar to that caused by guinea pig lymphocytes.

Mackaness and Collins observed a close relationship between delayed hypersensitivity and resistance of experimental animals to infection with facultative intracellular parasites (11, 21, 22). They showed that in mice enhanced resistance to infection with *Listeria, Brucella,* and *Salmonella* was correlated with delayed hypersensitivity and activation of host macrophages, the cells responsible for the destruction of these parasites. Mackaness (23) suggested that macrophages may be activated by a humoral factor released by immunologically committed lymphocytes upon contact with specific antigen.

Two recent studies with mice have failed to demonstrate a relationship between delayed hypersensitivity and acquired resistance to infection with another facultative intracellular parasite, *Mycobacterium tuberculosis* H37Rv. Youmans and Youmans (30) reported that mice protected against intravenous (iv) challenge by intraperitoneal (ip) inoculation of a ribosomal preparation of *M. tuberculosis* H37Ra did not react to footpad challenge with purified protein derivative (PPD) or the ribosomal fraction. We (3, 4) found that mice inoculated with cell walls of *M. bovis* BCG by the intradermal or subcutaneous routes gave strong delayed responses to footpad challenge with PPD but exhibited little or no enhanced resistance to airborne infection with H37Rv. In contrast, mice vaccinated iv gave weaker footpad reactions but were protected far better against infection.

Since the footpad test may not necessarily indicate the ability of the mouse to produce a delayed reaction in the lung, we sought a test method which would enable us to measure directly the hypersensitivity state of lung cells. The lung is a particularly important organ in our studies because (i) it is the organ first exposed to the airborne mycobacteria and (ii) there is a very significant accumulation of lymphocytes and macrophages in lungs of mice vaccinated iv with BCG cell walls (5). Youmans and Youmans (29) have suggested that these granulomatous cells are related to the immune state.

To measure delayed hypersensitivity, we
adopted the macrophage migration inhibition (MI) test, a test first developed by George and Vaughan (18) and now used by many workers. In this test, migration of macrophages from a capillary tube placed in a tissue culture chamber is inhibited in the presence of antigen and lymphocytes from animals with delayed hypersensitivity to this antigen. Ordinarily peritoneal exudate cells from guinea pigs are tested, but Al-Askari et al. (1) have shown that this in vitro test can be performed with peritoneal cells from mice which have received homografts. Lymphocytes and macrophages washed from guinea pig lung have also been successfully used (6, 19).

In our studies, we found that migration of macrophages is inhibited in the presence of lymphoid cells from mice vaccinated with oil-treated BCG cell walls. Results of our experiments to define certain parameters of this experimental system and to characterize the factor responsible for inhibition are presented in this report. Our studies of the relationship between delayed hypersensitivity, as assayed by the MI test, and acquired resistance to airborne infection with H37Rv are described in the accompanying report (28).

**MATERIALS AND METHODS**

**Mice.** Four- to 6-week-old female CAF1 mice from the Animal Production Unit of the National Institute of Allergy and Infectious Diseases, Bethesda, Md., and 3- to 4-week-old white mice reared at the Rocky Mountain Laboratory (RML) were employed.

**Sensitization of mice.** Mice were inoculated iv with BCG cell walls prepared and treated with mineral oil as described previously (10, 25).

**MI test.** The MI test was performed basically by the method of David et al. (15). Peritoneal cells were collected in Hanks balanced salt solution 3 to 5 days after ip injection of 2 ml of 12% sodium caseinate. Lung cells were obtained by sieving minced lung tissue through a 100-mesh stainless-steel screen. Cells of both kinds were washed twice in Hanks solution and resuspended to a concentration of 10% in Eagle minimum essential medium (Grand Island Biological Co., Grand Island, N.Y.) to which had been added 85 units of penicillin, 85 μg of streptomycin, 0.3% glutamine, and 15% fetal calf serum/ml. Approximately 2% of the nucleated cells in Wright-stained smears of suspensions prepared from lungs of vaccinated mice were identified as lymphocytes and 6% were identified as macrophages. Suspensions prepared from lungs of control mice contained about 1% lymphocytes and 4% macrophages. Suspensions of peritoneal exudate cells contained about 30% lymphocytes and 60% macrophages. The suspensions were centrifuged in capillaries at 200 × g, and the portion of the capillary containing the packed cells was then attached with silicone to the bottom cover slip of a Sykes-Moore tissue culture chamber. The chambers were then filled with the Eagle medium described above with or without 15 to 30 μg of PPD (Parke, Davis & Co., Detroit, Mich.) per ml and were incubated at 37 C for 24 hr. Since in early experiments a concentration of 30 μg of PPD per ml did not inhibit migration significantly more than did 15 μg of PPD, the latter concentration was used in the remaining experiments.

Areas of migration of the cells from the open end of the capillary were traced on paper after projection and enlargement and measured with a planimeter. Per cent migration was calculated by dividing the area of migration of test cells, multiplied by 100, by the area of migration of control cells.

**Cultivation of lung cells.** Lung cells, collected and washed as described above, were incubated as 5% suspensions in Eagle medium, with or without 15 μg of PPD/ml, for 24 hr at 37 C. The suspensions were then centrifuged at 17,300 × g for 20 min at 4 C, and the supernatant fluids were removed for further study.

In certain instances, actinomycin D (K & K Laboratories, Inc., Plainview, N.Y.) and puromycin dihydrochloride (Nutritional Biochemical Corp., Cleveland, Ohio) were added to the culture medium.

**Gel filtration.** Tissue culture supernatant fluid was concentrated six-fold by ultrafiltration at 4 C and filtered through a column (2.5 by 80 cm) of Sephadex G-200 (Superfine) equilibrated with glycine-NaCl buffer, by the method of Heise et al. (19). Absorbancy of the fractions at 280 nm was determined with a Beckman DU-2 spectrophotometer, and appropriate fractions were pooled, dialyzed against two changes of distilled water at 4 C, and lyophilized.

**Immunological analyses of fractions.** Portions of the lyophilized pools were dissolved in saline (5 mg/ml) and tested against goat anti-mouse gamma globulin and anti-mouse serum (Hyland Laboratories, Los Angeles, Calif.) by double diffusion in gel. Other portions were subjected to electrophoresis by the method of Scheidegger (26) and examined with the above antisera.

**Antibody titrations.** Antibody levels to a crude lipopolysaccharide fraction isolated from defatted BCG by phenol extraction were determined with the Middlebrook-Dubos hemagglutination test (24). Antigen for the Boyden hemagglutination test (9) was prepared from the culture filtrate of M. tuberculosis Aoyama B grown in Sauton medium; the filtrate was precipitated with ammonium sulfate (twice at 80% saturation) at pH 7.0 and dialyzed. Both of the preparations were generously supplied by A. Sasaki, Hokkaido University, Sapporo, Japan.

**RESULTS**

**Competency of lung cells in MI test.** Since appreciable migration of cells from capillaries containing lung cells from normal and cell wall-vaccinated mice did not occur in several experiments, cell mixtures prepared by combining equal volumes of 10% suspensions of lung cells from vaccinated mice and peritoneal
(or indicator) cells from normal mice were then tested. In nine experiments, in the presence of 15 to 30 μg of PPD/ml, the areas of migration of cells from capillaries containing mixtures of normal peritoneal cells and lung cells from CAF₁ mice vaccinated iv 4 to 7 weeks earlier with 300 μg of BCG cell walls averaged 15.0 ± 3.7% of those of similar mixtures of cells from normal mice. Representative examples of migration and inhibition are shown in Fig. 1. In the absence of PPD, migration of cells from these same mice was 20.8 ± 7.3% of that of control cells. This phenomenon of inhibition in the absence of externally supplied antigen will be discussed below. Similar degrees of inhibition were obtained in two experiments employing cells from RML mice. Since these results indicated that the MI test might be of value in our studies of the relationship of delayed hypersensitivity and immunity, additional experiments were then conducted to characterize this response in the mouse and to determine the nature of the inhibition.

**Effect of dose of cell walls.** CAF₁ mice in several groups were vaccinated with a single dose of cell walls varying from 37.5 to 300 μg, and the inhibitory activity of lung cells obtained 5 and 11 weeks later was determined (Table 1). When lung cells from mice vaccinated 5 weeks earlier and normal peritoneal cells were added to the capillaries at a ratio of 1:1, migration inhibition was approximately proportional to the vaccine dose, both in the presence and absence of PPD. However, inhibition was greater in the presence of PPD. When lung and indicator cells were mixed at a ratio of 1:4, appreciable inhibition was detected only when PPD was included in the medium, and then only with cells from mice vaccinated with the highest dose of cell walls.

Similar results were obtained 11 weeks after vaccination, except that inhibitory activity was reduced. At both time intervals, therefore, the degree of migration inhibition was dose-dependent.

**Development of inhibitory capacity of mouse cells.** To determine the time of appearance of cells reactive in the MI test, lung and peritoneal cells from CAF₁ mice were tested at various intervals after injection of BCG cell walls. Cells recovered from lungs and peritoneal cavity 2 and 3 weeks after vaccination, respectively, significantly affected macrophage migration (Table 2). Greatest inhibitory activity was found in lung cells obtained 4 to 7 weeks after immunization.

**Effect of ratio of lung to peritoneal cells.** David et al. (16) reported that peritoneal cells from sensitized guinea pigs, when mixed at concentrations as low as 2.5% with cells from normal animals, would inhibit the whole population in the presence of antigen. To determine the requirements for cell types in our system, various ratios of normal peritoneal cells and cells from lungs of mice vaccinated with BCG cell walls 5 and 10 weeks earlier were tested. The data presented in Table 3 demonstrated that 5

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**Table 1. Inhibitory activity of lung cells from CAF₁ mice vaccinated with different doses of BCG cell walls**

<table>
<thead>
<tr>
<th>Dose of BCG cell walls</th>
<th>Ratio of normal peritoneal cells to lung cells in capillary 1:1</th>
<th>Ratio of normal peritoneal cells to lung cells in capillary 4:1 (results 5 wk postvaccination)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Results 5 wk postvaccination</td>
<td>Results 11 wk postvaccination</td>
</tr>
<tr>
<td>μg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>150</td>
<td>39</td>
<td>20</td>
</tr>
<tr>
<td>75</td>
<td>60</td>
<td>42</td>
</tr>
<tr>
<td>37.5</td>
<td>103</td>
<td>97</td>
</tr>
</tbody>
</table>

³ Controls consisted of the appropriate ratio of normal peritoneal and normal lung cells incubated in medium without PPD.

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**Fig. 1. Migration of a mixture of mouse (CAF₁) peritoneal and lung cells from capillary tubes placed in tissue culture medium containing 15 μg of PPD/ml.**

(A) Lung and peritoneal cells from normal mice. (B) Lung cells from CAF₁ mice vaccinated 4 weeks earlier with 300 μg of BCG cell walls and normal peritoneal cells.
TABLE 2. Inhibitory activity in the presence of 15 µg of PPD/ml of peritoneal and lung cells obtained from CAF1 mice at various intervals after vaccination with 300 µg of BCG cell walls

<table>
<thead>
<tr>
<th>Interval after vaccination</th>
<th>Per cent migration of normal peritoneal cells (1:1)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Peritoneal cells</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>2 hr</td>
<td>106</td>
</tr>
<tr>
<td>1 wk</td>
<td>102±</td>
</tr>
<tr>
<td>2 wk</td>
<td>94±</td>
</tr>
<tr>
<td>3 wk</td>
<td>60±</td>
</tr>
<tr>
<td>4 wk</td>
<td>65±</td>
</tr>
<tr>
<td>7 wk</td>
<td>83±</td>
</tr>
</tbody>
</table>

*Control consisted of peritoneal cells from vaccinated mice incubated in medium without PPD.

*Control consisted of normal lung and peritoneal cells incubated in medium with 15 µg of PPD/ml.

*Average per cent migration from six capillaries representing six mice in pools of two mice per group, except two pools of two used for 2-hr sample.

weeks after immunization migration was inhibited in the presence of PPD when only one-sixth of the cells were derived from sensitized animals. A lower concentration of lung cells perhaps would also have been effective. Probably fewer competent cells were present in the lung 10 weeks after inoculation, because a higher proportion of lung cells was necessary to inhibit cell migration.

**A**ttended recovery of inhibitory principle from unstimulated lung cells. The above results demonstrated that migration of normal macrophages could be reproducibly inhibited by lung cells from BCG cell wall-vaccinated mice but did not indicate the mechanism by which inhibition occurred. Several experiments were then performed to determine whether the migration inhibitory factor (MIF) could be isolated directly from lung cells of sensitized mice. First, an attempt was made to elute MIF from cells by heating them at 56 C for 1 hr. Secondly, cells were subjected to 10 cycles of freezing in an ethanol-dry ice bath and thawing in an effort to release MIF from the cells. In neither instance was MIF found in the supernatant fluids.

**P**roduction of MIF by stimulated lung cells. Bloom and Bennett (8) and David (13) have
**TABLE 6. Effect of heat on migration inhibitory factor in supernatant fluid**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Per cent Migration&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heated at 56°C for 1 hr</td>
<td>43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>None</td>
<td>55</td>
</tr>
</tbody>
</table>

<sup>a</sup> Migration of normal peritoneal cells in supernatant fluid of lung cells from five CAF<sub>1</sub> vaccinated mice (300 µg of BCG cell walls) incubated with 15 µg of PPD/ml as compared to migration of normal peritoneal cells in medium without PPD.

<sup>b</sup> Average per cent migration from two capillaries.

**TABLE 7. Effect of actinomycin D and puromycin on production of migration inhibitory factor by lung cells from CAF<sub>1</sub> mice inoculated with 300 µg of BCG cell walls**

<table>
<thead>
<tr>
<th>Inhibitor added to culture medium</th>
<th>Normal lung and peritoneal cells (1:1)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sensitized lung and normal peritoneal cells (1:1)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12</td>
</tr>
<tr>
<td>Actinomycin D, 0.8 µg/ml</td>
<td>53</td>
<td>57</td>
</tr>
<tr>
<td>Puromycin, 1.0 µg/ml</td>
<td>38</td>
<td>46</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average of four tests; 15 µg of PPD/ml in all chambers.

<sup>b</sup> Normal lung cells obtained from a pool of six lungs.

<sup>c</sup> Sensitized lung cells obtained from a pool of three lungs from mice inoculated 8 weeks earlier with 300 µg of BCG cell walls.

<sup>d</sup> Area of migration from these capillaries taken as 100%.

shown that lymphoid cells from guinea pigs exhibiting delayed sensitivity elaborate a soluble protein substance, MIF, into the medium when incubated with the specific antigen. To determine whether lung cells from CAF<sub>1</sub> mice vaccinated with BCG cell walls similarly produce a soluble inhibitory factor, supernatant fluids obtained after incubation of lung cells with PPD were added to chambers containing capillary tubes packed with normal peritoneal exudate cells. Soluble MIF was produced by lung cells from mice vaccinated 2 to 7 weeks earlier (Table 4). Maximum activity was attained 3 weeks after sensitization; this same level of activity was also observed at the 4- and 7-week intervals.

The concentration of MIF in one supernatant fluid was estimated by determining the inhibitory activity of the fluid after it was diluted with supernatant fluid obtained after incubation of lung cells from normal mice in Eagle medium. These results (Table 5) showed that inhibitory activity was marked even after fourfold dilution of the active supernatant fluid.

**Effect of heat on MIF.** A supernatant fluid obtained after incubation with PPD of lung cells from CAF<sub>1</sub> mice, vaccinated 6 weeks earlier with 300 µg of BCG cell walls, was heated at 56°C for 1 hr. The results presented in Table 6 indicated that MIF was unaffected by this treatment.

**Effect of metabolic inhibitors on synthesis of MIF.** David (12) has shown that both actinomycin D, an inhibitor of deoxyribonucleic acid-dependent ribonucleic acid synthesis, and puromycin, an inhibitor of protein synthesis, depress the ability of sensitized lymphocytes to inhibit cell migration. To determine the effect of these two inhibitors upon synthesis of MIF by lung cells from CAF<sub>1</sub> mice vaccinated with BCG cell walls, we added these substances to the medium in which the various cell mixtures were incubated (Table 7). Although these metabolic inhibitors somewhat reduced migration of normal cells, migration of macrophages in a medium containing both sensitized lung cells and metabolic inhibitors was significantly greater than that of macrophages in a medium devoid of actinomycin D or puromycin. These results suggest that mouse MIF is (i) newly synthesized by lymphocytes upon exposure to antigen and (ii) protein in nature.

**Fractionation of active supernatant fluids.** Lung cells from 10 CAF<sub>1</sub> mice, vaccinated 6 weeks earlier with 300 µg of BCG cell walls,
were washed, suspended in medium containing 15 µg of PPD/ml and normal CAF₁ serum rather than the usual fetal calf serum, and incubated for 24 hr at 37 C. A portion of the supernatant fluid obtained after centrifugation at 17,300 ×g was concentrated by ultrafiltration and filtered through a Sephadex G-200 (Superfine) column. The elution profile of one of two similar runs is shown in Fig. 2. Since preliminary tests had shown that under these conditions gamma globulin did not form a peak distinct from that of macro-globulin, the first peak was separated into two pools on the basis of results from earlier immunoelectrophoretic and immunodiffusion analyses. Fractions under the second peak were also pooled, and the three pools were dialyzed against distilled water at 4 C and lyophilized.

Each pool was studied by immunoelectrophoretic and immunodiffusion methods with commercial preparations of goat anti-mouse gamma globulin and goat anti-mouse serum. Gamma globulin was observed in the immunoelectrophoretic patterns of the first two pools but not in the last pool (Fig. 3). A faint band was detected in the pattern of P-3, but the electrophoretic mobility of this component is greater than that of gamma globulin. Gamma globulin was also found in the first two pools by immunodiffusion analysis (Fig. 4); the component found in the P-3 pool probably was the same one noted in the immunoelectrophoretic pattern. These results indicated that the contamination of the third pool with gamma globulin was negligible.

Portions of the lyophilized preparations were reconstituted in Eagle’s medium and assayed for MIF (Table 8). Most of the MIF was found in pool 3, the pool containing the least gamma globulin. Since little MIF was detected in the first

<table>
<thead>
<tr>
<th>Pool of column</th>
<th>Conc. of pool as ml of original supernatant fluid</th>
<th>Per cent migration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>94b</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>99</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>78</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>71</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>47</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>38</td>
</tr>
</tbody>
</table>

*a Migration of normal peritoneal cells in the presence of supernatant fraction as compared to migration of normal peritoneal cells in medium without PPD.

*b Average of duplicate tests.
two pools, it is unlikely that antibody was primarily responsible for the inhibition of migration observed in this experimental system.

**Hemagglutinating antibody in supernatant fluids.** The original supernatant fluid and fractions P-1, P-2, and P-3 were also tested for the presence of antibody to mycobacterial antigens by the Middlebrook-Dubos and Boyden hemagglutination tests. With the Boyden test, a low titer (1:20) of antibody was found in the unfraccionated supernatant fluid only. Antibody to the polysaccharide antigen (Middlebrook-Dubos test) was not detected in any of the samples.

**DISCUSSION**

An in vitro test for the demonstration of delayed hypersensitivity, the MI test, has been adapted to the study of cells from mice. Peritoneal and lung cells from mice vaccinated iv with oil-treated BCG cell walls, upon exposure to PPD, can inhibit the migration of normal macrophages. Maximum inhibitory activity was detected with these cells 3 to 4 weeks after immunization, and significant activity persisted for at least 11 weeks. Delayed hypersensitivity, as determined by the footpad response to PPD, developed at about the same rate in mice vaccinated similarly (4). Because of this agreement between our results with the footpad and MI tests and because others have shown with guinea pigs that the MI test is positively correlated with the dermal test for delayed hypersensitivity (14, 17), we assume that the MI test also measures delayed hypersensitivity in cell wall-vaccinated mice.

MIF produced by mouse cells appears to be very similar to that produced by guinea pig lymphocytes (7, 14). In our system, MIF is not found preformed in cells but is elaborated upon incubation of the sensitized cells in tissue culture. Active synthesis precedes the appearance of MIF, since little or no MIF is found in supernatant fluid when puromycin or actinomycin D is added to the tissue culture medium. Also, like guinea pig MIF, mouse MIF elutes from a column of Sephadex G-200 with albumin-sized molecules. Others (2, 19) have reported that antibody may play a role in MI, but our results do not indicate that such is the case in this system.

Lung cells from vaccinated mice, but not peritoneal cells, were observed to inhibit migration of normal macrophages in the absence of externally supplied PPD. However, cell wall antigen probably was present within the macrophages of the lung-cell suspension, since an earlier study had shown that radioactivity persists for more than 4 weeks in lungs of mice vaccinated iv with 14C-labeled cell walls (3). Possibly, release of antigen by the lung macrophages in tissue culture stimulated sensitized lymphocytes to produce MIF.

The mechanism by which migration of mouse macrophages is inhibited was not revealed by this study. Presumably, either a cytotoxic factor (20) or a chemotactic factor (27) produced by tuberculin-sensitive lymphocytes would prevent migration of cells. Possibly, other products, such as the postulated macrophage-activating factor (23), are synthesized by the sensitized lymphocyte upon contact with specific antigen and affect migration.

Our data demonstrated that lung cells from mice vaccinated iv with BCG cell walls produce consistent and significant inhibition of migration of normal peritoneal macrophages. With this defined system, we were able to study the relationship in mice of immunity to airborne infection with H37Rv and the ability of lung cells to participate in the MI reaction associated with delayed hypersensitivity. The results of this study are presented in the accompanying report (28).

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