# VIRAL-INDUCED ANERGY OF CELL-MEDIATED IMMUNITY AS DETECTED BY THE MACROPHAGE MIGRATION INHIBITION TEST

by

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ABSTRACT

This study has shown that in vitro virus-treated peritoneal exudate cells from mice and peritoneal exudate cells obtained from virus-infected mice demonstrated viral-induced "anergy" of cellular immunity as detected by the macrophage migration inhibition test.

Preliminary studies showed that optimal conditions for eliciting maximum in vitro macrophage migration inhibition of peritoneal exudate cells obtained from mice were: 1) sensitization of mice to Mycobacterium tuberculosis, $H_{37Rv}$ for 3-4 weeks and then skin testing with the appropriate antigen, and 2) culturing the peritoneal exudate cells in vitro with 2.5 mg to 25 mg of old tuberculin or 1.5 µg to 15 µg of purified protein derivative (PPD).

Viral studies conducted in vitro showed that different viruses altered peritoneal exudate cell migration as compared to the migration of nonvirus-treated peritoneal exudate cells. Some viruses enhanced macrophage migration while other viruses inhibited the macrophage migration.

In vitro and in vivo virus studies conducted on sensitized peritoneal exudate cells demonstrated an altered cellular response to specific antigen as compared to controls, nonvirus-treated sensitized peritoneal exudate cells. Possible reasons for this in vitro and in vivo viral-induced "anergy" of cell-mediated immunity are discussed.

# INTRODUCTION

Viral effects on immunological competency have been demonstrated by detecting changes in the expression of humoral and cellular immunity. Viral-induced immunodepression of the humoral immune response has been demonstrated by decreased antibody production (1-3), decreased primary and secondary antibody responses (4-5), and depressed 19S and 7S antibody levels (6-7). The number of cells producing antibody to specific antigens such as sheep erythrocytes and E. coli lipopolysaccharide is also suppressed in mice infected with leukemic virus (1, 8-9). Not all viruses cause depression of the humoral immune response. Viruses such as Venezuelan Equine Encephalitis, Aleutian Disease, and Lactic Dehydrogenase virus (LDV) have an adjuvant effect on the humoral immune response as indicated by increased antibody production (10-12).

Viral effects on immunological competency of cell-mediated immunity (CMI) were first noted by von Pirquet (13) when he found that infection of tuberculin positive children with measles virus resulted in depression of delayed-type hypersensitivity (DTH) as measured by the tuberculin skin reaction. Bloomfield and Mateer (14) in 1919 demonstrated suppression of tuberculin sensitivity in human beings during attacks of influenza virus. Recent investigations have confirmed the above results (15-17).

Parameters of CMI include homograft rejection, graft versus host reaction (GVHR), tumor growth, and DTH. Prevention of homograft rejection was demonstrated by Dent, Peterson, and Good (18) when they infected leukemia resistant $C_3H$ newborn mice with Gross leukemia virus. Grafting was accomplished across a weak non-H-2 histocompatibility barrier. Häyry, Rago, and Defendi (19) and Salaman (5) have confirmed these observations. Investigations by Howard, Notkins, and Mergenhagen (20) showed that infection of mice with LDV caused a delay in their rejection of skin allografts, and that (BALB/c x AL/N)$F_1$ mice infected with LDV 3 to 7 days before receiving BALB/c spleen cells showed a definite inhibited graft versus host reaction (GVHR). Contrary to the above results, Purchase, Chubb, and Biggs (21) demonstrated that chickens with Marek's disease showed enhanced GVHR, whereas homograft rejection was delayed. Laboratory mice infected simultaneously or successively with leukemic and nonleukemic viruses were demonstrated to promote the growth of viral and chemically induced tumors (22-25). The exact mechanism for enhanced tumor growth by these viruses is still in question today.

The phenomenon of DTH has been known since 1891 when Koch (26) demonstrated an immunological reaction by injecting tuberculin into guinea pigs, and then observing ulceration at the site of the tuberculin injection within 24 to 48 hours following skin testing. In 1908 von Pirquet (13) noted that tuberculin positive individuals, when infected with measles virus, showed depression of DTH as measured by the tuberculin skin reaction. After the disease subsided and the

individual was skin tested again, a positive reaction to tuberculin was seen. Other investigators have reported diminished reactivity to tuberculin in human beings infected with influenza, chickenpox, and polio viruses (14, 27-28). The cause for this loss of response to tuberculin in the virus-infected tuberculin-sensitive individual is still not known today.

Investigators have used in vivo techniques to assist them in understanding the mechanism(s) involved in DTH. The in vivo technique of skin testing has been routinely used in human beings to detect DTH to tuberculosis, coccidiosis, mumps, and measles (29-32). Skin testing for tuberculin sensitivity in guinea pigs (33-36) has been successful, although results from skin tested Mycobacterium-sensitized mice have been inconsistent because this species does not always develop the classic skin test reaction. There are reports stating that mice do not show a positive skin test due to their high natural resistance to artificial infection and a failure to develop tubercle allergy (37-38). On the contrary, results from Swedberg (39), Dubos, Schaefer, and Pierce (40), and Gray and Jennings (41) have shown that mice do acquire resistance to tuberculosis as demonstrated by the tuberculin skin test. Extensive studies by Crowle (42-43) using different in vitro techniques have shown that mice do develop allergy to the tubercle bacilli. The most practical in vivo technique used by Crowle for detecting DTH in mice was the footpad test which was first developed by Gray and Jennings (41).

In the last 5 to 10 years, in vitro methods such as the lymphocyte blastogenic transformation test and the macrophage migration inhibition (MMI) test have been accepted as methods to test for the in vitro correlates of DTH. These procedures allow one to obtain information on reactions which would not be possible in an in vivo situation due to the complex biological and chemical reactions which occur in the intact animal. Olson et al. (44) showed that lymphocytes from infants infected with rubella virus were depressed in their ability to undergo blast transformation, and that viral-infected leucocytes obtained from normal human beings demonstrated a reduced macromolecular synthesis and transformation when stimulated in vitro with general mitogens (PHA and pokeweed mitogen) and specific antigens (diptheria toxoid and tetanus toxoid). Investigations by Smithwick and Berkovich (45) demonstrated that the addition of measles virus to peripheral lymphocytes from Mantoux positive children could inhibit in vitro PPD-induced transformation. A similar response using NDV infection of mycobacterial-sensitized peripheral leucocytes from guinea pigs was seen by Belehu and Olson (35) who established an in vitro guinea pig model for further study of the virus-lymphocyte relationship.

Rudimentary work on the MMI technique actually started in 1928 when Rich and Lewis (46) demonstrated that cells of an animal sensitized to tuberculin were much more sensitive to the effects of tuberculin than were the cells of a normal animal. A plasma clot technique was used for exposing washed cells of normal and allergic guinea pigs to different dilutions of tuberculin. It was concluded that cellular

injury and necrosis associated with allergy in tuberculosis caused a cellular change rendering the cells more sensitive to the tubercle bacillus products. In addition, no humoral factors were found to be involved in the local effects associated with the tuberculosis allergy.

A classic experiment by Rich and Lewis (47) in 1932 demonstrated that explants of spleens and lymph nodes from tuberculin sensitive guinea pigs showed inhibition of migration of allergic cells from the explant into the surrounding plasma containing tuberculin. The sensitized cells were able to migrate out in the absence of tuberculin. Washed cells taken from the tuberculous-sensitized guinea pig were shown to retain their hypersensitivity to tuberculin when isolated from the body and maintained in tissue culture.

More recently, George and Vaughan (34) have presented studies which provide further information on the use of macrophages in an in vitro model for DTH. Modification of the Rich and Lewis technique allowed for an easier quantitation of results using cells from sensitized animals cultured in medium containing specific antigen. The specificity of this in vitro system paralleled that of the in vivo skin test reaction to detect DTH. George and Vaughan cultured peritoneal exudate (PE) cells from tuberculous-sensitized animals in capillary tubes and studied their ability to migrate in the presence of the specific antigen tuberculin-purified protein derivative (PPD). The PE cells failed to migrate in the presence of PPD while control cells from nonsensitized animals in the presence of PPD migrated out from the capillary tube during an incubation time of 24 to 48 hours at 37 C. Other

individuals have used this technique to demonstrate MMI using antigens such as tuberculin (35), brucella (48), conjugates of picryl guinea pig albumin and bovine gamma globulin (49), beta-lactoglobulin in Freund's complete adjuvant (FCA) (50), crystalline human serum albumin (51), and mumps and influenza virus vaccines emulsified in FCA (52).

Bloom and Bennett (36) demonstrated that the immunologically active cells in this system were peritoneal lymphocytes, and the peritoneal macrophages were merely indicator cells. Separation of the peritoneal lymphocytes and macrophages allowed Bloom and Bennett to determine the individual immunological activity of these cells. It was found that purified macrophages were not inhibited in their migration when specific antigen was added to the tissue culture medium, but MMI was demonstrated even when 2-2.5% of sensitized lymphocytes were mixed with sensitized or nonsensitized macrophages.

Since the presence of a very few sensitized lymphocytes caused migration inhibition of normal macrophages, it was thought that a soluble material was released by the lymphocytes. To investigate this possibility, Bloom and Bennett cultured purified peritoneal lymphocytes from tuberculin-sensitized guinea pigs in the presence or absence of PPD (36). Supernatants from sensitized lymphocytes cultured with PPD produced marked inhibition of normal PE cells while supernatants from sensitized lymphocytes incubated without PPD failed to inhibit the migration of normal PE cells. Further characterization of this biologically active substance showed that it was soluble, nondialyzable, heat stable, had a molecular weight of 70,000, accelerated cutaneous DTH

reactions, was highly antigen specific, was newly synthesized by the cell, and its production was blocked by mitomycin C and puromycin (53-55). Bloom and Bennett (53) called this biologically active substance the migration inhibition factor (MIF), because it inhibited, in vitro, the migration of PE cells from capillary tubes. Other investigators have characterized MIF and have received similar results (56-58).

Increased awareness of the importance of cellular response in host resistance to viral infection have led investigators to explore further the effects of viral infection on CMI by the use of virus infected PE cells. Glasgow (59) and Glasgow and Habel (60) performed in vitro experiments to determine the interferon production in normal mouse PE cells and in virus-infected PE cells. It was found that vaccinia virus-infected PE macrophages showed increased interferon production. They postulated that PE macrophages provided a restrictive host environment after virus infection, and also produced interferon as a host defense mechanism against viral infections. Feinstone, Beachy, and Rytel (52) used PE cells from animals infected with influenza or mumps virus and the MMI technique to demonstrate that the development of DTH to these viral antigens was altered. They also showed that the mouse is an excellent model to further study the role of DTH in resistance to viral infection. Similar investigations by Hall and Kantor (33), and Friedman and Ceglowski (61) showed depression of DTH to viral antigens with PE cells and lymphoid tissues (spleen, lymph nodes, thymus, and bone marrow) obtained from mice infected with mumps and Friend leukemia virus. It was hypothesized that virus-infected macrophages

lost their in vitro migratory function, and also lost their capacity to normally "process" antigens or other particles (61). Further investigations by Friedman and Ceglowski (62) showed impaired cellular response to PPD of PE cells obtained from Friend leukemia virus-infected mice which were sensitized to Freund's complete adjuvant one week after virus infection. The results of this study were interpreted as demonstrating an absence of "sensitized" lymphocytes in the peritoneal cells of leukemic mice, and therefore no MIF was present to inhibit macrophage migration.

The purpose of this investigation is to demonstrate that PE cells obtained from virus-infected tuberculin-sensitized mice do not show inhibition of migration when incubated with PPD, whereas PE cells from nonvirus-infected tuberculin-sensitized mice do show inhibition of migration in the presence of specific antigen. A defect of cellular immunity in virus-infected mice will be assessed by a macrophage migration inhibition assay.

## MATERIALS AND METHODS

### Animals

Outbred Swiss Webster (SW) male mice, 8 to 9 weeks old, were obtained from the Department of Microbiology and housed in metal cages in a controlled environment. All mice were fed Purina Mouse Chow (Ralston Purina Company, Checkerboard Square, St. Louis, Missouri) and water ad lib.

### Reagents

#### Mycobacterium tuberculosis, H37Rv

One hundred mg of dried, heat-killed (100 C for one hour) tubercle bacilli was suspended in 40 ml of mineral oil (California Mineral Oil, Rabin-Winters Corp., El Segundo, California) and mixed with 10 g melted vaseline (White Petroleum Jelly, Chesebrough-Pond's, Inc., N.Y., N.Y.) (63). Two-tenths mg of the tubercle bacilli suspension was used.

#### Purified Protein Derivative

Sterile tuberculin-purified protein derivative (PPD, Connaught Medical Research Laboratories, Toronto, Canada, A8, PPD-CT 68) when used for skin testing was diluted in tissue culture medium-199 (TC-199) to a final concentration of 15 µg/ml. Fifteen-hundredths ml of this dilution was injected intradermally into the footpad of each recipient mouse.

Purified protein derivative when used as a specific antigen in cell culture was diluted to 1.5, 15, and 150 μg/ml in TC-199 with 15% fetal calf serum (FCS).

Old Tuberculin

Sterile old tuberculin (OT, Connaught Medical Research Laboratories, Toronto, Canada, Lot: 58-1) was diluted for skin testing and for cell culture medium to final concentrations of 2.5, 25, and 250 mg/ml.

Lactic Dehydrogenase Virus (LDV)

Lactic Dehydrogenase virus was received from Dr. Abner Notkins of the National Institutes of Health, Bethesda, Maryland. A virus stock was prepared in SW male mice by IP injection of 0.1 ml LDV diluted 1:100 in 20% veal infusion broth (VIB). One day later, blood was collected from each mouse by heart puncture. Serum was removed from the clotted blood, centrifuged, and the virus-serum stock was stored in small volumes at -70 C. The stock virus (Arizona LDV stock) was titrated by making tenfold dilutions from $10^{-1}$ to $10^{-2}$ using 20% VIB as diluent. Five mice served as recipients for each dilution and received 0.1 ml by IP injection (64). Serum was collected by retro-orbital bleeding after 96 hours and then assayed for serum lactate dehydrogenase using a spectrophotometric method (64). A five to tenfold increase in the activity of the plasma LDH enzyme of LDV-infected mice served as the basis for detecting the virus (65). The final titer was $1 \times 10^{9}$ $ID_{50}$/ml (the dose that infected 50% of the animals as calculated by the

method of Reed and Muench (66). Arizona LDV stock was used for all experiments.

Lactic Dehydrogenase virus samples recovered from infected PE cells were either diluted 1:5 or a series of dilutions (1:5, 1:10, 1:50, 1:100, 1:250) were made. One-tenth ml of each sample was injected IP into SW mice. After 96 hours, serum was collected and assayed for LDH as described above.

Sendai Virus

Purified Sendai virus was received from Dr. Olson. The concentration of the virus was determined by injecting dilutions of virus into embryonated eggs ($EID_{50}$) (67) and the hemagglutination (HA) procedures (68). The $EID_{50}$ was determined by making tenfold dilutions of Sendai virus and injecting 0.1 ml of each dilution into 10-day-old embryonated chicks. Eggs were incubated for three days after which 0.5 ml of allantoic fluid from each egg was mixed with 0.5 ml of 1% chicken red blood cells. The mixture was incubated at room temperature for one hour and then observed for evidence of hemagglutination. The final titer was $1 \times 10^8$ $EID_{50}$/ml as calculated by the Reed and Muench method (66). For the HA determination, twofold dilutions of Sendai virus in TC-199 with 3% FCS were made using the microtiter method (69). After a one hour incubation period at room temperature the HA titer was found to be 8,192.

Sendai virus recovered from infected PE cells was titered for $EID_{50}$ as described above. The virus samples were diluted 1:10 and

1:100 or by a geometric twofold dilution scheme beginning with a 1:10 dilution.

Mengovirus

Mengovirus (large plaque variant) at a concentration of 1.8 x $10^9$ PFU/ml was received from Dr. Olson.

Reovirus

Reovirus at a concentration of 2 x $10^9$ PFU/ml was received from Dr. Olson.

Schwartz Virus

Schwartz virus received from Dr. Olson was in a 20% mouse spleen cell suspension.

Newcastle Disease Virus (NDV)

Newcastle Disease virus Cal-1 received from Dr. Olson was titered by the $EID_{50}$ method, and the titer was 1 x $10^9$ $EID_{50}$/ml.

## Sensitization of Animals

Swiss Webster male mice, 8 to 9 weeks old, were sensitized to tuberculin by one IP injection of 0.1 ml volume containing 0.2 mg *M. tuberculosis*, $H_{37Rv}$ strain (39).

Skin tests were done 3 weeks after sensitization using the footpad technique of Crowle (70). Each animal received approximately 0.015 ml PPD into the footpad area of the left hind foot. The footpad was checked at 6, 24, and 48 hours for induration and erythema and results were recorded as being either positive or negative.

## Collection of Peritoneal Exudate (PE) Cells

Three days before the start of an experiment, 4 ml of sterile mineral oil was injected IP into the tuberculin-sensitized and non-sensitized mice to induce peritoneal exudates. One day later the mice were skin tested with 0.015 ml PPD. Peritoneal exudate cells were collected and washed 3 times in Hanks Balance Salt Solution (HBSS) containing 10 units/ml of sodium heparin (U.S.P. Rabin Winters Pharmaceuticals, El Segundo, California).

After the final wash, the individual cell buttons were resuspended in 15 ml HBSS and total white cell counts were done. All samples were centrifuged at 600 x g once more and then resuspended in the appropriate amount of TC-199 (Grand Island Biological Company, Grand Island, N.Y.) containing 15% FCS, heat-inactivated 30 minutes at 56 C, 100 units/ml of penicillin and 100 µg/ml of streptomycin, 1.0 ml/100 ml Hepes buffer 100X (Calbiochem, Los Angeles, California, Lot 010168, A grade), and 1N NaOH.

Slide preparations of each cell suspension were stained with Wright-Giemsa stain and differential counts were done by standard procedures (71). The cell preparations contained approximately 60% macrophages, 13% lymphocytes, and 27% polymorphonuclear cells.

## Macrophage Migration Inhibition Test

The MMI test (34) was performed by filling capillary tubes (0.7 mm diameter and 75-100 mm long, Drummond Scientific Co., U.S.A.), with normal or test PE cell suspensions (PE cells without antigen, PE cells with antigen, PE cells with virus, PE cells with virus and

antigen) followed by 2% Noble's agar and then flame sealed. The capillary tubes were centrifuged at 100 xg for 5 minutes after which the capillary tubes were cut with a file at the interface between the packed cells and the supernatant. The broken ends containing the cells (4 replicates per treatment) were placed in cell culture chambers (Linbro Chemical Co., Inc., New Haven, Conn.). Appropriate medium was added and the chambers were incubated for 24 hours at 37 C in 100% air. The area of cellular migration from individual capillary tubes was determined by projecting the cell-fan image onto a sheet of paper (Simpson Lee Paper Co., San Francisco, Cal., 8½ x 11, Sub. 20) using a Leitz-Prado Universal slide projector. Each fan image was cut out and weighed on a Mettler balance. The weight of the paper was recorded in grams and used to determine the areas of cell migration (62). Percent migration was determined as:

$$\left( \frac{\text{mean fan weight of test cell population}}{\text{mean fan weight of control cell population}} \right) \times 100$$

The percent migration inhibition (MI) was calculated as:

$$\% \text{ MI} = 100 - \% \text{ migration}$$

In vivo virus-infected PE cell response (%) was determined as:

$$\left( \frac{\text{mean fan weight of PE cells with PPD}}{\text{mean fan weight of PE cells without PPD}} \right) - 1 \times 100$$

Experimental Protocol

1. To determine the optimum conditions required for the MMI test. The parameters include the time period required for optimal sensitization of cells in vivo and the optimal concentration of OT needed to give maximum in vitro response. Peritoneal exudate cells were obtained from nonsensitized animals and from animals sensitized to M. tuberculosis, $H_{37Rv}$ for 1, 2, and 4 weeks. One-tenth ml of 1 x $10^7$ cells/ml from each of the above groups was aliquoted into tubes with 0.1 ml TC-199 or 0.1 ml of the appropriate OT dilution (0.025, 0.25, 2.5, 25 mg/ml). All cells were incubated for one hour at 37 C, then placed in capillary tubes and then into chambers with the appropriate medium for 24 hours at 37 C in 100% air. Data were analyzed according to the procedure stated above.

2. To determine the MI response of PE cells from sensitized and sensitized, skin-tested mice when tested in vitro with optimal concentrations of OT and PPD. From each of 6 mice per group 1 x $10^7$ PE cells were obtained. Cells were washed and diluted in TC-199, then aliquoted into 5 test tubes with each tube containing 2 x $10^6$ PE cells in 0.5 ml volume plus 0.02 ml of OT (2.5 and 25 mg/ml), PPD (1.5 and 15 μg/ml), or TC-199. All cells were incubated for one hour at 37 C, then placed in capillary tubes and then into chambers with the appropriate medium for 24 hours at 37 C in 100% air. Data were analyzed as stated above.

3. To determine the MI response of normal PE cells treated with various viruses, in vitro. Peritoneal exudate cells were

collected, pooled, and then 0.5 ml of 1 x $10^7$ cells was distributed into test tubes. The cells were incubated 3 hours with 0.1 ml of appropriate virus dilution [Mengovirus (L) MOI 10:1 and 1:1, Reovirus 1:1 and 0.1:1, LDV 10:1 and 1:1, NDV Cal-1 10:1 and 1:1, Sendai virus 1:1 and 0.1:1, Schwartz virus 1:1 and 0.1:1], and then put up in capillary tubes. Macrophage migration inhibition studies were completed as stated previously.

4. To determine the presence of Sendai virus (MOI 1:1 and 0.1:1) and LDV (MOI 10:1 and 1:1) in normal PE cells. Mouse PE cells were collected, pooled, and then distributed 0.2 ml of 5 x $10^7$ cells/ml to get 1 x $10^7$ cells/tube after which 0.1 ml of the appropriate virus dilution was added. Cells were incubated at 37 C for 3, 12, and 24 hours. All samples were freeze-thawed 3 times and the supernatant solutions were assayed for presence of virus as stated previously.

5. To determine the MI response of sensitized PE cells following antigen stimulation and treatment with viruses, in vitro. Peritoneal exudate cells collected from sensitized, skin-tested animals were divided into 3 test tubes each containing 5 x $10^6$ cells/ml. Cells in tube 1 were incubated with 0.1 ml TC-199, cells in tube 2 were incubated with 0.1 ml LDV MOI 10:1, and cells in tube 3 were incubated with 0.1 ml Sendai virus MOI 1:1. After 2 hours of incubation at 37 C the cells in each tube were divided in half, so that PE cells in medium only received another 0.1 ml of medium or 0.1 ml 15 µg/ml PPD. Virus-infected PE cells received either 0.1 ml of medium or 0.1 ml of 15 µg/ml PPD. All tubes were incubated one more hour, and then assayed

for MMI as stated previously. Control cells from nonsensitized, non-skin-tested mice were treated the same way as cells from the sensitized, skin-tested animals. Because LDV was prepared and stored in mouse serum, some of the control cells were treated with normal mouse serum to determine if it had an effect on the PE cells.

6. To determine the MI response of sensitized PE cells obtained from mice following viral infection for 36 or 72 hours. Two different procedures were used. In procedure A, sensitized, skin-tested mice were oiled and then 36 hours later received an IP injection of 0.1 ml of the appropriate virus [Mengovirus (L) $1 \times 10^3$ PFU/mouse, Reovirus $1 \times 10^4$ PFU/mouse, Sendai virus $5 \times 10^9$ $EID_{50}$/mouse, LDV $1 \times 10^7$ $ID_{50}$/mouse]. One and one-half days later, PE cells collected from individual mice were aliquoted into 3 test tubes with $3.3 \times 10^6$ cells/ml. One tube received 0.1 ml medium, second tube received 0.1 ml 90 µg/ml PPD, and the third tube received 15 µg/ml PPD. After one hour incubation at 37 C, all cells were assayed for MMI as previously stated. Controls for this experiment included PE cells from sensitized, skin-tested, nonvirus-infected animals treated with 15 µg/ml and 90 µg/ml PPD.

For procedure B, sensitized, skin-tested mice were injected IP with 0.1 ml of the appropriate virus [Mengovirus (L) $1 \times 10^3$ PFU/mouse, Reovirus $1 \times 10^4$ PFU/mouse, Sendai virus $5 \times 10^9$ $EID_{50}$/mouse, LDV $1 \times 10^7$ $ID_{50}$/mouse] and then 3 hours later received 4 ml of sterile mineral oil, IP. Three days later PE cells were collected and cultured as described above.

7. To determine the MI response of sensitized PE cells obtained from virus-infected mice. Sensitized, skin-tested mice were injected IP with 0.1 ml of the appropriate virus [LDV 1 x $10^7$ $ID_{50}$/mouse, Sendai virus 5 x $10^9$ $EID_{50}$/mouse, Mengovirus (L) 1 x $10^3$ PFU/mouse], and then 3 hours later received 4 ml of sterile mineral oil, IP. Three days later PE cells from individual mice were aliquoted into 2 test tubes each containing 5 x $10^6$ cells/0.5 ml. One tube received 0.1 ml TC-199; the other tube received 0.1 ml 15 µg/ml PPD. Cells were incubated one hour at 37 C, and then assayed for MMI as stated before. Cells were also assayed for the presence of virus as described earlier.

## Analysis of Data

All data were analyzed statistically by an analysis of variance program (Anova) (72) and a discrimination program (73). Statistical programs were run on The University of Arizona CDC 6400 computer.

## RESULTS

Optimal conditions required for maximum in vitro OT-induced macrophage migration inhibition responses were determined by using PE cells from Mycobacterium-sensitized mice. Table I summarizes the macrophage migration response of nonsensitized PE cells and PE cells from M. tuberculosis-sensitized mice collected 1, 2, and 4 weeks after injection of the heat-killed tubercle bacilli. No migration inhibition of cells was observed with PE cells obtained one week after sensitization. Migration inhibition of PE cells, however, was seen in cells procured after 2 and 4 weeks of sensitization. Twenty-five mg of OT caused 44% MI and 39% MI, respectively. Control, nonsensitized PE cells showed no migration inhibition for the first two test periods but did show varying degrees of macrophage migration inhibition to OT at the third test period (week 4).

The dose response of sensitized PE cells to various OT concentrations as seen in Table I shows that an increase in OT (0.025 mg OT to 25 mg OT) was paralleled by an increase in percent migration inhibition. For example, at week 2 sensitized PE cells showed 0% migration inhibition at 0.025 mg OT while at 25 mg OT 44% migration inhibition was demonstrated. Overall, the in vitro response of PE cells sensitized for 2 and 4 weeks to various concentrations of OT was similar.

The macrophage migration inhibition response of PE cells from Mycobacterium-sensitized and Mycobacterium-sensitized, skin-tested mice

Table I. In Vitro Response of Nonsensitized and Sensitized Mouse PE Cells to Concentrations of OT after 1, 2, and 4 Weeks Sensitization.

| Cell treatment | 1 Week | | | 2 Weeks | | | 3 Weeks | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean fan weight (g) | % Mig. | % MI | Mean fan weight (g) | % Mig. | % MI | Mean fan weight (g) | % Mig. | % MI |
| Nonsensitized cells plus: | | | | | | | | | |
| TC-199 | 0.469[a] | 100 | 0 | 0.286 | 100 | 0 | 1.248 | 100 | 0 |
| 0.025 mg OT | 0.681 | 146 | 0 | 0.306 | 107 | 0 | 1.154 | 93 | 7 |
| 0.25 mg | 0.550 | 117 | 0 | 0.423 | 148 | 0 | 1.187 | 95 | 5 |
| 2.5 mg | 0.595 | 127 | 0 | 0.325 | 114 | 0 | 0.789 | 63 | 37 |
| 25 mg | 0.473 | 101 | 0 | 0.361 | 126 | 0 | 0.944 | 76 | 24 |
| Sensitized cells plus: | | | | | | | | | |
| TC-199 | 0.167 | 100 | 0 | 0.918 | 100 | 0 | 0.872 | 100 | 0 |
| 0.025 mg OT | 0.706 | 114 | 0 | 1.011 | 110 | 0 | 0.818 | 94 | 6 |
| 0.25 mg | 0.835 | 135 | 0 | 0.823 | 90 | 10 | 0.555 | 64 | 36 |
| 2.5 mg | 0.809 | 131 | 0 | 0.732 | 80 | 20 | 0.653 | 75 | 25 |
| 25 mg | 0.709 | 115 | 0 | 0.511 | 56 | 44 | 0.535 | 61 | 39 |

a. Mean fan weight of four replicates per treatment.

was tested in vitro with optimal concentrations of OT and equivalent concentrations of PPD (Table II). Peritoneal exudate cells collected from nonsensitized mice demonstrated 7-11% inhibition of migration in the presence of 2.5 and 25 mg OT or 1.5 and 15 µg PPD. A fourfold increase in percent migration inhibition was noted when sensitized PE cells were cultured in the presence of these antigens, and a fivefold increase in percent migration inhibition was observed when PE cells obtained from sensitized, skin-tested mice were compared to nonsensitized PE cells.

Statistical analysis of these data using the analysis of variance program showed a significant difference between nonsensitized PE cells and PE cells from sensitized or sensitized, skin-tested animals at the 99% confidence limit (72). However, there was no significant difference between PE cells from sensitized animals and PE cells from sensitized, skin-tested animals, and there was also no significant difference in drug treatment at the 95% confidence limit.

The previous data obtained from pooled PE cells and PE cells tested on an individual mouse basis showed that 3 to 4 weeks of sensitization and 2.5 mg to 25 mg OT or its equivalent dose of PPD were optimal conditions for eliciting maximum in vitro macrophage migration inhibition. Consequently, the following optimal conditions were selected for subsequent experiments: 25 mg OT or 15 µg PPD was used in cell culture chambers after PE cells were obtained from mice sensitized to *M. tuberculosis*, $H_{37Rv}$ for three weeks and then skin-tested with the appropriate antigen (OT or PPD).

Table II. Percentage Migration Inhibition of Nonsensitized, Sensitized, and Sensitized, Skin-tested Mouse PE Cells Treated with Optimal Concentrations of OT and PPD.

| Cell type | % Migration inhibition following treatment with | | | |
|---|---|---|---|---|
| | OT | | PPD | |
| | 2.5 mg | 25 mg | 1.5 µg | 15 µg |
| Nonsensitized cells[a] | 8[b] | 11 | 7 | 11 |
| Sensitized cells | 37 | 39 | 38 | 39 |
| Sensitized, skin-tested cells | 47 | 55 | 51 | 54 |

a. Statistical analysis of variance at the 99% confidence limit showed a significant difference between nonsensitized PE cells and PE cells from sensitized and sensitized, skin-tested mice. No significant difference was observed between PE cells from sensitized and sensitized, skin-tested mice (73).

b. Mean % MI of six mice per treatment.

The effect five different classes of viruses had on the macrophage migration inhibition response of nonsensitized PE cells is summarized in Table III. Mengovirus (L) did not inhibit PE cell migration but instead enhanced macrophage migration. Lactic Dehydrogenase virus enhanced macrophage migration approximately threefold that of control PE cells and twofold that of Mengovirus (L) treated PE cells. Reovirus tested at two different concentrations caused 11% and 12% migration inhibition while Schwartz virus caused 47% and 61% macrophage migration inhibition. The paramyxoviruses, NDV Ca1-1 and Sendai, caused 45% migration inhibition and 63% migration inhibition, respectively. There was no correlation seen between virus dose and cellular response.

Table IV presents data demonstrating the presence of Sendai virus and LDV after nonsensitized PE cells were incubated in vitro for 3, 12, and 24 hours with these particular viruses. In both cases, cell associated and freeze-thawed released virus could be detected. This limited procedure, however, was not capable of indicating whether viral replication had occurred.

The macrophage migration inhibition responses of PE cells from control and sensitized animals following in vitro treatment with LDV and Sendai viruses were variable (Table V). A threefold increase in migration inhibition of sensitized PE cells in the presence of PPD was seen over nonsensitized PE cells in the presence of PPD.

Sendai-treated PE cells from nonsensitized and sensitized animals cultured in the absence of PPD showed 52% migration inhibition and 30% migration inhibition, respectively. Addition of PPD to virus

Table III. Cellular Response of Nonsensitized PE Cells after In Vitro Treatment with Six Different Viruses.

| Cell treatment | Virus dose (MOI) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | | 10:1 | | 1:1 | | 0.1:1 | |
| | Mean fan wt. (g) | Cell response | Mean fan wt. (g) | Cell response | Mean fan wt. (g) | Cell response | Mean fan wt. (g) | Cell response |
| Nonsensitized cells plus: | | | | | | | | |
| TC-199 | 0.625[a] | +100 | | | | | | |
| Mengovirus (L) | | | 0.827 | +132[b] | 0.846 | +135 | | |
| LDV | | | 1.650 | +260 | 1.750 | +280 | | |
| Reovirus | | | | | 0.553 | -12 | 0.556 | -11 |
| Schwartz virus | | | 0.328 | -47 | 0.242 | -61 | | |
| NDV Cal-1 | | | 0.362 | -42 | 0.330 | -47 | | |
| Sendai virus | | | | | 0.231 | -63 | 0.231 | -63 |

a. Mean fan weight from pooled PE cells with four replicates per treatment.

b. % Migration (+) = $\left(\frac{\text{mean fan weight of test cell population}}{\text{mean fan weight of control cell population}}\right) \times 100$.

% MI (-) = 100 - % migration.

Table IV. Detection of Sendai Virus and LDV in Nonsensitized PE Cells.

| Cell treatment | Titer of virus in PE cells at[a] | | |
|---|---|---|---|
| | 3 Hours | 12 Hours | 24 Hours |
| Nonsensitized cells plus: | | | |
| Sendai virus[b] | 1/320 | 1/80 | 1/160 |
| LDV[c] | 1/100 | 1/100 | 1/100 |

a. HA titer of Sendai virus obtained from cell cultures (68). Dilution of cell culture fluid giving a fivefold increase over controls in the LDH spectrophotometric assay procedure (64-65).

b. Sendai virus added to PE cells at a MOI of 1:1.

c. LDV added to PE cells at a MOI of 10:1.

Table V. In Vitro Response to PPD by Nonsensitized and Sensitized, Skin-tested Mouse PE Cells Incubated with and without Virus.

| Cell treatment | % Migration inhibition | | | |
|---|---|---|---|---|
| | No PPD | | 15 µg PPD | |
| | Nonsensitized cells | Sensitized cells | Nonsensitized cells | Sensitized cells |
| Cells only | 0[a] | 0 | 13 | 34 |
| Cells + Sendai | 52 | 30 | 48 | 19 |
| Cells + LDV | 3 | 3 | 0 | 10 |

a. Mean % MI calculated from 10 mice per treatment.

treated sensitized PE cells caused a decreased macrophage migration inhibition.

A 3% migration inhibition was observed when nonsensitized and sensitized PE cells were treated with LDV. Nonsensitized PE cells treated with LDV plus PPD were not inhibited in their migration, whereas addition of PPD to LDV-treated sensitized PE cells resulted in only 10% inhibition as compared to 34% inhibition of migration observed with antigen-treated sensitized PE cells.

Table VI shows the macrophage migration inhibition response of sensitized, skin-tested PE cells collected from mice 36 and 72 hours following an IP injection of virus inoculum. Sensitized PE cells obtained from virus-infected mice 36 hours postinfection showed very little or no inhibition of migration with PPD present in the chambers while PE cells obtained from nonvirus-infected, sensitized, skin-tested mice showed 37% and 33% migration inhibition, respectively.

Sensitized PE cells from virus-infected mice 72 hours after infection showed enhanced cellular migration in the presence of PPD. For example, PE cells obtained from sensitized, skin-tested LDV-infected mice migrated +112% and +104% at two different concentrations of PPD. Mengovirus (L) infected mouse PE cells migrated +17% in the presence of 15 µg PPD, but did show slight migration inhibition (-8%) with 90 µg of PPD in the cell culture chambers. Nonvirus-infected, sensitized, skin-tested mouse PE cells displayed 43% and 48% inhibition of migration in the presence of 15 µg and 90 µg of PPD, respectively.

Table VI. Percent Cell Response of PE Cells Obtained from Sensitized, Skin-tested Mice after 36 and 72 Hours of Virus Infection.

| Cell treatment | In vivo virus infection | | | |
|---|---|---|---|---|
| | 36 Hours | | 72 Hours | |
| | Mean fan wt. (g) | % Cell response | Mean fan wt. (g) | % Cell response |
| Cells plus: | | | | |
| TC-199 | 0.303[a] | | 0.258 | |
| 15 µg PPD | 0.191 | -37[b] | 0.146 | -43 |
| 90 µg PPD | 0.202 | -33 | 0.133 | -48 |
| Cells with Mengovirus (L) plus: | | | | |
| TC-199 | 0.212 | | 0.259 | |
| 15 µg PPD | 0.258 | +22 | 0.303 | +17 |
| 90 µg PPD | 0.211 | -1 | 0.237 | -8 |
| Cells with LDV plus: | | | | |
| TC-199 | 0.277 | | 0.356 | |
| 15 µg PPD | 0.269 | -3 | 0.754 | +112 |
| 90 µg PPD | 0.350 | +27 | 0.726 | +104 |
| Cells with Reovirus plus: | | | | |
| TC-199 | 0.478 | | 0.230 | |
| 15 µg PPD | 0.621 | +30 | 0.394 | +71 |
| 90 µg PPD | 0.771 | +61 | 0.291 | +26 |
| Cells with Sendai virus plus: | | | | |
| TC-199 | 0.199 | | 0.262 | |
| 15 µg PPD | 0.175 | -12 | 0.371 | +42 |
| 90 µg PPD | 0.157 | -21 | 0.315 | +20 |

a. Mean values calculated from 2 mice per treatment per time period.

b. % Cell response =

$$\left( \frac{\text{mean fan weight of PE cells with PPD}}{\text{mean fan weight of PE cells without PPD}} \right) - 1 \times 100$$

(+) indicates migration of cells, (-) indicates inhibition of migration of cells.

The altered cellular response observed above was greater in PE cells obtained from mice 72 hours after infection. In each case the virus prevented the normal inhibition of migration observed when sensitized PE cells were incubated with the appropriate antigen. The effect was more pronounced in cultures stimulated with 15 µg PPD but even 90 µg PPD was not capable of aborting the effect of the virus.

A second experiment to study the effect of virus on the migration of sensitized PE cells using the optimal conditions of 15 µg PPD and 72 hours postinfection was done. In each case 8 mice were infected with either LDV, Sendai virus, or Mengovirus (L). Results are given in Table VII. Peritoneal exudate cells incubated with PPD depicted the normal 46% inhibition of migration. However, PE cells obtained from LDV, Sendai virus, and Mengovirus (L) infected mice and incubated in the presence of 15 µg PPD did not show inhibition of migration as indicated by positive migration values of 50%, 8%, and 12%, respectively. The migration of PE cells from noninfected and virus-infected mice when cultured in the absence of antigen was not significantly different. Calculated experimental F-values of virus-infected mouse PE cells were: LDV F-value -29.0, Sendai F-value -29.22, and Mengovirus (L) F-value -21.21 (72).

Peritoneal exudate cells obtained from mice 72 hours postinfection were assayed for virus as described previously. Washed PE cells from each infected mouse demonstrated the presence of virus.

Table VII. Percent Cell Response of PE Cells Obtained from Sensitized, Skin-tested Mice after 72 Hours of Virus Infection.

| Cell treatment | Mean fan weight (g) | % Cell response |
|---|---|---|
| Cells plus: | | |
| TC-199 | 0.471[a] | |
| 15 µg PPD | 0.258 | -46[b] |
| Cells with LDV plus: | | |
| TC-199 | 0.508 | |
| 15 µg PPD | 0.759 | +50 |
| Cells with Sendai plus: | | |
| TC-199 | 0.503 | |
| 15 µg PPD | 0.545 | +8 |
| Cells with Mengovirus (L) plus: | | |
| TC-199 | 0.548 | |
| 15 µg PPD | 0.613 | +12 |

a. Mean values calculated from 8 mice per treatment.

b. % Cell response =

$$\left( \frac{\text{mean fan weight of PE cells with PPD}}{\text{mean fan weight of PE cells without PPD}} \right) - 1 \times 100$$

(+) indicates migration of cells, (-) indicates inhibition of migration of cells.

## DISCUSSION

The major question in this research was to ascertain if sensitized PE cells treated in vitro with viruses or PE cells obtained from sensitized, virus-infected mice would react differently in the MMI test. Proper evaluation of such a question requires a proper assessment of the delayed hypersensitive state and knowledge as to how virus-treated, nonsensitized macrophages migrate in the MMI test.

Delayed hypersensitivity to Mycobacterium tuberculosis, $H_{37Rv}$ was assessed in vivo using the footpad technique developed by Gray and Jennings (41). The skin test results showed no enduration or edema after 24 and 48 hours. These negative results were comparable to the negative skin test results of Feinstone et al. (52) and others (74,75).

In vitro detection of tuberculin sensitivity in PE cells from Mycobacterium-sensitized mice was successful using the MMI test of George and Vaughan (34). With this technique, tubercle sensitivity could be detected as early as two weeks with best migration inhibition occurring three to four weeks after sensitization, whereas negative skin tests were observed in three-week sensitized mice.

Peritoneal exudate cells from sensitized mice and from mice sensitized and skin-tested demonstrated similar migration inhibition percentages, 30-40% and 50-60%, respectively. Statistical analysis of these migration inhibition values showed no significant difference at the 95% confidence limit. There was a significant difference, however,

at the 99% confidence limit between nonsensitized mouse PE cell migration inhibition as compared to the migration inhibition of sensitized mouse PE cells and sensitized, skin-tested mouse PE cells. This demonstrates that the mice were sensitized to M. tuberculosis, $H_{37Rv}$.

From the above results it could be speculated that skin testing with such small quantities of OT and PPD, as was used here, did not induce immunological paralysis. There was no overloading of the immune system due to antigen received in vivo by IP injection and skin testing or due to antigen received in vitro during PE cell-antigen incubation.

Migration inhibition of PE cells from sensitized mice and sensitized, skin-tested mice in the presence of specific antigen was observed (Table II). It was also noted that 10-15% migration inhibition of nonsensitized PE cells occurred when they were exposed to OT or PPD in cell culture chambers. Reports by other investigators (52, 58, 76-78) showed that migration inhibition of nonsensitized PE cells to specific antigen ranged from 0-40%. An explanation for the migration inhibition of nonsensitized PE cells to specific antigen still is not readily apparent.

Viral studies conducted in vitro showed that different viruses altered PE cell migration as compared to the migration of nonvirus-treated PE cells. These experiments indicated that some viruses caused enhanced macrophage migration of PE cells, whereas other viruses caused inhibition of macrophage migration. Besides LDV causing enhanced migration of macrophages, it is known that LDV-infected mice have elevated levels of gamma globulin and plasma enzymes (lactic dehydrogenase,

malic dehydrogenase, glutamic-oxaloacetic transaminase) (10, 64). These enhanced metabolic activities of LDV-infected macrophages may account for the increased migration of the macrophages. To the contrary, Sendai virus-treated PE cells demonstrated inhibition of macrophage migration. Difficulty working in vitro with the paramyxovirus could be due to a protein component of the virus, hemagglutinin, which can agglutinate red blood cells and leucocytes (79).

It was demonstrated that sensitized PE cells treated in vitro with viruses and PE cells obtained from sensitized virus-infected mice did react differently in the MMI test. Such studies showed that tuberculin-sensitized PE cells treated with LDV migrated out in the presence of PPD while nonvirus-treated, sensitized PE cells demonstrated migration inhibition in the presence of PPD. This would suggest that LDV had produced an unresponsiveness or "anergy" in these sensitized PE cells to the specific antigen.

Sendai virus-treated, sensitized PE cells demonstrated the above phenomenon of inhibition of the inhibition of macrophage migration observed with LDV but did so to a lesser extent. This may be due to the viral-induced leukoagglutination which agglutinated the macrophages and prevented the detection of any altered specific immune response.

Due to the difficulty of working with Sendai virus in vitro, the phenomenon of inhibition of the inhibition of macrophage migration was studied in vivo. Results from the in vivo experiment showed that PE cells from sensitized mice infected with virus [LDV, Sendai virus,

Mengovirus (L)] demonstrated the same phenomenon as was detected in vitro.

These studies seem to provide evidence for at least two hypotheses to explain the viral induced alteration of macrophage migration. One possibility is that the presence of virus either decreases or stops the production of cell-mediated immunity (MIF for example) of the small lymphocyte. If MIF is not released to inhibit the macrophage migration, then the macrophages will migrate out even in the presence of virus. A second mechanism may be that the virus affects only macrophages by occupying receptor sites that MIF was supposed to have contacted; thus, the available receptor sites would have been "blocked" by the virus allowing for macrophage migration.

This study has shown that PE cells from virus-infected, tuberculin-sensitized mice demonstrated viral induced "anergy" of cellular immunity as detected by the macrophage migration inhibition technique. Peritoneal exudate cells from virus-infected, tuberculin-sensitized mice demonstrated inhibition of the inhibition of macrophage migration when incubated with PPD while PE cells from nonvirus-infected, tuberculin-sensitized mice showed inhibition of migration in the presence of specific antigen.

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