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INHIBITION OF MACROPHAGE MIGRATION
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by

Robert Thomas McCalmon, Jr.

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF MICROBIOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
WITH A MAJOR IN MOLECULAR BIOLOGY

In the Graduate College
THE UNIVERSITY OF ARIZONA

1 9 7 3
I hereby recommend that this dissertation prepared under my direction by Robert T. McCalmon, Jr. entitled INHIBITION OF MACROPHAGE MIGRATION BY SUBCELLULAR CONSTITUENTS be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy.

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To

Flemingism and Serendipidy

With a Little Help From My Friends
ACKNOWLEDGMENTS

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ABSTRACT

The present study was designed to determine whether or not a naturally occurring cell-mediated autoimmunity to liver subcellular constituents is present in normal adult rats. Macrophage migration inhibition was induced with Lewis rat peritoneal exudate cells and isologous liver mitochondria, inner and outer mitochondrial membranes and microsomes. Isologous liver plasma membrane and the cytosol did not cause inhibition of macrophage migration. The inhibition of macrophage migration was found to involve lymphocytes and monocytes from a peritoneal exude. Polymorphonuclear leucocytes did not appear to influence the inhibition of peritoneal exudate cells.

Further studies indicated that the inhibition of macrophage migration was caused by the specific release of an inhibitory factor from isologous lymph node cells. The cell-free supernatant from lymph node cell cultures incubated with isologous liver mitochondria, inner and outer mitochondrial membranes and microsomes caused the inhibition of peritoneal exudate cell migration, whereas, the supernatant from cultures with liver plasma membrane and the cytosol did not inhibit the migration of macrophages. However, in lymphocyte cultures, the same isologous subcellular constituents did not induce blastogenic transformation, but tuberculin PPD, PHA-P and Con A did stimulate mitogenic activity. Further studies indicated that the inhibitory factor released
into the lymph node culture supernatants had an estimated molecular weight between 20,000 and 55,000 (Sephadex). Also, the biological activity was stable at \(-20^\circ C\) and \(56^\circ C\), but labile at \(80^\circ C\).

Additional studies indicated that the release of the migration inhibitory factor was thymic dependent. Neonatally thymectomized Lewis rats were found to be unable to demonstrate inhibition of macrophage migration in either a direct test or an indirect test when challenged with isologous mitochondria in vitro. In contrast, non-thymectomized Lewis rats demonstrated both the direct and the indirect inhibition of macrophage migration to isologous mitochondria.
INTRODUCTION

Tolerance

"Immunologic tolerance" may be defined as a state of immunologic unresponsiveness toward a specific antigen as a consequence of a previous exposure to that antigen by the lymphoreticular system. Through a mechanism not completely understood, tolerance can be acquired by exposure to relatively large amounts of antigen or during periods of immunologic immaturity. Originally, the term "tolerance" was reserved for the classic example provided by Medawar (1) in which spleen cells transferred from a genetically dissimilar donor to a neonatal recipient produced a situation in which the recipient could later accept a skin graft from the spleen cell donor. In contrast, the control animals that did not receive spleen cells from the same histoincompatible donors, rejected the donors skin graft. The failure of mice to develop antipneumococcal antibody following the injection of a large dose of pneumococcal polysaccharide was called "paralysis" (2). The absence of antibody in adult rabbits following continued administration of large doses of protein antigen was termed "protein overloading" (3). The failure to develop sensitivity to picryl chloride in guinea pigs previously fed picryl chloride was termed "unresponsiveness" (4). It is now generally accepted that all of these phenomena represent aspects of the same basic process and that a common mechanism underlies all types of immunologic unresponsiveness produced by antigen (5).
At least two different cell populations are involved in the active immunization to an antigen (6). One, derived from bone marrow, consists of the antibody-producing cells and their precursors. The other consists of thymic-dependent cells. In addition, macrophages seem to play an important part, at least in some immunologic reactions, in the initial processing of antigen (6). The necessity for two lymphocyte populations to interact has been shown by Claman and Chapon (7) and by Miller and Mitchell (8). Both groups demonstrated independently that when mice were rendered immunologically incompetent by whole-body irradiation, restoration of immunological competency, with regard to the development of anti-sheep red blood cell antibody, could be achieved only by injecting the irradiated mice with both bone marrow and thymus cells from animals able to form antibodies to the antigen. Weigle (6) showed a similar effect between bone marrow and thymus cells with respect to the immunologic responses to aggregated human gamma globulin (HGG). Inbred mice were made unresponsive to HGG by injecting the antigen in a tolerogenic aggregate-free form. Such specifically tolerant animals were then used as donors of bone marrow and thymus cells in combination with cells from normal isogenic mice. The irradiated recipients produced antibody to aggregated HGG only when they had been reconstituted with bone marrow and thymus cells from normal animals. When reconstitution involved either cell type from tolerant donors, no antibody to HGG was elicited. The implication of these reports was that immunologic tolerance was created by tolerance in either of the two cell types required to initiate an antibody response.
Recent experiments by Chiller, Habicht and Weigle (9) have demonstrated a dichotomy in the kinetic behavior of the two cell populations with thymus-dependent cells becoming tolerant sooner and remaining unresponsive much longer than bone marrow cells. Additional experiments (9) showed that the tolerogenic dose response curves of the two cell populations also varied. The results presented in the above reports (7,8,9) are consistent with the possibility that, while in most cases tolerance to self antigens involves both thymus and bone marrow cells, there could be situations in which tolerance is actually existent in only one type of cell, or if induced in both types of cells, it may be maintained in only one cell type. Many autoimmune diseases involve a type of semisequestered antigen that circulate in the body in extremely small quantities. Therefore, the dose-tolerance relationships observed in the experiments just described (9) may well be pertinent to the question of tolerance being only partial and therefore, breakable.

Autoimmunity

With the exception of the autoantibodies directed against certain cells and tissue, e.g., red blood cells, kidney glomerular basement membrane and perhaps the sperm, there is a lack of convincing evidence that autoantibodies can damage cells in vivo. Weiss and Dingle (10) immunized rabbits with a lysosome-rich fraction prepared from rat liver cell homogenate and tested the resulting antisera for ability to release lysosomal hydrolases. With partially purified
lysosomal preparations, no release was seen. However, when antiserum
was added to living cell cultures of rat dermal fibroblasts, release
of intracellular acid phosphatase from the lysosomal granules occurred.
The effect was inhibited by heat inactivation of the antiserum and
by the addition of hydrocortisone. Dumonde, Roodyn and Prose (11),
studying the effects of antisera prepared against mixed mitochondrial
and lysosomal particles from rat liver, used ferroglobulin prepara­
tions of antibody for electron microscopy as well as investigating the
effects of antibody on the biochemical activity of subcellular particles.
They found that although incubation of mitochondrial and lysosomal
fractions of rat liver with rabbit antibody resulted in immune reactions
fixing large amounts of complement, the efficiency of mitochondrial
oxidative phosphorylation and the activity of lysosome acid phosphatase
were not adversely affected by these reactions at the particle surfaces.
Electron microscopy showed that antibody was localized mainly at the
outer mitochondrial membrane and on the lysosomal surface. Very little
penetration of the particles occurred, despite the presence of comple­
ment in the reaction mixture. These results seem to argue against a
primary role, in the pathogenesis of disease, for antibodies reacting
specifically with intracellular particles.

Weir, with his colleagues (in 12-16), developed an animal model
for study of autoantibodies produced in response to tissue damage.
They find that hepatocellular necrosis induced in rats by carbon tetra­
chloride (CCl₄) resulted in non-organ-specific IgM complement-fixing
autoantibodies. DeHeer, Pinckard and Olson (17) have recently shown that CCl₄ induced liver autoantibodies were associated with IgM immunoglobulins and react with all subcellular constituents tested. However, no autoantibody was observed directed towards liver plasma membrane. Weir and Elson (18) proposed that particulate subcellular antigens liberated by cell death induce antibody responses because the phagocytosis they undergo is a necessary step in "processing" antigen, at least for primary responses. Soluble cellular proteins, on the other hand, induce tolerance because they are better able to make direct contact with immunologically competent cells. However, an alternative hypothesis may be that subcellular membranes are sequestered from the lymphoreticular system during fetal and neonatal development. Therefore, the subcellular constituents are not accepted as "self" by the lymphoreticular system when released from an effete cell. This hypothesis is supported by the work of DeHeer et al. (17), that autoantibodies were not produced against the particulate plasma membrane, but they were produced against soluble proteins in the cytoplasm.

Naturally occurring anti-tissue antibodies have been detected in the sera of rabbits (19-21), rats (17,22), hamsters and mice (23) and dogs (24). These studies, coupled with the knowledge that CCl₄ induced liver injury results in the production of IgM autoantibodies and that the development of tolerance may be partial, raise the question as to the extent of tolerance to self. In order to answer this question, preliminary investigations were performed to determine whether lymphocytes would react with subcellular components. McCalmon and Pinckard (25)
reported that the migration of normal rat peritoneal exudate cells (PEC) from capillary tubes was inhibited by isologous Lewis rat liver mitochondria. Weir and Suckling (26) have recently published a similar finding. The latter workers reported that the migration of normal guinea pig, as well as PEC from guinea pigs with CCl₄ induced liver injury, was inhibited by guinea pig liver mitochondria. These findings were the first evidence that a cell-mediated immunity to subcellular particles might exist in normal adult animals. However, it was not clear if the inhibition of macrophage migration by mitochondria was identical to that induced by sensitized lymphoid cells when challenged by the sensitizing antigen. Therefore, it was necessary to compare the inhibition of macrophage migration by mitochondria with previous studies on macrophage migration inhibition and to consider the role of tissue autoantigens and delayed hypersensitivity in the inflammatory response.

**Delayed Type Immune Response**

Several mediators of delayed hypersensitivity, termed "lymphokines", have been found to be released in vitro from sensitized lymphocytes. The three most widely studied lymphocyte mediated events have been lymphocyte transformation (27–31), target-cell destruction (32–34) and inhibition of macrophage migration (35–37). Through an unknown mechanism, lymphocyte transformation reflects the interaction of antigen and receptor sites on "thymic-dependent" (T) lymphocytes that have previously become specifically sensitized towards the antigen with which they are interacting (38,39). This phenomenon is characterized by an
increase in the DNA, RNA and protein synthesis in the activated T lymphocytes (40-45). The biochemical events listed above are reflected morphologically in the transformation of sensitive T lymphocytes to "blast-cell" forms (46-52). Target-cell destruction, on the other hand, is an event produced as a consequence of antigen-sensitized lymphocyte interaction. Target-cell death may be mediated by direct contact with the sensitized lymphocytes or by a cell-free material liberated by these reactive lymphocytes into the culture medium when they are incubated with the specific antigen (53-54). Inhibition of macrophage migration is also an event arising as a consequence of sensitive lymphocyte-antigen interaction. One of the proteins elaborated by antigen-stimulated lymphocytes is a non-immunoglobulin, heat stable (56°C/30 minutes) component with an estimated molecular weight between 35,000 and 55,000 which inhibits the migration of normal macrophages (55,56).

**Lymphocyte Transformation**

Lymphocyte transformation to "blast-cell" forms is accompanied by numerous biochemical and morphological changes which take place within the small T lymphocyte after interaction with a mitogen. Acetylation of nuclear histones (57), phosphorylation of nuclear proteins (58) and an increase in phospholipid metabolism (59,60) take place within minutes after addition of the mitogen to the lymphocyte culture. These events are followed by increased protein production, RNA breakdown, new RNA formation (42-45), activation of lysosomes with
formation of new lysosomal enzymes (61-63) and finally, an increased DNA synthesis (40,41). Morphologically, lymphocyte transformation to "blast-cell" forms is reflected by cell enlargement accompanied by changes in the nuclear chromatin, the appearance of nucleoli, mitosis and formation of new daughter cells (52).

Substances that exert mitogenic action on T lymphocytes can be divided into three classes: (a) Non-specific mitogens can stimulate lymphocytes, from animals that have not been actively sensitized to the mitogen (46). The most widely used agent of this group is phytohemagglutinin (PHA). PHA is a plant lectin obtained from the red kidney bean, *Phaseolus vulgaris*; (b) Numerous viral, bacterial, fungal and other antigens are known as specific mitogenic stimulators. As a rule, they produce blastoid transformation only if the T lymphocytes have been previously exposed to these antigens. Therefore, specific lymphocyte transformation probably represents a memory phase in the response to an antigen (52); (c) Lymphocytes can also be stimulated with allogeneic white blood cells (64-66). This probably represents a cellular response due to histocompatibility differences between the cells (67,68). That is, a mitogenic response is detected only if the mixed cells are genetically different and, therefore, exhibit non-self antigens. A response cannot be elicited if cells from syngeneic animals or identical twins are mixed.
Migration Inhibition Factor

Migration Inhibition factor (MIF) is the best characterized of the lymphocytic mediators. Rich and Lewis (69) showed that if explants of spleen or lymph node cells from sensitized animals were placed in vitro with a specific antigen, cell migration from these lymphoid explants was inhibited. George and Vaughn (35) packed peritoneal exudate cells (PEC) from sensitized animals in small capillary tubes and measured their migration out of the capillary tubes onto glass when cultured in the presence of antigen. With this system, it became possible to quantitate the cells migration after a certain period of time. Further research suggested two cell types participating in the hypersensitivity reaction. Bloom and Bennett (70), and independently David et al. (36), took PEC from sensitized guinea pigs, separated the lymphocytes from the macrophages and added antigen to the macrophage fraction. These cells were no longer inhibited by antigen unless the lymphocytes were returned to the preparation.

Thor et al. (71), and later Rocklin, Meyers and David (72), were able to show migration inhibition of non-sensitive guinea pig PEC with a non-dialyzable fraction from a cell-free supernatant of sensitized human lymphocytes cultured with PPD. Further characterization of MIF came as the result of experiments by Remold et al. (73). He showed that MIF was heat stable at 56°C for 30 minutes and confirmed Thor's observation that MIF was non-dialyzable. Remold et al. (73) also showed, by using Sephadex filtration and acrylamide disc gel electrophoresis, that MIF from hapten-BGG conjugate sensitized guinea pig
lymphocytes had a molecular weight between 35,000 and 55,000 and that it was not an immunoglobulin. Further, Remold and David (74) have shown that if supernatants containing guinea pig MIF were pretreated with an insoluble chymotrypsin or an insoluble neuraminidase, the MIF activity was lost. From these experiments, they suggest that MIF may be a glycoprotein.

Recently, Nathan et al. (75) showed that after 72 hours of incubation, MIF-treated macrophages exhibited an increase in adhesiveness to glass surfaces, a three to four fold increase in phagocytosis of starch granules and a four to eight fold increase in glucose oxidation. The elevated glucose oxidation was found to represent increased metabolism through the hexose monophosphate shunt, which may well be related to a potential heightening of bactericidal capability (75). More recently, Bloom, Gaffney and Jimenez (76) and later Rocklin (77) have shown that MIF release from sensitized lymphocytes was dissociated from cell proliferation.

**Statement of the Problem**

Understanding the role of lymphocytes in cell-mediated reactions have become more clear since the description of a factor inhibiting normal macrophage migration (MIF), released by sensitized lymphocytes after contact with antigen (35,36). Other non-antibody factors have been found to be released at the same time (78). These include mitogenic, chemotactic, skin reactive and cytotoxic factors. Recent evidence has shown that MIF can be released from normal guinea pig lymphocytes by the plant mitogens PHA-P and concanavalin A (79).
Previous studies have shown that hepatocellular injury induced in rats by CCl$_4$ or other liver damaging agents results in the production of IgM autoantibodies directed at various subcellular components (13,17). Similar naturally-occurring IgM anti-tissue antibodies have been reported in various species by a variety of immunological techniques (17,19,23,24) and it was proposed that rats did not develop neonatal tolerance to the subcellular antigens (80). Other studies have indicated a dichotomy in the kinetic behavior of lymphoid cells to tolerogenic antigens (9). These observations raise the question as to the extent of the anti-tissue immunity and whether it is completely restricted to an IgM response or includes other aspects of immunity including cell-mediated immunity. Indeed, McCalmon and Pinckard (25) and Weir and Suckling (26), have reported that a macrophage migration inhibitory effect occurs when PEC are exposed to mitochondria.

The finding of a direct macrophage migration inhibitory effect described for mitochondria was the first evidence that cell-mediated immunity might exist with respect to subcellular constituents. However, the direct inhibition of PEC by subcellular particles could be due to (a) non-specific aggregation or agglutination of granulocytes by the subcellular membrane material added which then inhibit the migration of the macrophages (81), (b) a macrophage cytophilic antibody (82), (c) antigen-antibody complexes (83), or (d) the specific release of a migration inhibiting factor from lymphocytes. Therefore, it was necessary to compare the phenomenon of macrophage migration by subcellular constituents with previous studies on MIF and to consider
the role of tissue antigens on macrophage migration in relation to the inflammatory response.

The objective of this research was to study the possible existence of a naturally occurring cell-mediated immunity toward subcellular constituents in normal isogenic adult rats. This study consisted of (a) determining if the normal migration of PEC was inhibited by isologous subcellular membraneous constituents from normal adult rats; (b) characterizing the inhibition of PEC induced by the subcellular constituents by determining if the responsive cells were lymphocytes which, when cultured in the presence of the isologous subcellular fractions, underwent blastogenesis and/or liberated factors which would cause the inhibition of cellular migration; (c) determining if the cells involved in the response observed were "T" lymphocytes; and finally, (d) estimating the molecular weight of the membrane induced MIF along with determining its stability between -20° and 80°C.
MATERIALS AND METHODS

Animals

Adult, 150 gm female isogenic Lewis rats obtained from Simonsen Laboratories, Inc. (Gilroy, Calif.) and Charles River Breeding Laboratories, Inc. (Wilmington, Mass.) were used throughout these experiments. Neonatally thymectomized Lewis rats and non-thymectomized control Lewis rats were obtained from Charles River Breeding Laboratories at 21 days of age and were used before they were 28 days old. All rats were cared for by the Division of Animal Resources at the University of Arizona Medical Center. They were caged in pairs and fed water and Purina Rat Chow ad libitum.

Bleeding and Collection of Rat Serum

The rats were anesthetized with ether and bled by cardiac puncture using a 21-gauge needle and a 5 ml syringe. The blood was allowed to clot at room temperature and subsequently centrifuged at 1,000 x g for 15 minutes at 4°C. Following centrifugation, the serum was pooled and heated in a water bath at 56°C for 30 minutes. All sera was either used immediately as a supplement for tissue culture medium, or was divided into aliquots and frozen at -20°C until needed.


**Media**

Two media were utilized throughout this study. One is termed a "holding" medium; i.e., a medium which is used to maintain the viability of cells for only a very short period of time. This type of medium does not contain all of the essential ingredients necessary for cell survival and growth. The other medium was a "maintenance" or tissue culture medium. This type of medium contains all the essential ingredients for cell survival and growth. This medium was used for all experiments requiring in vitro cultivation of cells.

Hank's Balanced Salt Solution (HBSS; Grand Island Biological Co. [GIBCO], Grand Island, N.Y.) buffered with sodium bicarbonate to pH 7.4 and supplemented with 0.6% Dextran (Cutter Laboratories, Inc., Berkeley, Calif.) and 5 Units of heparin/ml (HBSS-DH) was the "holding" medium used. The tissue culture medium was Minimal Essential Medium with Eagle Salts (GIBCO) supplemented with 10% normal heat inactivated isologous rat serum, 100 Units of Penicillin/ml, 100 µg of Streptomycin/ml and 200 mM L-glutamine (MEM). MEM was the tissue culture medium used in all in vitro experiments.

**Mitogens**

The mitogens used were the plant lectins, phytochemagglutinin (PHA-P; Difco, Detroit, Mich.), concanavalin A (Con A; Calbiochem, San Diego, Calif.) and pokeweed mitogen (PWM; GIBCO). Tuberculin purified protein derivative without preservatives (PPD; Parke Davis Co., Kalamazoo, Mich.) was used as a specific mitogen in experiments with rats
previously sensitized to *Mycobacterium tuberculosis* H37Ra in Freund's adjuvant (Bacto-Adjuvant Complete H37Ra, Difco).

**Antigens**

In order to determine if there was a population of lymphocytes in the normal adult rat sensitized to subcellular membraneous constituents, isologous liver plasma membranes, microsomes, mitochondria, inner and outer mitochondrial membranes and cytosol were used as antigens. The mitochondria, inner and outer mitochondrial membranes, microsomes and cytosol were isolated by differential centrifugation in 0.25 M sucrose according to a modification of the procedure of Parsons, Williams and Chance (84). Plasma membrane was isolated according to Ray (85). All membranes were finally resuspended in HBSS (without Dextran or heparin) suplimented with 100 Units of Penicillin/ml and 100 µg Streptomycin/ml (HBSS-PS), dispensed in 1 ml vials and frozen at -70°C.

**Mitochondria**

Rat liver mitochondria was prepared according to a modification of the procedure of Parsons et al. (84). Eight to ten rats were stunned and decapitated. The livers were rapidly removed, washed in 0.25 M sucrose to remove blood then they were minced with scissors. The minced liver was placed into a 50 ml Potter-Elvehjem homogenizing vessel filled to the neck with 0.25 M sucrose and homogenized with a Teflon pestle a maximum of four times. The homogenizing vessel was then filled with 0.25 M sucrose and the contents poured into four
plastic 50 ml centrifuge tubes. The homogenate was centrifuged at 480 x g for 10 minutes to sediment unbroken cells, cellular debris, nuclei, etc. The supernatant, containing mitochondria, microsomes and cytoplasm, was poured into another 50 ml centrifuge tube and spun at 5,090 x g for 20 minutes to sediment the mitochondria. The floating fatty layer was removed with tissue paper and the supernatant decanted and saved for isolation of the microsomal membranes and the cytoplasm. The mitochondria were washed by combining the pellets in every two centrifuge tubes and resuspending the mitochondria in 15 ml of 0.25 M sucrose. The suspension was then centrifuged at 7,710 x g for 10 minutes. The washing procedure was repeated once again.

**Inner and Outer Mitochondrial Membranes**

The washed mitochondria, after having been prepared as described above, were resuspended in 300 ml of cold 0.02 M potassium phosphate buffer, pH 7.2, and were gently shaken for 20 minutes in a 1,000 ml Erlenmeyer flask packed in ice. The contents of the flask were dispersed into eight 50 ml plastic centrifuge tubes and spun at 34,800 x g for 20 minutes. The supernatants were discarded and the pellets were resuspended in the phosphate buffer by drawing the contents in and out of a 10 ml pipette. The suspension was then gently homogenized by hand in a 50 ml Potter-Elvehjem homogenizing vessel with a teflon pestle. The homogenate was brought to a final volume of 300 ml, dispersed into eight 50 ml plastic centrifuge tubes and spun at 1,935 x g for 15 minutes to sediment the inner mitochondrial membranes. The supernatant containing the crude outer mitochondrial membrane was decanted and saved for further processing.
The pellet was washed twice by resuspending each of the pellets in 25 ml of phosphate buffer followed by centrifugation at 3,020 x g for 15 minutes. The inner mitochondrial membrane pellet was resuspended in HBSS-PS and aliquoted as previously described.

The supernatant containing the outer mitochondrial membrane was centrifuged at 27,000 x g for 30 minutes. The supernatant was discarded and the pellets were resuspended in phosphate buffer to a final volume of 15 ml. Five ml of the homogenate was layered on each of three 1" x 3" cellulose nitrate tubes containing a sucrose gradient prepared as follows:

- **Bottom:** 6 ml of 51.3% (w/v) sucrose in phosphate buffer.
- **Middle:** 10 ml of 37.7% (w/v) sucrose in phosphate buffer.
- **Top:** 6 ml of 25.2% (w/v) sucrose in phosphate buffer.

The sucrose gradient was centrifuged at 56,250 x g for 90 minutes in a Beckman Model L ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) using a SW 25.1 rotor. The bands on the sucrose gradients were designated as "A", "B", "C", and "D" from top to bottom and were removed by lateral puncture with a 10 ml syringe fitted with an 18-gauge needle. Each band was placed in a 50 ml centrifuge tube and brought to a total volume of approximately 40 ml with phosphate buffer. The tubes were then centrifuged at 34,800 x g for 45 minutes. According to the data reported by DeHeer et al. (17), only band B was used in this study.

**Microsomes and Cytoplasm**

After the supernatant containing mitochondria, microsomes and cytoplasm was centrifuged, during the mitochondria preparation procedure,
the floating fatty layer was removed with tissue paper and the supernatants containing the microsomes was decanted. The supernatant was then spun at 23,500 x g for 20 minutes to sediment mitochondria, cell debris, etc. The pellet was discarded and the supernatant was centrifuged at 80,800 x g for 90 minutes in a Beckman Model L ultracentrifuge using a Type 40 rotor. The supernatant contained the cytoplasm and the pellet contained the microsomes. The cytoplasm, or cytosol, was decanted and aliquoted into 1 ml vials. The pellet containing the microsomes was resuspended in HBSS-PS and aliquoted as previously described.

**Plasma Membrane**

Plasma membrane was isolated according to Ray (85). Eight to ten rats were stunned and decapitated. The livers were removed, washed in cold homogenizing medium containing 1.0 mM NaHCO$_3$ and 0.5 mM CaCl$_2$, pH 7.5. The livers were then minced with a scissors. Each liver was homogenized in 40-50 ml of homogenizing medium using a Potter Elvehjem homogenizing vessel. The homogenates were pooled and diluted to a final volume of 2,000 ml and allowed to stand for 5 minutes. The pooled homogenate was then passed through 4 layers of cheese-cloth into 250 ml plastic bottles in order to remove any connective tissue and centrifuged at 1,500 x g for 30 minutes (pellet I). The supernatants were discarded and the pellets resuspended in 1,000 ml (1/2 the previous volume) of homogenizing medium and centrifuged at 1,250 x g for 15 minutes (pellet II). Pellet II was resuspended in 500 ml of homogenizing buffer and
poured into sixteen 50 ml plastic centrifuge tubes and spun at 1,250 x g for 15 minutes (pellet III). Pellet III was resuspended in 6 ml of homogenizing buffer and subsequently mixed with 12 ml of 70% (w/w) sucrose giving a final sucrose concentration of 48% (w/w). Of the 48% sucrose-membrane homogenate 5.5 ml was placed into each of three 1" x 3" cellulose nitrate tubes and overlayed with 8 ml of 45% (w/w) sucrose, 10 ml of 41% (w/w) sucrose and 4 ml of 37% (w/w) sucrose. The sucrose gradients were centrifuged at 63,580 x g for 2 hours in a Beckman Model L ultracentrifuge using a SW 25.1 rotor. The plasma membrane was found as a continuous fraction throughout the 37% sucrose solution. The membrane was removed by lateral puncture with a 10 ml syringe fitted with an 18-gauge needle. The 37% sucrose-plasma membrane containing fraction was diluted with approximately 25–30 ml of homogenizing medium and centrifuged at 3,020 x g for 30 minutes. The supernatant was discarded and the pellet resuspended in HBSS-PS and aliquoted as previously described.

**Enzyme Assays**

The relative purity of each membrane fraction can be assessed by either electron-microscopy or enzymatically. For this study, the relative purity of each subcellular fraction was assessed enzymatically due to the relative ease in carrying out the assays and the speed at which the assays could be completed compared to the procedures necessary for electron-microscopy. The enzymes used were glucose-6-phosphatase for microsomes (86,87); Mg++-adenosinetriphosphatase for plasma
membrane (88); cytochrome oxidase for inner mitochondrial membrane (89) and monoamine oxidase for outer mitochondrial membrane (90). Protein concentrations were determined according to the technique of Lowry et al. (91).

All spectrophotometric enzyme assays were performed at room temperature. Oxygen consumption was measured using a Clark-type oxygen electrode at 28°C and spectrophotometric assays were performed using a Beckman DB spectrophotometer. The rate of each reaction, except those assays requiring phosphate determinations, were determined by calculation of the rate of change of absorbance or the rate of oxygen consumption. ATPase and glucose-6-phosphatase assays were performed by assaying spectrophotometrically for phosphate released from appropriate substrates. The rates of all reactions were expressed as nmoles of substrate converted to product per minute.

**Cytochrome Oxidase**

Cytochrome oxidase was assayed according to the method of Schnaitman and Greenawalt (89) by measuring the rate of oxygen consumption using an oxygen electrode. The reaction mixture consisted of 1.63 ml of 0.15 M potassium phosphate buffer, pH 7.2, 0.84 ml of deionized water, 0.30 ml of 3.0 x 10^{-4} M cytochrome c, 0.10 ml of 0.375 M ascorbate and 0.03 ml of 0.03 M tetramethyl-p-phenylenediamine (TMPD). The baseline was established after the addition of TMPD and prior to the addition of 0.10 ml of the sample which had previously been activated by a 1:1 dilution with 1% (w/v) Lubrol.
Monoamine Oxidase

Monoamine oxidase was assayed according to the procedure of Deitrich and Erwin (90). The rate of the conversion of p-dimethylaminobenzylamine (DAB) to p-dimethylaminobenzaldehyde was measured as a change in absorbance at 355 nm. The reaction mixture consisted of 1.5 ml of 0.10 M potassium phosphate buffer, pH 7.6, 0.30 ml of 3.0 x 10^{-2} M DAB, 1.1 ml of deionized water and 0.10 ml of a 1:1 1% (w/v) Lubrol activated sample.

Glucose-6-Phosphatase

Glucose-6-phosphatase was assayed according to the method of Swanson (86) and inorganic phosphate was estimated by the procedure of Gamori (87). In order to estimate inorganic phosphate at zero time and after 15 minutes, all reactions were carried out in duplicate.

Procedure. 0.30 ml of 0.09 M maleate buffer, pH 6.5, and 0.10 ml of 0.10 M glucose-6-phosphate were added to a 12-15 ml glass tube and brought to 37°C in a water bath. To one of the duplicate tubes, 1.0 ml of 10% (w/v) trichloroacetic acid (TCA) was added and marked time zero. To all tubes, 0.10 ml of sample was added, gently mixed and allowed to incubate in a 37°C water bath for 15 minutes. After 15 minutes, 1.0 ml of 10% TCA was added to the time-15 tubes to stop the reaction and all tubes were chilled in ice. After 5 minutes, the mixture was diluted to a final volume of 2.5 ml with deionized water and centrifuged at 1,000 x g for 10 minutes. Following centrifugation, two ml of the reaction mixture were added to a test tube
containing 2.5 ml of a molybdate-sulfuric acid reagent, prepared by mixing 2 volumes of 5% (w/v) sodium molybdate and 1 volume each of 10 N H₂SO₄ and deionized water and 1 ml of a reducing agent, prepared by dissolving 1.0 gm of p-methylaminophenol sulfate (Elon) in 100 ml of 3% (w/v) sodium bisulfite. The solution was, then, brought to a final volume of 15 ml. After 60 minutes, at room temperature, the solutions were assayed spectrophotometrically at 600 nm. The concentration of inorganic phosphate was determined from a standard curve using dilutions of a 0.01 mg monobasic potassium phosphate standard solution.

\[
\text{Mg}^{++}-\text{Adenosinetriphosphatase}
\]

\[
\text{Mg}^{++}-\text{adenosinetriphosphatase (ATPase) was assayed according to the method of Emmelot and Boss (88). All reactions were performed in duplicate as in the glucose-6-phosphatase assay. The assay was performed by mixing 1.60 ml of 0.05 M Tris buffer, pH 7.2, containing 0.10 M KCl, 0.005 M MgCl₂ and 0.005 M CaCl₂ with 0.10 ml of 0.10 M ATP and 0.10 ml of sample. The reaction mixture was incubated at 37°C, as in the glucose-6-phosphatase assay, prior to the addition of the sample. The reaction was stopped by the addition of 1.0 ml of 10% TCA, chilled in ice and centrifuged at 1,000 x g for 10 minutes. Two ml of the centrifuged mixture were assayed for inorganic phosphate as in the glucose-6-phosphatase assay.}
\]
**Tuberculin PPD Sensitization**

Rats were sensitized to tuberculin purified protein derivative (PPD) by a modification of the technique of Graf and Mather (92). One tenth ml Freund's complete adjuvant (FCA) containing 500 µg *M. tuberculosis* H37Ra (Difco) was injected subcutaneously into the hind footpad and 0.9 ml was injected into the nuchal region. The injection schedule was repeated two weeks later. Ten days after the secondary immunization, the lymph node cells and/or the PEC were tested for mitogenic activity and production of MIF.

**Direct Test for Migration Inhibition Factor**

A modification of the method of George and Vaughn (35) was used. Four days after the injection of 10 ml of sterile white paraffin oil (Matheson Coleman and Bell, East Rutherford, N.J.) into the peritoneal cavity, 2-6 rats were decapitated and the peritoneal exudate cells (PEC) were harvested from the peritoneum in 50 ml of HBSS-DH. The exudate was centrifuged at 200 x g and the pellet was washed three times in HBSS-DH. The resulting pellet was resuspended in 10 times the HBSS-DH. The resulting pellet was resuspended in 10 times the packed volume of the pellet with MEM containing either 100 µg membrane protein/ml, 10 µg PPD/ml or 10 µg Con A/ml. The PEC suspension was, then, drawn into untreated capillary tubes, sealed at one end with Seal Ease Clay (Clay Adams, Inc., N.Y., N.Y.) and centrifuged at 200 x g to pack the PEC. The capillary tubes were broken at the cell-fluid interface and the packed cell end placed into a Sykes-Moor chamber (Bellco Glass, Inc.,
Vineland, N.J.) and secured in place with a small amount of silicone stopcock grease (Dow Corning, Midland, Mich.). The Sykes-Moore chamber was closed and medium containing antigen was added. The area of migration was viewed after 18-24 hours of incubation at 37°C in a humidified 5% CO₂ in air environment. The area of migration was quantitated by projecting the image in the chambers onto the wall and tracing the image on standardized paper. The images were cut out and weighed. The migration index, or the relative area of migration of the test chambers to the area of migration in the control chambers was calculated as follows:

\[
MI = \frac{\text{the weight of migration}}{\text{of the experimental}} \times 100
\]

\[
= \frac{\text{the weight of migration}}{\text{of the control}}
\]

Each chamber contained two capillary tubes and each test had at least two chambers. This, then, allowed four observations per test. The control chambers contained medium without any antigen. In those experiments testing the ability of PPD to inhibit the migration of PEC, the PEC were obtained from rats previously sensitized to *M. tuberculosis* H37Ra.

**Ficoll-Sodium Metrizoate Density Gradient Fractionation of Peritoneal Exudate Cells**

Peritoneal exudates from six rats were separated into three cellular components: granulocytes, monocytes and lymphocytes, by a modification of a method described by Boyum (93). Ficoll-Sodium Metrizoate (solution 1) was prepared by mixing 29.4 ml of Sodium Metrizoate (32.8% solution, density = 1.200 gm/ml at 20°C, Nyegaard
& Co., Nycoveien, Oslo, Norway) to 70.6 ml of Ficoll (Pharmacia Fine
Chemicals, Inc., Piscataway, N. J.). The resulting mixture had a
final density of 1.077 gm/ml at room temperature. Solution 2 was
prepared by adding 2.0 ml HBSS (without supplements) to 8.0 ml of
solution 1.

Four day PEC were induced and obtained from 6 rats as pre-
viously described. The pooled PEC were suspended in 5 ml of HBSS-H
(without Dextran), and 2.5 ml of the suspension was layered on each
of two 10 ml glass tubes containing a Ficoll-Sodium Metrizoate
gradient prepared as follows: three ml of solution 1 was placed into
the tubes followed by carefully layering 3 ml of solution 2. This,
in effect, produced a three phase density gradient. The gradient was
centrifuged at 400 x g for 40 minutes. The fractions were designated
as "A", "B" and "C" (pellet) from top to bottom. Fraction A com-
prised those cells found at the HBSS-solution 2 interface and within
solution 2. Fraction A was removed with a Pasteur pipette. Fraction
B contained the cells at the solution 1-solution 2 interface and
within solution 1. Fraction B was removed by decantation. Fraction C,
the pellet, was removed by resuspension in HBSS-DH and subsequently
decanted. Each fraction was washed 3 times by resuspending the cells
in HBSS-DH and centrifuging at 200 x g. The pellets were resuspended
in MEM and used in a direct test for MIF as previously described.
Each fraction was tested against 100 μg mitochondrial protein/ml of
medium.
Induction and Assay of Mitogenesis

Rat lymph node cells (LNC) were assayed for mitogenic activity according to a modification of Colley, Wu and Waksman (94). Rats were anesthetized with ether and exanguinated by cardiac puncture. This allowed for the use of isologous serum as a medium supplement and reduced the red blood cell contamination in the in vitro LNC cultures. The popliteal, mesenteric, axillary, submaxillary and cervical lymph nodes were removed and placed into HBSS-DH. The cells were teased from the lymph nodes into HBSS-DH. The cell suspension was drawn into a 10 ml syringe through an 18-gauge needle and passed through a 50 mesh stainless steel screen (0.009 d wire, Pacific Metals, Los Angeles, Calif.) into a 50 ml conical glass centrifuge tube. A total viable cell count was performed by diluting the cell suspension 1:20 in 3% acetic acid and counting the cells in a Neubauer counting chamber. The cells were centrifuged at 200 x g for 10 minutes and resuspended at a cell concentration of 2.5 x 10^6 cells/ml in MEM. All mitogenic experiments were performed in triplicate with a volume of 2 ml in each glass culture tube (Kimble Products, Toledo, Ohio). The appropriate mitogen was added and the cultures incubated at 37°C in a 5% CO₂ atmosphere. Control cultures were void of any mitogen. The PHA-P, Con A and PWM stimulated experiments, except when otherwise designated, were cultured for 72 hours and the PPD and mitochondria stimulated tests were incubated for 96 hours. Twenty-four hours before the cultures were terminated, 1 µCi of tritiated thymidine (³H-TdR, thymidine-methyl-³H, specific activity of 6.7 Ci/mM, New England Nuclear, Boston, Mass.)/ml culture fluid was
added to each test tube. The cultures were terminated and the incorporation of radioactive thymidine determined by a method of Mans and Novelli (95). One hundred µl of a well mixed cell suspension from each tube was distributed onto a 2.3 cm Whatman 3 MM filter pad (W. and R. Balson, Ltd., England) and allowed to air dry. Nucleic acid material was precipitated by placing the dried filter pads in 10% trichloroacetic acid (TCA) for one hour. The pads were then washed for 15 minutes in each of 5% TCA, 95% ethanol, 95% ethanol–acetone (1:1) at 37°C and twice in acetone. After the pads were allowed to air dry, each pad was placed into a glass scintillation vial and 5 ml of Omnifluor (New England Nuclear) was added. Each vial was counted for 5 minutes or to a preset error of 2% in a Beckman LS250 spectrometer. The counting efficiency was found to be between 15% and 20%.

**Indirect Test for Migration Inhibition Factor**

Cell-free supernatants from lymph node cell cultures were assayed for macrophage migration inhibition activity by an adaptation of techniques described by Thor et al. (71) and Falk, Collste and Moller (96). Lymph node cells were obtained and processed as described for the mitogenic experiments. After determining the viable cell count, the cell concentration was adjusted to 25.0 x 10^6 cells/ml in MEM. Ten ml of the cell suspension were put into glass prescription bottles and 100 µg membrane protein, 10 µg PPD or 10 µg Con A/ml were incubated for 72 hours at 37°C in a 5% CO₂ atmosphere. The cultures were
terminated by centrifuging at 200 x g for 10 minutes to pellet the cells and cellular debris. The supernatant was again centrifuged at 105,000 x g for 60 minutes in a Beckman Model L ultracentrifuge using a Type 40 rotor. The supernatants were millipore filtered through a 0.45 μ Swinnex-25 millipore apparatus (Millipore Corporation, Bedford, Mass.) and either frozen at -20°C or tested immediately for migration inhibitory activity. The MIF supernatants were tested in a similar way as described for the direct test for MIF except the PEC were resuspended in the MIF supernatants and the chambers were filled with the test supernatants. The chambers were incubated for 18-24 hours and the MI calculated as previously described. Two types of control cultures were established and treated as described above. The first type was an antigen control, i.e., the appropriate antigen in MEM and incubated for 72 hours without LNC. The second type was a cell control, i.e., LNC in MEM incubated without antigen for 72 hours. The cell control was always used to determine the MI.

**Bovine Serum Albumin Density Gradient Fractionation of Lymph Node Cells**

Lymph node cells were obtained as previously described and separated by differential flotation in discontinuous bovine serum albumin (BSA) density gradients. The method of Raidt, Mishell and Dutton (97) was used with slight modification. One hundred grams of BSA (Sigma Chemical Co., St. Louis, Mo.) was allowed to dissolve in 200 ml of MEM (without supplement) for approximately 48 hours. The
BSA solution was sterilized by passage through a 0.45 μm millipore filter. The refractive index (RI) of the BSA was determined by using an Abbe 3 refractometer (Bausch and Lomb, Rochester, N.Y.) and the concentration calculated as follows:

\[ C = \frac{\text{RI (BSA)} - \text{RI (MEM)}}{0.19} \]

The stock BSA was stored at 4°C. \(1.4 \times 10^9\) LNC were suspended in sufficient MEM (without supplement) to dilute the stock BSA to a final concentration of 30% in a total volume of 5.5 ml. The BSA-cell suspension was placed in the bottom of a 1" x 3" cellulose nitrate tube for use in the Beckman SW 25.1 rotor. The cell mixture was carefully overlaid with 7 ml each of 27%, 24%, 20% and finally 4 ml of 10% BSA. Centrifugation was performed at 4°C for 30 minutes at 20,000 x g. Discrete bands of cells formed at the density interfaces and were harvested by lateral puncture using a 10 ml syringe fitted with an 18-gauge needle. The fractions, designated "A", "B", "C" and "D" beginning at the top and "P" (pellet), were diluted with HBSS-DH and centrifuged at 400 x g for 10 minutes. The pellets were resuspended in HBSS-DH and washed twice by centrifuging at 200 x g for 5 minutes. The fractions were then treated accordingly, depending on the type of experiment performed, as previously described for mitogenesis and the indirect test for MIF.

Molecular Weight Estimation of Migration Inhibition Factor

The molecular weight of the MIF produced by rat lymph node cells was estimated by ascending gel-filtration according to a method
of Remold et al. (73). A 2.5 cm x 100 cm glass column was packed with Sephadex gel-filtration media G-100 (Pharmacia Fine Chemicals, Inc.) which had been equilibrated in phosphate buffered saline (PBS; 0.01 M sodium phosphate in 0.15 M sodium chloride), pH 7.4. The flow rate was established at 24 ml/hour by pumping PBS through the column against gravity. All experiments were performed at 4°C.

The column void volume (V₀) was determined with 1 ml of 0.3% Blue Dextran 2000 (Pharmacia Fine Chemicals, Inc.) and the column calibrated with the following marker proteins: bovine serum albumin, M.W. 67,000; ovalbumin, M.W. 45,000; carbonic anhydrase, M.W. 29,000 and cytochrome c, M.W. 12,400. Five mg of each of the known protein markers was dissolved in 1 ml PBS and applied to the column by allowing the sample to be pumped into the column. Collection of the column effluent in 2.5 ml fractions began when half the Blue Dextran 2000 was judged to have entered the column. The elution volumes of the known samples were estimated spectrophotometrically in a cuvette with a 1 cm light-path. Blue Dextran 2000 was estimated at 630 mµ. Bovine serum albumin, ovalbumin and carbonic anhydrase were estimated at 280 mµ and cytochrome c was estimated at 408 mµ.

The lymph node cell supernatants containing the migration inhibitory activity induced by 10 µg Con A/ml and 100 µg mitochondrial protein/ml along with a control cell culture supernatant were prepared for gel-filtration by concentrating the supernatants to one-tenth their original volume by vacuum dialysis. One ml of the concentrate was applied to the column and collected in 2.5 ml fractions. The
fractions were pooled into 5 test samples and concentrated to 1 ml by negative pressure dialysis. The test samples were designated as follows: F-1, the void volume (fraction 1-90); F-2, from the void volume through the peak BSA elution volume (fractions 91-105); F-3, from just past the BSA elution peak through OA peak (fractions 106-115); F-4, from the OA elution peak through the end of the carbonic anhydrase elution curve (fractions 116-135) and F-5, corresponded with the cytochrome c elution curve (fractions 136-160).

The test samples were added to 9 ml of MEM and tested against 4 day PEC in an indirect test for migration inhibition as described previously.
RESULTS

Direct Inhibition of Macrophage Migration by Mitochondria

The first objective of this study was to determine whether rat liver subcellular constituents could inhibit the normal migration of isologous PEC. The subcellular antigens were isolated by fractionating Lewis rat liver into mitochondria, inner and outer mitochondrial membranes, microsomes, cytosol and plasma membranes. Analysis for marker enzymes was performed on the isolated subcellular constituents to determine the relative purity of each preparation. The results of these enzymes analyses are shown in Table 1. Based on the relative specific activity of each marker enzyme, most of the subcellular fractions were found to be free of membrane cross-contamination. However, there appeared to be a certain amount of cytochrome oxidase and glucose-6-phosphatase activity contaminating the plasma membrane preparation. Glucose-6-phosphatase activity was also found in the outer mitochondrial membrane preparation and the cytosol preparation.

Due to the relatively low yield of each subcellular constituent, as compared with the yield of mitochondria, the majority of this study was performed using Lewis liver mitochondria as the antigen. Also, it was necessary to isolate each component several times throughout this work. Analyses for marker enzymes were performed after each isolation
Table 1. Activities of Marker Enzymes in Various Subcellular Fractions of Rat Liver.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Marker Enzyme</th>
<th>Mitochondria</th>
<th>Inner Membrane</th>
<th>Outer Membrane</th>
<th>Microsomes</th>
<th>Cytosol</th>
<th>Plasma Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer mitochondrial</td>
<td>Monoamine Oxidase</td>
<td>1.51</td>
<td>0.95</td>
<td>42.35</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>membrane</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inner mitochondrial</td>
<td>Cytochrome Oxidase</td>
<td>1042.60</td>
<td>813.56</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>33.60</td>
</tr>
<tr>
<td>membrane</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomes</td>
<td>Glu-6-Pase</td>
<td>7.95</td>
<td>15.04</td>
<td>52.50</td>
<td>156.21</td>
<td>9.38</td>
<td>11.25</td>
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<tr>
<td>Plasma membrane</td>
<td>Mg$^{++}$-ATPase</td>
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<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>89.64</td>
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</table>
procedure. The data shown on Table 1 was representative of the results obtained after each membrane fractionation.

The amount of mitochondrial membrane necessary to cause a consistent inhibition of Lewis PEC was determined by a titration of mitochondria from 0 to 400 μg of membrane protein/ml (Figure 1). The data obtained indicated that at 100 μg/ml, the maximum inhibition of migration was obtained. Less than 100 μg/ml did not give significant results and concentrations above 100 μg/ml caused a reduction in PEC viability after 24 hours of incubation, as determined by trypan blue exclusion.

The data on Table 2 show that 100 μg/ml of a subcellular constituent, i.e., isologous mitochondria, caused the inhibition of normal isologous macrophage migration in all experiments. Using the "Student's t-test", the results showed statistically significant PEC migration inhibition with a "p" value of less than 0.05.

Characterization of the Cellular Response to Mitochondria

The second objective of the present study was to characterize the inhibition of macrophage migration by mitochondria. That is, (a) was the inhibition of macrophage-migration due to a lymphocyte-monocyte response to mitochondria, or was this response due to a non-specific aggregation, or agglutination of granulocytes by the particulate mitochondria? (b) Would mitochondria induce blastogenic transformation in isologous lymphocytes, as is thought to occur when the cell-mediated immune mechanism is intact? (c) Can the inhibitory activity be detected in cell-free antigen induced lymphocyte cultures?
Figure 1. Mitochondria Dose Response for Inhibition of Macrophage Migration in a Direct Test.
Table 2. Direct Test for Inhibition of Macrophage Migration by Mitochondria

<table>
<thead>
<tr>
<th>Experiment</th>
<th>( \bar{X} ) Weight of Migration</th>
<th>( S^2 ) (Variance)</th>
<th>( M_I )</th>
<th>( t_s )</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.6253</td>
<td>0.002750</td>
<td>59.89</td>
<td>3.6230</td>
</tr>
<tr>
<td>Test</td>
<td>0.3745</td>
<td>0.01642</td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>0.7948</td>
<td>0.01867</td>
<td>62.44</td>
<td>4.0950</td>
</tr>
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<td>Test</td>
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<td>0.002581</td>
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<td></td>
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<tr>
<td>Control</td>
<td>2.7763</td>
<td>0.3930</td>
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<td>0.06634</td>
<td></td>
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</tr>
<tr>
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<td>0.0006215</td>
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<td>Test</td>
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<tr>
<td>Control</td>
<td>0.0526</td>
<td>0.0002271</td>
<td>48.48</td>
<td>3.0783</td>
</tr>
<tr>
<td>Test</td>
<td>0.0256</td>
<td>0.00008065</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.6235</td>
<td>0.01333</td>
<td>79.29</td>
<td>2.1784</td>
</tr>
<tr>
<td>Test</td>
<td>0.4944</td>
<td>0.0007185</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.0549</td>
<td>0.0002032</td>
<td>58.47</td>
<td>2.7316</td>
</tr>
<tr>
<td>Test</td>
<td>0.0321</td>
<td>0.00007550</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. 100μg mitochondrial protein/ml medium.
b. All experiments were performed in quadruplicate.
c. See Materials and Methods for an explanation.
d. \( M_I = \frac{\bar{X}_{migration\ of\ experimental}}{\bar{X}_{migration\ of\ control}} \times 100 \)
e. Students' t-test was the test of significance.
Tables 3 and 4 show the results of PEC subpopulations after separation on a discontinuous gradient of Ficoll-Sodium Metrizoate. There was no apparent inhibition of cellular migration by mitochondria in fractions A and C. Fraction A contained at least 95% lymphocytes while fraction C, the pellet, was comprised of cells equally divided between neutrophils and monocytes with some lymphocytes contaminating the preparation. However, the cellular migration in fraction B, which was composed of approximately 75% monocytes with the rest being equally distributed between lymphocytes and neutrophils, was inhibited by mitochondrial membranes. The inhibition compared favorably with the inhibition seen using the non-fractionated original PEC population and with the data on Table 2.

The next aspect in characterizing the cellular response to mitochondrial membranes was to determine whether mitochondria would induce lymphocyte transformation as detected by in vitro mitogenic activity. The results are seen on Table 5. Triplicate cultures of $5 \times 10^6$ LNC/2 ml were incubated in the presence of PHA-P, Con A, PPD, mitochondria or mitochondrial outer membrane for 96 hours at $37^\circ$C. Twenty-four hours before the cultures were terminated, the cultures were pulsed with 2 $\mu$Ci $^3$H-TdR. The increase in incorporation of $^3$H-TdR into the LNC nucleic acids after stimulation with PHA-P and Con A indicated that the rat lymphocytes were capable of blastogenic transformation and that the culture conditions were adequate for mitogenic activity. The increase in counts per minute observed in the PPD stimulated cultures of LNC from previously immunized rats indicated
Table 3. Yield and Distribution of Peritoneal Exudate Cells Separated on a Ficoll-Sodium Metrizoate Density Gradient.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Cell Yield(^b)</th>
<th>Neutrophils</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
<th>Other(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td>109 x 10(^6)</td>
<td>3-5</td>
<td>13-14</td>
<td>80-83</td>
<td>1</td>
</tr>
<tr>
<td>A</td>
<td>3.6 x 10(^6)</td>
<td>0-1</td>
<td>95-97</td>
<td>3-4</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>13.8 x 10(^6)</td>
<td>9-12</td>
<td>14-15</td>
<td>73-77</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>23.0 x 10(^6)</td>
<td>44-46</td>
<td>7-10</td>
<td>42-44</td>
<td>3-4</td>
</tr>
</tbody>
</table>

a. The values of 500 cells counted on each slide from each fraction in two experiments.
b. Representative total cell count in each fraction.
c. Other granulocytes, such as basophils and eosinophils.
Table 4. Direct Test for Inhibition of Migration of Subpopulations of PEC by Mitochondria\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Fraction\textsuperscript{b}</th>
<th>MI Experiment 1</th>
<th>MI Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td>58.35</td>
<td>72.67</td>
</tr>
<tr>
<td>A</td>
<td>101.86</td>
<td>97.61</td>
</tr>
<tr>
<td>B</td>
<td>44.98</td>
<td>67.67</td>
</tr>
<tr>
<td>C</td>
<td>106.11</td>
<td>98.79</td>
</tr>
</tbody>
</table>

\textsuperscript{a} 100 \mu g whole mitochondrial protein/ml medium.

\textsuperscript{b} Refer to Table 3.
Table 5. Incorporation of $^3$H-thymidine Into Lymph Node Cells After Mitogenic Stimulation.

<table>
<thead>
<tr>
<th>Mitogen</th>
<th>$^3$H-TdR Incorporation (X cpm)</th>
<th>S.I. c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>48.06 (±19.91)</td>
<td>1.00</td>
</tr>
<tr>
<td>PHA-P (1:1000)</td>
<td>2748.07 (±1120.52)</td>
<td>57.18</td>
</tr>
<tr>
<td>Con A (10μg/ml)</td>
<td>2680.31 (±2008.19)</td>
<td>55.77</td>
</tr>
<tr>
<td>PPD (10μg/ml)</td>
<td>259.52 (±93.20)</td>
<td>5.40</td>
</tr>
<tr>
<td>Mitochondria (100μg/ml)</td>
<td>48.81 (±2.56)</td>
<td>0.87</td>
</tr>
<tr>
<td>Outer Mitochondrial Membrane (100 μg/ml)</td>
<td>42.77 (±5.21)</td>
<td>0.89</td>
</tr>
</tbody>
</table>

a. All cultures were pulsed with 1 μCi $^3$H-TdR/ml 18-24 hr. prior to termination. All cultures were terminated after 96 hr. of incubation.

b. Mean values of four experiments performed in triplicate. (± one standard deviation).

c. S.I. = Stimulation index which is a ratio of the $\bar{X}$ cpm in the experimental cultures to the $\bar{X}$ cpm in the control cultures.
that the sensitized lymphocytes were capable of recognizing and reacting to a specific antigen. The data obtained from the cultures containing the mitochondria suggested that there were no cells, or at least not enough cells, present that were sufficiently sensitized to the membranes to undergo mitogenic activity.

The next step in characterizing the cellular response to mitochondria was to determine whether migration inhibitory activity could be recovered from cell-free LNC cultures that had previously been incubated with mitochondria. However, before the macrophage migration inhibitory affect of rat LNC cultures incubated with isologous liver mitochondria could be determine, the PEC migration inhibiting activity of each of the LNC culture components, i.e., the medium, the cells and the antigen individually, had to be determined. Therefore, five experimental treatments representing four different cell-free supernatants and one mixture of two of the cell-free supernatants, were tested on normal isologous PEC for migration inhibitory activity. The 5 treatments were as follows: treatment 1, the centrifuged and millipore filtered supernatant from a 72 hour culture of MEM by itself; treatment 2, the supernatant from a culture containing MEM and 100 μg mitochondria protein/ml without cells; treatment 3, the supernatant from a culture containing medium and \(2.5 \times 10^8\) LNC without antigen; treatment 4, an equal volume mixture of treatment 2 and treatment 3; treatment 5, the test treatment, the supernatant from a culture of LNC and antigen. Six observations were made for each treatment and the
data was subjected to analysis of variance. Any differences among treatment means was assumed to be due to the fixed treatments previously established by the investigator. The purpose of the analysis of variance was to estimate the differences among the treatment means. The analysis of variance is summarized on Table 6. From the results, using the F-test as the test of significance, one can conclude that there was a highly significant (P<0.001) added component due to treatment effects in the mean square among treatments. That is, the different supernatants clearly do not have the same affect on isologous PEC.

At this point one was not able to determine whether each treatment had a different affect on the migration of PEC from every other treatment or whether only the test treatment (treatment 5) was different from the control treatments (treatments 1-4), but the control treatments were not different from each other. Therefore, the data were subjected to a priori comparisons, as seen on Table 7. This was done by subdividing the treatment sum of squares and the treatment degrees of freedom into separate comparisons, using F-tests as tests of significance. First, treatment 5, the test treatment, was compared to the four control treatments as a single group. This comparison was significant (P<0.05), showing that the cell-free supernatant from a culture which had been incubated with both mitochondria and LNC significantly inhibited the migration of isologous PEC. Next, treatment 3 was compared to treatments 1, 2 and 4 as a single group. This comparison was performed to test the assumption that the affect of a
Table 6. Variance Analysis on the Indirect Test for Inhibition of Macrophage Migration Performed on Isologous Peritoneal Exudate Cells.

<table>
<thead>
<tr>
<th>Treatments (a=5)</th>
<th>(1) Medium</th>
<th>(2) Medium + Ag(^a)</th>
<th>(3) Medium + Cells(^b)</th>
<th>(4) Medium + Ag: Medium + cells(^c) + Ag</th>
<th>(5) Medium + Cells + Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \bar{X} )</td>
<td>0.0761</td>
<td>0.0912</td>
<td>0.0826</td>
<td>0.0881</td>
<td>0.0689</td>
</tr>
<tr>
<td>( \sum X )</td>
<td>0.4569</td>
<td>0.5477</td>
<td>0.4956</td>
<td>0.5288</td>
<td>0.4136</td>
</tr>
<tr>
<td>( n )</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

Analysis of variance table

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>Degrees of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>( F^t )</th>
</tr>
</thead>
<tbody>
<tr>
<td>X - ( \bar{X} ) (Among Treatments)</td>
<td>4</td>
<td>0.00196</td>
<td>0.00491115</td>
<td>20.808 (P&lt;0.001)</td>
</tr>
<tr>
<td>X - ( \bar{X} ) (Among Replicates)</td>
<td>25</td>
<td>0.005901</td>
<td>0.00023604</td>
<td></td>
</tr>
<tr>
<td>X - ( \bar{X} ) Total</td>
<td>29</td>
<td>0.007866</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. 100 \( \mu g \) mitochondrial protein/ml medium.
b. Lymph node cell concentration = 25.0 cells/ml.
c. Treatment (4) was equal volumes of the centrifuged and filtered supernatants from treatment (2) and treatment (3).
d. The F-test was the test for significance.
Table 7. Variance Analysis on the Indirect Test for Inhibition of Macrophage Migration Performed on Isologous Peritoneal Exudate Cells. Analysis of Variance Table with Treatment Sum of Squares Decomposed into Planned Comparisons.

<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>Sum of Squares</th>
<th>Degrees of Freedom</th>
<th>Mean Squares</th>
<th>F b</th>
<th>a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments (1-5)</td>
<td>0.0019645</td>
<td>4</td>
<td>0.0049115</td>
<td>20.808 (P&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td>Test (5) a vs. Others (1-4)</td>
<td>0.0011693</td>
<td>1</td>
<td>0.0011693</td>
<td>4.954 (P&lt;0.05)</td>
<td></td>
</tr>
<tr>
<td>Cell Control (3) vs. Other Controls (1,2 &amp; 4)</td>
<td>0.00000080</td>
<td>1</td>
<td>0.00000080</td>
<td>0.003 (NS)</td>
<td></td>
</tr>
</tbody>
</table>

a. Refer to Table 6.

b. The F-test was the test for significance.
cell-free supernatant from a culture of only LNC in medium did not inhibit the migration of PEC significantly from cell-free supernatants from cultures of MEM, MEM plus mitochondria or a 1:1 mixture of supernatants from cultures of LNC and mitochondria cultures. The comparison was not significant and, therefore, the choice of LNC cultures without antigen was a valid control. Consequently, LNC cultures without antigen were used throughout all subsequent indirect tests for migration inhibition factor.

To summarize the results on Tables 6 and 7, three conclusions can be drawn: (a) The supernatant from the test culture significantly inhibited the migration of PEC as compared to a control; (b) The use of a supernatant from LNC cultures not containing subcellular constituents was an adequate control; (c) The supernatant which had been incubated only with the antigen, isologous Lewis rat liver mitochondria, did not have any significant activity on the PEC. This indicated that if there was any solubilization of the mitochondrial antigens, the soluble material was not, by itself, inhibitory.

**Direct and Indirect Tests for Inhibition of Macrophage Migration by Subcellular Constituents**

The next objective of this study was to determine whether other subcellular membranes could cause the direct inhibition of isologous PEC migration. This aspect of the research was approached in two ways. First, by determining what membranes caused the direct inhibition of macrophages and secondly, by examining the cell-free
supernatants from LNC cultures that had been incubated with various subcellular constituents for their macrophage migration inhibitory activity.

Table 8 gives the data obtained from a number of experiments testing inner and outer mitochondrial membranes, microsomes, plasma membranes and cytosol for their ability to cause the direct inhibition of isologous PEC migration. Ten μg PPD/ml was added to chambers whose capillary tubes were packed with PEC obtained from M. tuberculosis H37Ra sensitized rats. Control chambers contained no antigen. The results indicate that Lewis rats are capable of becoming sensitized to a specific antigen, e.g., tuberculin, and that the specific antigen would cause the direct inhibition of macrophages in a peritoneal exudate obtained from a sensitized animal. The mean MI for PPD was 55. The results also show that both inner and outer mitochondrial membranes were inhibitory in a direct test for MIF along with microsomal membranes. The mean MI for inner mitochondrial membrane, outer mitochondrial membrane and microsomes were 59, 59 and 57 respectively. In contrast, cytosol and plasma membranes were not inhibitory.

Table 9 gives the data obtained from indirect tests for MIF with the various subcellular membranes and PPD used in Table 8. The migration indices were calculated using supernatants from cultures containing LNC alone. The antigen controls, labelled control on Table 9, were supernatants from cultures containing antigen alone. The results using PPD as antigen and LNC from previously sensitized
Table 8. Direct Test for Inhibition of Macrophage Migration by Subcellular Constituents.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>MI</th>
<th>Range of MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria (7)^b</td>
<td>59.77</td>
<td>43.77 - 79.29</td>
</tr>
<tr>
<td>Outer Mitochondrial Membrane (4)</td>
<td>59.20</td>
<td>48.87 - 66.87</td>
</tr>
<tr>
<td>Inner Mitochondrial Membrane (3)</td>
<td>59.24</td>
<td>54.69 - 64.70</td>
</tr>
<tr>
<td>Microsomes (3)</td>
<td>57.25</td>
<td>53.98 - 62.19</td>
</tr>
<tr>
<td>Cytosol (2)</td>
<td>96.03</td>
<td>95.63 - 96.42</td>
</tr>
<tr>
<td>Plasma Membrane (4)</td>
<td>96.50</td>
<td>80.76 - 108.73</td>
</tr>
<tr>
<td>Tuberculin PPD^c (4)</td>
<td>54.62</td>
<td>44.68 - 65.23</td>
</tr>
</tbody>
</table>

a. The concentration of all subcellular constituents was 100µg protein/ml medium.

b. The number of experiments performed in quadruplicate.

c. The peritoneal exudate cells were obtained from PPD sensitized rats. The antigen concentration was 10µg PPD/ml medium.
Table 9. Indirect Test for Inhibition of Macrophage Migration by Subcellular Constituents.

<table>
<thead>
<tr>
<th>Antigen a</th>
<th>( \overline{X} ) MI b</th>
<th>Range of MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria (4) c</td>
<td>55.37</td>
<td>30.24-75.47</td>
</tr>
<tr>
<td>Control d</td>
<td>105.24</td>
<td>96.85-118.26</td>
</tr>
<tr>
<td>Outer Mitochondrial Membrane (4)</td>
<td>72.27</td>
<td>70.50-76.99</td>
</tr>
<tr>
<td>Control</td>
<td>98.62</td>
<td>89.32-111.20</td>
</tr>
<tr>
<td>Inner Mitochondrial Membrane (4)</td>
<td>66.68</td>
<td>54.12-80.40</td>
</tr>
<tr>
<td>Control</td>
<td>98.59</td>
<td>88.84-107.75</td>
</tr>
<tr>
<td>Microsomes (4)</td>
<td>72.06</td>
<td>49.95-92.75</td>
</tr>
<tr>
<td>Control</td>
<td>104.14</td>
<td>100.45-115.01</td>
</tr>
<tr>
<td>Cytosol (4)</td>
<td>98.07</td>
<td>91.29-107.32</td>
</tr>
<tr>
<td>Control</td>
<td>100.52</td>
<td>87.87-113.23</td>
</tr>
<tr>
<td>Plasma membrane (4)</td>
<td>83.74</td>
<td>68.43-99.29</td>
</tr>
<tr>
<td>Control</td>
<td>97.50</td>
<td>88.76-105.83</td>
</tr>
<tr>
<td>Tuberculin PPD e (4)</td>
<td>41.50</td>
<td>36.00-45.87</td>
</tr>
<tr>
<td>Control</td>
<td>99.06</td>
<td>83.03-116.60</td>
</tr>
</tbody>
</table>

a. The concentration of all subcellular constituents was 100μg protein/ml medium.

b. The relative index of migration as compared to the migration of PEC in supernatants from cultures containing LNC only.

c. The number of experiments performed in quadruplicate.

d. Centrifuged and filtered supernatants from cultures incubated with antigen alone.

e. The lymph node cells were obtained from PPD sensitized rats. The antigen concentration was 10μg PPD/ml medium.
rats indicate that sensitized lymphocytes, when challenged by the specific antigen to which they are sensitized, will release MIF into the supernatant. The data also show that both mitochondrial membranes along with microsomes caused the same phenomenon to occur when the membranes were cultured in the presence of normal isologous LNC. The supernatants from cultures which had contained subcellular constituents without cells showed no inhibiting affect of the PEC. Therefore, a soluble product of the subcellular particles was not causing the inhibition of macrophage migration. MIF was released only when LNC and subcellular membranes were cultured together. Cytosol did not induce the release of MIF into the supernatants. Plasma membrane gave somewhat inconsistent results with MI of 99, 68, 74 and 94 for the four experiments utilizing plasma membrane as antigen.

Initial experiments demonstrated that the inhibition of PEC by subcellular constituents is organ specific and possibly limited to subcellular constituents isolated from rat liver. Isologous rat heart mitochondria was found to be unable to directly inhibit PEC or induce the release of MIF from LNC. Heart mitochondria caused a direct MI of 88 and an indirect MI of 86. In contrast, liver mitochondria caused a direct MI of 51 and an indirect MI of 45.

**Thymic Dependency of MIF Release**

In order to further characterize the inhibition of macrophage migration by subcellular constituents experiments were performed to determine whether the release of MIF from isologous LNC was thymic-dependent.
Following separation and repeated washing of LNC on a discontinuous BSA density gradient, LNC were recovered as subpopulations among four bands and a pellet. As shown on Table 10, approximately 50% of the cells were found in the pellet. Approximately 20% of the cells were recovered in both fraction C and in fraction D with 8% in fraction B and only 1% in fraction A. Although individual yields varied, these patterns remained constant throughout four separate experiments. Viability, as determined by trypan blue dye exclusion, in the four bands was always greater than 90%. The cells from the pellet were found to be 80-85% viable.

Differential counts of the subpopulations, with determination of cell size by use of an ocular micrometer, are also shown on Table 10. While the BSA gradient separates cells primarily on the basis of density, the data indicate a relationship between density and size. The less dense fractions, A and B, contained the majority of large cells. Band C contained predominantly small lymphocytes, but also a moderate number of medium-sized cells. Fraction D and P were composed almost entirely of small, very dense lymphocytes.

The mitogenic activity of the LNC subpopulations to mitogens and the mitochondria induced MIF activity released into the LNC supernatants is shown on Table 11. PHA-P stimulated cells in fractions A, B, C and especially fraction D. Con A had the same stimulatory pattern, however, Con A did not stimulate any one specific subpopulation as did PHA-P. PWM showed a very different mitogenic stimulatory pattern. The peak activity appeared to be in fraction A with some
Table 10. Cellular Yield and Size Distribution of LNC Following BSA-Discontinuous Density Gradient Fractionation.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yield (percent recovered)</th>
<th>Size Distribution (range in percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (±1-SD)</td>
<td>8μ</td>
</tr>
<tr>
<td>Original</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1.08 (±0.45)</td>
<td>93-100</td>
</tr>
<tr>
<td>B</td>
<td>8.10 (±4.60)</td>
<td>84-93</td>
</tr>
<tr>
<td>C</td>
<td>23.32 (±4.16)</td>
<td>90-95</td>
</tr>
<tr>
<td>D</td>
<td>18.15 (±5.47)</td>
<td>95-99</td>
</tr>
<tr>
<td>P</td>
<td>49.32 (±4.56)</td>
<td>95-100</td>
</tr>
</tbody>
</table>

a. Data collected from four experiments.
Table 11. Mitogenic Response and Production of MIF by LNC Subpopulations after Fractionation on a BSA-Density Gradient.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>PHA-P</th>
<th>Con A</th>
<th>PWM</th>
<th>PPD</th>
<th>MI&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6.12</td>
<td>5.85</td>
<td>4.73</td>
<td>0.85</td>
<td>ND</td>
</tr>
<tr>
<td>B</td>
<td>9.70</td>
<td>9.08</td>
<td>2.32</td>
<td>3.47</td>
<td>64.15</td>
</tr>
<tr>
<td>C</td>
<td>7.82</td>
<td>4.14</td>
<td>0.94</td>
<td>0.87</td>
<td>59.06</td>
</tr>
<tr>
<td>D</td>
<td>35.77</td>
<td>8.80</td>
<td>1.03</td>
<td>0.97</td>
<td>59.72</td>
</tr>
<tr>
<td>P</td>
<td>0.85</td>
<td>0.93</td>
<td>0.89</td>
<td>0.82</td>
<td>84.97</td>
</tr>
<tr>
<td>Original</td>
<td>26.08</td>
<td>22.39</td>
<td>5.12</td>
<td>3.12</td>
<td>54.79</td>
</tr>
</tbody>
</table>

<sup>a</sup> The MI was determined by testing the centrifuged and filtered supernatants from each fraction on peritoneal exudate cells from normal Lewis rats. The antigen was 100μg mitochondrial protein/ml medium.
activity in band B. There was no detectable mitogenic activity in bands C, D and P. Tuberculin PPD exhibited a different pattern from any of the mitogens. The only fraction to show any increase in 
$3^H$-TdR incorporation was band B with a stimulation index of 3.47.

The MIF activity induced by mitochondria as assayed on normal isologous PEC was found in fractions B, C and D. Due to the relatively low yield of cells in fraction A, the test for MIF activity was not performed.

Neonatally thymectomized Lewis rats and non-thymectomized normal Lewis rats were obtained from Charles River Breeding Laboratories. The PEC from both the thymectomized and the non-thymectomized rats were tested for the ability to be inhibited by mitochondria. The LNC from similarly treated animals were, also, tested for a mitogenic response to PHA-P and MIF release as induced by mitochondria. The data are given on Table 12. The non-thymectomized controls showed LNC mitogenic activity and MIF release. Also, their PEC were directly inhibited by mitochondria. In contrast, the neonatally thymectomized animals showed no response in any of the three categories tested.

**Estimation of MIF Molecular Weight**

The last phase of this study was to estimate the molecular weight of the membrane induced MIF. A 2.5 x 100 cm Sephadex G-100 column was calibrated with bovine serum albumin, ovalbumin, carbonic anhydrase and cytochrome c. Con A and mitochondria induced LNC cultures containing MIF were concentrated 10 times the original volume by vacuum
Table 12. The Mitogenic Activity and Direct and Indirect Inhibition of Macrophage Migration by Cells from 23-28 Days Old Neonatally Thymectomized and Non-thymectomized Lewis Rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SI^a</th>
<th>dMI^b</th>
<th>iMI^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-thymectomized</td>
<td>18.88</td>
<td>50.55</td>
<td>45.06</td>
</tr>
<tr>
<td>Thymectomized</td>
<td>0.95</td>
<td>83.80</td>
<td>85.89</td>
</tr>
</tbody>
</table>

^a. SI = Relative incorporation of $^3$H-TdR (E/C) into lymph node cells. The mitogen was PHA-P (1:1000).

^b. dMI = Migration index in a direct test for inhibition of macrophage migration. The antigen was 100μg mitochondrial protein/ml medium.

^c. iMI = Migration index in an indirect test for inhibition of macrophage migration. The antigen was 100μg mitochondrial protein/ml medium.
dialysis. One ml of each of the concentrates was placed on the column and eluted against gravity at a flow rate of 24 ml/hour. The eluates were pooled into fractions approximating the void volume and the elution curves of BSA, OA, carbonic anhydrase and cytochrome c. Each of these pooled fractions was concentrated to 1 ml by vacuum dialysis, diluted 1:10, millipore filtered and tested for MIF activity. The data is shown on Figure 2. The Con A induced LNC supernatant showed MIF activity in both the pooled fractions approximating ovalbumin and carbonic anhydrase, with a relative elution volume (V_e/V_o) between 1.40 and 1.79. This corresponded to an estimated molecular weight between 19,000 and 55,000. The mitochondria LNC induced supernatant showed a slightly different elution pattern. MIF activity was found only in fraction 3, or that which approximated ovalbumin. The V_e/V_o was between 1.40 and 1.54 which corresponded to an estimated molecular weight between 32,500 and 55,000.

**Stability of MIF**

In order to determine the stability of MIF over a wide temperature range, three aliquotes from each of the MIF positive column fractions were treated by freezing at -20°C, heating at 56°C for 30 minutes and heating at 80°C for 30 minutes. The results, as included on the summary table, Table 13, indicated that MIF activity was stable to freezing at -20°C and to heating for 30 minutes at 56°C. However, the biological activity was lost after heating at 80°C for 30 minutes.
Figure 2. Estimation of the Molecular Weight of Rat MIF by Ascending G-100 column Chromatography.

The $V_e/V_o$ Values for MIF Activity Induced by Con A were 1.40 to 1.79 and those Induced by mitochondria, 1.40 to 1.54.
DISCUSSION

The development of autoimmune states has been demonstrated with respect to circulating antibody (17) and to delayed or cell-mediated immunity (98). Although, it is generally felt that many autoimmune states can cause tissue pathology, the cause and effect relationships between the development of the autoimmune state and tissue pathology remain, for the most part, a matter of speculation. Acquired hemolytic anemia is one well documented autoimmune state which has been demonstrated to be caused by circulating antibody directed against the host erythrocytes (99). Conversely, the autoantibodies seen in systemic lupus erythematosus, although responsible for the renal and vascular lesions, are not thought to cause the initial cell destruction which leads to the release of the detrimental DNA autoantigens (100).

Regarding cell-mediated autoimmunity, only experimental allergic encephalomyelitis has been conclusively demonstrated to be caused by cell-mediated immunity directed against basic proteins of the myelin sheath (101). However, the cause and effect relationship of cell-mediated immunity seen in other autoimmune diseases, such as Hashimoto's Thyroiditis, still remains unclear (102).

Much of the confusion of the cause and effect relationships of autoimmune states arises due to an apparently normal autoimmune response occurring after tissue degeneration. It is well known that following tissue damage autoantibodies are produced against subcellular
organelles of various tissues (17). Whether this normal development of autoimmunity is a result of a sequestered antigen or the inability of an animal to become immunologically tolerant to subcellular membranous organelles remains to be answered. However, Weir and Pinckard (103) did demonstrate the inability to induce acquired immunological tolerance towards liver subcellular constituents in neonatal rats.

Recently, interest has developed with respect to the development of cell-mediated immunity in renal disease (104) and particularly in liver disease (105). Rocklin, Lewis and David (104) have reported in vitro evidence for cell-mediated immunity to human glomerular basement-membrane. Hardt, Nerup and Bendixen (105), using liver mitochondria as antigen demonstrated inhibition of migration of leucocytes from patients with chronic active hepatitis and primary biliary cirrhosis. These above reports are intriguing and one must wonder whether these purported cell-mediated forms of immunity are responsible for the renal and liver pathology or whether they are simply present due to the result of tissue necrosis.

The present experiments were designed to determine whether or not a naturally occurring cell-mediated autoimmunity to subcellular constituents is present in normal adult rats. Studies on delayed hypersensitivity in the rat present problems with respect to measuring the degree of cell-mediated immunity. In unpublished data, skin testing for cell-mediated immunity in the rat was found to be an extremely difficult procedure to reproduce. Rats sensitized to PPD and demonstrating positive lymphocyte transformation and MIF production did not
demonstrate consistent skin test results to PPD. Therefore, it was decided to study the presence of cell-mediated autoimmunity by utilizing an in vitro correlate of delayed hypersensitivity. The method of choice was that of an inhibition of macrophage migration.

Rat PEC were packed into capillary tubes and were allowed to migrate in the presence of isologous subcellular constituents or tuberculin PPD. The results (Table 8) demonstrated that PEC from PPD sensitized rats were inhibited from migrating when they came in contact with PPD. The results also demonstrate the inhibition of PEC from normal adult rats by mitochondria (Table 2), both inner and outer mitochondrial membranes and microsomes (Table 8). However, there was no observable inhibition induced by either the particulate cell constituent plasma membrane, or the soluble proteins of the cytosol. David et al. (36), showed that inhibition of normal migration of PEC from PPD sensitized guinea pigs required the presence of at least 2% sensitized lymphocytes among the population of macrophages. Therefore, one could conclude that the inhibition of migration when PEC were challenged by subcellular liver constituents was due to the presence of lymphocytes sensitized to antigenic determinant(s) on the subcellular organelles. However, the observation that the subcellular constituents, mitochondria, inner and outer mitochondrial membranes and microsomes, caused the direct inhibition of isologous PEC does not, in itself, prove that the inhibition observed was identical to that produced by sensitized lymphoid cells. The direct inhibition of macrophage migration is a mechanical phenomenon. It does not demonstrate
the production of MIF from lymphocytes as a consequence of a specific immunologic challenge. Therefore, experiments were performed to demonstrate that the direct inhibition of macrophage migration by isologous subcellular constituents was due to the release of MIF from lymphocytes and was not due to a non-immunologic mechanism which caused the inhibition of PEC migration.

Read and Zabriskie (81), studying the function of each of the cellular components in the leucocyte migration test, separated peripheral leucocytes from tuberculin positive patients into granulocytes, monocytes and lymphocytes. Each of these cell fractions and mixtures of the cell fractions were then challenged with the particulate antigen BCG. These investigators found that BCG did not inhibit the migration of any of the individual cell fractions. However, inhibition of migration was observed when any two cell fractions were mixed. According to Read and Zabriskie (81), the migrating cell mixture of granulocytes and monocytes was possibly inhibited due to the aggregation of polymorphonuclear leucocytes with the particulate antigen. The aggregated cells then formed a barrier which impeded the migration of monocytes causing the total cell migration to be less than the migration of identical cells in a control chamber. In order to investigate this possibility in the present study, peritoneal exudates were separated into three subpopulations (Table 3). Fraction A contained approximately 95% lymphocytes with the remaining cells primarily monocytes. There was virtually no difference in the areas of fraction A cell migration between the control chambers and the mitochondria containing test chambers as seen
in Table 4. Fraction B contained approximately 75% monocytes with the remaining cells distributed almost evenly between neutrophils and lymphocytes. This cell fraction showed a significant inhibition (45% and 68% of control) of cell migration when incubated with mitochondria. However, fraction C, even though approximately 90% of the cells were equally distributed between neutrophils and monocytes with approximately 10% lymphocytes comprising the fraction, was not inhibited from migrating by mitochondria. If neutrophils non-specifically aggregated with the particulate subcellular membranes impeding the migration of all cells within a peritoneal exudate, the migration of fraction C, comprised of 45% neutrophils, would be expected to be inhibited. However, since there was no inhibition observed, the data from fraction C indicate, as does the data showing no inhibition of PEC migration induced by plasma membrane (Table 8), that the inhibition of PEC migration was not due to aggregation of the neutrophils with the particulate subcellular constituents. However, since fraction C also contained 10% lymphocytes and approximately 45% monocytes, the reasons for its not being inhibited through the same mechanism inhibiting fraction B was puzzling. An explanation for this enigma may be due to the relatively large population of migrating neutrophils which "masked" the inhibition of mononuclear cells. Another explanation may be that the lymphocytes were separated by the Ficoll-Sodium Metrizoate gradient into lymphocytes capable of producing MIF (fraction B) and lymphocytes not capable of producing MIF (fraction C).
Thor et al. (71), showed that when human peripheral leucocytes from tuberculin positive patients were cultured in the presence of PPD, MIF was released into the tissue culture medium. This was demonstrated by the observation that the cell-free supernatants from cultures which had contained tuberculin positive peripheral blood leucocytes and PPD caused the inhibition of normal guinea pig PEC migration. Falk et al. (96), in similar experiments, showed that the migration of rat spleen cell cultures from capillary tubes was inhibited by supernatants from spleen cell cultures incubated with histocompatibility antigens. The data obtained in this study similarly indicate that an MIF-like factor was released into the supernatant of LNC from PPD sensitized rats when cultured for 72 hours with PPD (Table 9). These data coupled with the results showing the direct inhibition of migration of PEC from PPD sensitized rats (Table 8) indicates that the adult Lewis rat was capable of becoming sensitized to M. tuberculosis H37Ra and that the sensitized lymphoid cells released, after incubation with PPD, a factor or factors which caused the inhibition of normal rat PEC. Therefore, since the data on Table 2 show, without exception, the direct inhibition of PEC by isologous mitochondria, and the data on Tables 6 and 7 show statistically that only supernatants from lymphocytes cultured with mitochondria inhibit macrophage migration, the only conclusion that can be made is that lymphocytes from Lewis rats are autosensitized to isologous liver mitochondria. No other conclusion can be made since the data on Tables 6 and 7 show statistically that there was no inhibitory affect on PEC
by the tissue culture medium, a soluble component of mitochondria or a product of non-stimulated LNC.

Bergstrand and Kallen (106) statistically evaluated the macrophage migration inhibition assay and found that, if the variation within chambers was ignored, a migration index of 80 was the maximum migration allowable and still have a statistical value of P<0.05. Therefore, using an MI of less than 80 as significant inhibition, it was interesting to note that the same subcellular constituents, mitochondria, inner and outer mitochondrial membranes and microsomes, which caused a direct inhibition of macrophage migration (Table 8) also induced the release of an MIF-like factor or factors from normal isologous LNC. The exception to these observations was plasma membrane. The data obtained from assaying the cell-free supernatants from LNC cultures incubated with plasma membrane on normal rat PEC was inconsistent with respect to both that membranes ability to cause the direct inhibition of macrophage migration and its ability to consistently induce the release of an MIF-like factor. The results with plasma membrane gave MI of 99, 68, 74 and 94. Therefore, plasma membrane induced the release of an MIF-like activity in two of the four experiments completed. These results could be explained by contamination of the plasma membrane preparation with inner mitochondrial membrane and/or microsomes as the enzyme data indicates (Table 1). Since the cultures were incubated for 72 hours, as opposed to only 24 hours in the direct MIF test, the extra 48 hours may have been sufficient time to allow sensitized lymphocytes
to react with the relatively small concentration of MIF-inducing subcellular constituents to release MIF in biologically detectable amounts. However, the inconsistency in the MI obtained from repeated experiments with plasma membrane was difficult to explain. It could possibly be due to variations in the extent of homogenization between aliquots of plasma membrane. Therefore, since a different aliquot of plasma membrane was used in each experiment, the degree of contamination with other subcellular constituents varied. That the inhibition of PEC in the above experiments was due to an MIF-like factor and not due to the tissue culture conditions was shown on Table 9. The data show clearly that there was no inhibitory affect on PEC by any of the antigen control cultures.

The direct and indirect MIF data presented above indicated that inhibition of PEC by subcellular constituents did occur by a mechanism identical to that produced by lymphocytes sensitized to a specific antigen or lymphocytes challenged by Con A or PHA-P. Further support for this conclusion was derived from experiments designed to compare the molecular weight of mitochondria and Con A induced MIF with previously reported molecular weights for MIF. MIF positive LNC supernatants from Con A and mitochondria stimulated cultures were fractionated by ascending gel-filtration. Cellular migration inhibiting activity was found over a large range (Figure 2). Con A induced supernatants showed positive activity in the fractions between 20,000 and 55,000 M.W. Whereas, mitochondria induced supernatants showed inhibition in fractions between 37,000 and 55,000 M.W. Remold et al. (73),
found that guinea pig MIF induced by a hapten-bovine gamma globulin conjugate eluted from a Sephadex G-100 column over a large range with molecular weights between 35,000 and 55,000. Other reports (107) estimate the molecular weight of both guinea pig MIF and human MIF to be between 25,000 and 55,000. These results indicate that MIF may be several proteins with a variety of molecular weights, all having a similar biological activity. Also, as the data obtained from Con A supernatants and the mitochondria supernatants indicates (Figure 2), the molecular weight(s) of MIF may be dependent, to some extent, on the inducer. This may also be an explanation for the differences in molecular weights obtained for MIF by other investigators (107). However, the difference in results may be due to the fact that Con A, a non-specific inducer, acts upon a greater number of cells than mitochondria. Therefore, one is detecting only the "spill-over" of MIF activity into the lighter fraction. Indeed, such a "spill-over" would be amplified since the eluates were pooled into fractions corresponding to the elution curves of BSA, OA, carbonic anhydrase and cytochrome c. Therefore, the true molecular weight of rat MIF may be 45,000 and that the Con A induced MIF activity "trailed" into the fraction corresponding to the carbonic anhydrase elution curve.

Finally, in order to further compare the mitochondria induced rat MIF with other known criteria for MIF, supernatants showing MIF activity were frozen at -20°C and heated to 56°C and 80°C. The mitochondria induced MIF, as well as MIF induced by Con A, was found to be
stable at -20°C and 56°C, but labile at 80°C after 30 minutes. These data are in accord with previously published reports for both guinea pig and human MIF (107).

Recently, a controversy has developed concerning whether MIF is released from dividing cells. Papageorgiou et al. (108) have reported an MIF-like factor present in the supernatants of lymphoid cell lines. However, supernatants from non-lymphoid cell lines, including HeLa, fetal lung tissue and malignant brain tumor tissue from hamsters also were found to contain MIF-like activity. Papageorgiou et al. (108) found that the MIF produced by non-lymphoid cells appeared in the same Sephadex eluates as that produced by lymphoid cell lines. Their findings indicate that MIF is produced by any lymphoid or non-lymphoid continuously dividing cell. Similarly, Tubergen et al. (109) reported that MIF was released by both lymphoid and fibroblast cell lines in continuous growth. The release of MIF was associated with activation of cells from a resting state into the mitotic cycle, particularly on cells in S-phase. However, Bloom et al. (76) and independently Rocklin (77) have reported the dissociation of MIF production and cell division. Bloom et al. (76), showed that culturing LNC from guinea pigs sensitized to tuberculin with either vinblastine, an inhibitor of mitosis, or cytosine arabinoside, an inhibitor of DNA synthesis, had no effect on the production of MIF. Rocklin (77) prevented lymphocyte proliferation in response to streptokinase-streptodornase in human lymphocytes by treatment with
5-bromo-2-deoxyuridine and light; however, upon antigenic stimulation of the surviving cells, MIF production occurred.

The data presented in the present study lend support to the hypothesis that the cells involved in MIF production may be distinct from those cells undergoing mitogenic transformation. The data on Table 5 show that the subcellular constituents, rat liver mitochondria and outer mitochondrial membrane did not stimulate lymphocyte transformation. In contrast, the data on Table 9 clearly show that both mitochondria and outer mitochondria membrane induced the release of MIF. However, as these same tables indicate, along with the results on Figure 2, the dissociation of MIF release and lymphocyte transformation may be dependent upon the type of sensitizing antigen and its interaction with the lymphocytes. These results demonstrated that general mitogens, e.g., Con A, as well as the specific antigen PPD cause both the release of MIF from LNC as well as induce mitogenic activity in LNC. Nerup et al. (98) reported a similar finding. He was able to show positive migration inhibition with leucocytes from patients with Idiopathic Addison's disease, but was unable to induce lymphocyte transformation with the same adrenal cortex mitochondrial antigen. It would appear that either the LNC reacting with mitochondria and producing MIF are different from LNC that are capable of undergoing mitosis or the mechanism of antigenic stimulation of lymphocyte blastogenesis is different from the mechanism of antigenic induction of MIF release. Probably, both mechanistic differences occur.
In order to study further the lymphoid cells involved in producing MIF after incubation with subcellular constituents, LNC were separated on a discontinuous BSA density gradient. The cellular size distribution and mitogenic activity towards PHA-P parallels that of Colley et al. (94) (Tables 10 and 11). However, there was a significant mitogenic response towards PHA-P in all fractions, except the pellet, as was also found with Con A stimulated cells. PWM, on the other hand, induced peak mitogenic responses in fractions A and B while PPD stimulated blastogenesis only in fraction B. These observations are important since MIF was found to be released in all cell fractions tested, except the pellet. Janossy and Greaves (110), studying the mitogenic response of mouse spleen cells, reported that B cells responded well to PWM and poorly, if at all, to PHA-P. Whereas, mitogenic responsiveness to PHA-P appeared to be a property of T cells. Therefore, if rat LNC respond to PHA-P and PWM in the same manner as mouse spleen cells, the data obtained from the BSA gradient experiment seen in Table 11 show B cells to reside in fractions A and B while T cells were found in all fractions. The mitogenic response to PPD may require both B and T cells. This statement is based on the observation that if PPD induced only a B cell response, blastogenesis would be expected in both fractions A and B, as was found with PWM. Similarly, if PPD stimulated mitosis in T cells exclusively, one would expect to find lymphocyte transformation in fractions C and D, especially, fraction D where PHA-P induced such a large mitogenic response.
No conclusion could be made from the data obtained from the BSA gradient as to whether MIF induction by mitochondria was a B cell function or a T cell function. However, MIF production probably was a T cell dependent function, since MIF was found to be released in fractions B, C and especially in fraction D where there was no evidence of B cell mitogenic activity (Table 11). A mechanism of MIF release could be dependent on both B and T cells. If both cell types were needed for MIF release, the percentage of B cells contaminating fraction D must have been sufficient to cause MIF release but insufficient to have been detected by $^3$H-TdR incorporation into dividing lymphocytes.

In order to try and resolve the question of thymic dependency with regard to MIF production in rats, Lewis rats were neonatally thymectomized and their LNC were later tested for PHA-P stimulated lymphocyte transformation and mitochondria induced MIF activity. Neonatally thymectomized rats have been shown (111) to have been rendered immuno-incompetent with respect to cell-mediated immunity. There is no mitogenic response to PHA-P and allogeneic skin grafts are tolerated. There is, also, a reduced ability to form specific antibodies and immunoglobulin levels are quantitatively reduced. The data presented on Table 12 show no mitogenic activity was induced by PHA-P nor was there any mitochondria induced MIF activity from 23-28 day old neonatally thymectomized rats. In contrast, the 23-28 day old non-thymectomized control rats showed a positive LNC mitogenic response to PHA-P ($SI = 19$) as well as exhibiting positive MIF activity.
to mitochondria. Isologous mitochondria caused both the direct inhibition of 23-28 day old non-thymectomized rat PEC and the release of MIF from 23-28 day old non-thymectomized rat LNC. Therefore, since neonatal thymectomy abolishes the mitogenic response to PHA-P (111), a T cell function (110), the data would suggest that MIF release in the rat is a thymic-dependent function. However, the data does not indicate the cell type responsible for MIF production. The possibility exists that B cells after having received a "message" from T cells, produces MIF. However, this appears unlikely since it was shown that MIF was produced in the BSA fraction D where no B cell mitogenic activity was detected.

In conclusion, the evidence presented shows that in Lewis rats, the addition of isologous liver subcellular constituents prevents the normal migration of isologous PEC. That the migration inhibition was brought about by the release of MIF in the same way as was reported for antigen sensitized cells (56) or for mitogen stimulated cells (79) was suggested by several pieces of evidence: (a) a monocyte-lymphocyte population of cells was inhibited and neutrophils did not appear to have any affect on the migration of cells; (b) migration inhibiting activity was recovered from cell-free LNC supernatants which had been incubated with subcellular constituents; (c) the estimated molecular weight of rat MIF approximated the molecular weights previously reported for MIF from other species; and (d) the stability of rat MIF parallels that reported for other species. The release of
<table>
<thead>
<tr>
<th>Species</th>
<th>Rat (Lewis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell source</td>
<td>Lymph node</td>
</tr>
<tr>
<td>Culture time</td>
<td>72 hr.</td>
</tr>
<tr>
<td>Inducers</td>
<td>ConA, PPD and rat liver mitochondria, inner and outer mitochondrial membrane and microsomes</td>
</tr>
<tr>
<td>Target cell</td>
<td>Isologous Peritoneal Exudate Cells</td>
</tr>
<tr>
<td>Heat stabile</td>
<td>$-20^\circ C$ and $56^\circ C$</td>
</tr>
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<td>Heat labile</td>
<td>$80^\circ C$</td>
</tr>
<tr>
<td>Molecular Weight (Sephadex)</td>
<td>20 - 50,000</td>
</tr>
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</table>
MIF from rat LNC stimulated by subcellular constituents is believed to be a cell-mediated immune response because: (a) the production of MIF was a specific response induced by certain subcellular constituents, as was shown by the inability of rat liver plasma membrane to cause MIF release from LNC; and (b) MIF production was shown to be a thymic-dependent response.

Since the cell-mediated immune function of MIF production was found to exist in normal adult rat LNC as a consequence of exposure to isologous subcellular constituents in vitro, the question arises as to whether or not this cell-mediated immunity was naturally occurring. Data obtained from a single experiment indicated that the induction of MIF production was not a naturally occurring phenomenon, but was due to previous exposure of isologous liver subcellular constituents to the lymphoreticular system. This statement was based on preliminary evidence that showed isologous heart mitochondria unable to either directly inhibit the migration of rat PEC, or stimulate the production of MIF from rat LNC. MIF production is a specific cell-mediated immune response (56) occurring only when lymphocytes from a previously immunized animal are incubated with the sensitizing antigen. Therefore, the lack of MIF release from LNC incubated with isologous heart mitochondria suggests that normal adult Lewis rats had not been previously immunized with heart mitochondria. This would be expected since normal heart tissue having no history of disease or trauma does not undergo degenerative changes or necrosis with the subsequent liberation of subcellular constituents (112). Evidence presented
by Friedman et al. (113) indicated that when myocardial damage occurred in the rat, the LNC obtained from rats with myocarditis showed a cytotoxic affect on rat heart cell cultures. The liver, on the other hand, is known to undergo parenchymal cell degenerative changes with subsequent regeneration (114). Therefore, the liver is probably releasing subcellular constituents that are phagocytized by the lymphoreticular system. The phagocytosis of subcellular constituents probably leads to the sensitization of lymphocytes capable of MIF production through a mechanism not understood at the present time. If, indeed, after further study heart mitochondria is demonstrated not to induce the release of MIF from lymphocytes, this data would indicate antigenic specificity. The lack of cross-reactivity between liver mitochondria and mitochondria isolated from other organs was indicated by the report of Nerup et al. (98). He was able to demonstrate MIF activity induced in leucocytes from patients with Idiopathic Addison's disease by mitochondria isolated from the adrenal cortex. However, no MIF release was induced by mitochondria isolated from other organs, e.g., fetal liver.

The particular manifestation of migration inhibition described in these studies could be significant as part of a process of inflammation and repair. The process of macrophage migration inhibition would enable the localization of macrophages at the site of tissue breakdown. It may also explain the latent infiltration of mononuclear cells into an area of inflammation. Recently, Simpson and Ross (115) reported a study on the progress of wound repair in guinea pigs rendered
neutropenic by treatment with anti-neutrophil serum. They observed no differences between the wounds of the control animals and the neutropenic animals relative to the rate of wound debridement or the extent of repair. They found the wounds from the two groups of animals were identical in cellularity and in the degree of connective tissue formation. Their observations lend support to the hypothesis that a neutrophil infiltration in early wounds is not an essential antecedent to the infiltration of monocytes.

In proposing the generic term "lymphokine" to describe non-antibody products of lymphocyte activation, Dumonde et al. (78), suggested that the lymphokine factors represent the very molecules by which sensitized lymphocytes take action in the peripheral expression of cellular immune responses. Their discussion carries two main suggestions. (a) Single mediators or combinations of factors may be expressed independently of one another. (b) Not all of the soluble biological activities need be generated together by a given event of lymphocyte activation in vivo. A biological prediction is that different subpopulations of sensitized lymphocytes may govern the generation of individual mediators (116) or, more likely, certain groups of mediators. The clinical corollary would be two fold. First, that the results of cellular hypersensitivity tests in clinical diseases may not run parallel with one another, even in clinical states where widespread cellular immune reactions are taking place (116), e.g., organ graft rejection or chronic intracellular infection. Second, that assay of
the separate mediator activities may reveal selective activation or
depression of three principal expressions of cellular immune responses
in vivo, i.e., the inflammatory, surveillance and adjuvant functions (116).

The multiple actions of these mediators on lymphocytes and
macrophages suggest that they may act to amplify and regulate the
responses of populations of lymphoid cells to specific antigen. Their
physiological function may be to assist cellular co-operation and to
regulate cellular traffic through vascular and lymphoid tissue. In
clinical allergy, this implies that in assaying the extent of mediator
activity, one may be examining the integrity of an important component
of immunological homeostasis. For example, the production of IgE by
pollen antigen-stimulated lymphocytes in reagenic hypersensitivity
may require the participation of lymphocyte-activating factors genera-
ted by stimulation of other antigen-sensitive lymphocytes which do
not generate antibody. This hypothesis allows the generation of a
mitogenic factor in circumstances where an inflammatory mediator of
delayed hypersensitivity need not be produced. In other circumstances,
the association of MIF with desensitization therapy and with milder
forms of reagenic hypersensitivity (116), or its association with
autoimmune diseases not exhibiting lymphocyte blastogenesis (98) suggests
that MIF may possibly act in vivo as an internal adjuvant for the
production of blocking antibody, and/or a blocking agent of mitogenic
factors.

In organs, e.g., the liver, where there is cell death, the
actions of the lymphokines may be to aid the host in "clearing" cellular
debris. Mononuclear chemotactic factor, macrophage activating factor and MIF would be of value, however, mitogenic factor or a cytotoxic factor need not be generated. Whereas, in diseases that are caused by certain intracellular agents, e.g., certain viral diseases, amplification of the cell-mediated immune mechanism may be necessary with the subsequent generation of mitogenic factor, cytotoxic factor, chemotactic factors, MIF and a viral growth inhibiting factor, e.g., interferon.

In conclusion, the results presented in this dissertation indicate the need for studies into the clinical significance of mediators of cellular immunity. There is growing interest in the extent to which various mediators of cellular immune responses may be associated with neoplasia, chronic granulomatous disease, connective tissue disorders and chronic infectious diseases. Quite frequently, there is a clinical association of depressed cellular immune responses to intrinsic antigens with a tendency to enhanced antibody and autoantibody production, suggesting a dissociation of deficiencies in inflammatory and surveillance functions of cellular immune responses. The identification of selective deficiencies or abnormalities in the mediators may add a further dimension to the study of impaired immunological reactivity, whether caused by tolerance or antigenic competition, enhancing antibody or other serum factors, genetic defects, severe virus infection, purposeful immunosuppression or lymphoreticular neoplasia.
REFERENCES


