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TABLE OF CONTENTS

LIST OF TABLES

Table | Page

Table | Page

LIST OF ILLUSTRATIONS

IN VITRO STUDIES ON DELAYED HYPERSENSITIVITY TO TUBERCULIN

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Delayed-type hypersensitivity, or cell-mediated immunity, has recently become the object of intense investigation both in the laboratory and in the clinic. The apparent association of the homograft reaction, certain auto-immune diseases, and tumor immunity with specific cell-mediated immune processes has stimulated progress in rescuing this type of reaction from the obscurity into which it had fallen.

The main criteria that have been used to dissociate delayed-type hypersensitivity from humoral antibody mediated, immediate-type hypersensitivity have been that, in the former, (1) a characteristic delay in appearance of the local skin reaction is observed when the specific sensitizing antigen is introduced into the appropriately sensitized host, and (2) passive sensitization can be accomplished only with cells of lymphoid origin and not with immune serum. More recently, the concept of dissociation of cellular and humoral immunity has been supported by the observation that certain

agammaglobulinemic patients who are incapable of developing a normal humoral antibody response are not compromised in their ability to respond normally to delayed-type stimuli (Good and Gabrielson, 1964). Further study of the ontogeny of the immune response using experimental animal models has resulted in the concept that the two types of immunity evolve independently during embryonic development (Cooper et al. 1965). Thus, delayed hypersensitivity is thought to be thymus-dependent, while humoral antibody is dependent upon the bursa of Fabricius in the chicken experimental model, or upon some corresponding, but as yet unknown, central lymphoid tissue in man (Cooper et al. 1966).

Delayed-type responses to infectious agents, various proteins, and innumerable chemical agents appear to be rather common in man. However, since the original observations of Koch, tuberculin hypersensitivity has served as the model for delayed-type reactions to microbial agents. The response to proteins of the tubercle bacillus has been studied in detail because of its possible role in the pathogenesis of tuberculosis. Koch observed that viable or heat killed tubercle bacilli innoculated subcutaneously into guinea pigs evoked a much more intense inflammatory response in previously infected than in uninfected animals. Subsequently, he found that culture filtrates of Mycobacterium tuberculosis were also effective in eliciting a delayed-type inflammatory response in infected animals.

Histologically, the delayed-type response is characterized by a massive accumulation of inflammatory cells. Initially, granulocytes are abundant about small blood vessels, but by 24 hr the lesions are populated almost exclusively by mononuclear cells (Dienes and Mallory, 1932). Crowle (1962) described the reaction as a mononuclear cell invasion of the effected area with the accumulation of these cells around blood vessels to form perivascular islands. Infiltrating cells may be found in the epidermis, dermis, and sub-dermal areolar areas. If invading mononuclear cells involve the epidermis, necrosis ensues, followed by infiltration of the necrotic area by polymorphonuclear leukocytes. In a more exhaustive histological study in the guinea pig, Spector (1967) described in detail the sequence of cellular events in the tuberculin reaction. It was evident from his observations that whether the tuberculin reaction is predominantly polymorphonuclear or chiefly mononuclear depends upon the time at which the skin test site is examined. The tuberculin reaction was clearly different from most inflammatory reactions in that it presented a biphasic pattern. A minor peak, predominantly polymorphonuclear in nature, occurred at 3-4 hr, probably as a non-specific response to the injection of tuberculin. Subsequently, a major peak occurred at 8-12 hr due to specific sensitization. During this biphasic process, the polymorphonuclear leukocytes preceded the mononuclear cells in their migration from the vessels. In each

instance, the granulocytic cells were unimpeded after extravasion and migrated freely throughout the intravascular spaces. In contrast, the mononuclear cells appeared to be immobilized just beyond the vessel walls to form perivascular islands. Ebert et al. (1948) studied the vascular changes in the tuberculin-sensitive rabbit using ear chambers. They noted that introduction of viable tubercle bacilli into the vessels resulted in progressive vascular damage characterized by dilation of blood vessels, sticking of leukocytes to the vascular endothelium, and diapedesis of leukocytes. Vascular damage was followed by hemoconcentration, stasis, and thrombosis of small blood vessels with infarction of tissue and necrosis. The necrotizing response occurred coincidentally with the development of dermal reactivity to tuberculin.

One of the major contributions to the field of delayed hypersensitivity was the recognition of the fundamental role of the leukocyte. Landsteiner and Chase (1942) reported the passive transfer of delayed-type skin sensitivity to a contact sensitizing chemical (picryl chloride) through the use of peritoneal exudate leukocytes from sensitive donor guinea pigs. In 1945, Chase demonstrated further that delayed skin sensitivity to tuberculin was transferable to normal guinea pigs by means of leukocytes of peritoneal exudates, spleen, lymph node, or blood from tuberculin-sensitive animals. Lawrence (1949) reported the cellular transfer of cutaneous hypersensitivity to tuberculin in man by means of

intact peripheral blood leukocytes from tuberculin-positive donors. In further studies (Lawrence, 1954 and 1956), it was found that leukocyte extracts were as capable as viable cells in the transfer of delayed hypersensitivity in humans. Jeter et al. (1954) reported successful passive transfer of sensitivity to 2,4,-dinitrochlorobenzene (DNCB) in guinea pigs using cells disrupted by sonic vibration. Cummings et al. (1956) used similar techniques to accomplish transfer of tuberculin sensitivity. Some workers (Chase, 1953 and Eisen, 1959) have been unsuccessful in confirming these findings using the guinea pig as an experimental model. More recently, Guthrie et al. (1966) reported that the time after sensitization that cells were collected from peritoneal exudates and lymph node was critical in the successful transfer of contact sensitivity to 1-fluoro-2,4-dinitrobenzene (DNFB) in the guinea pig. Moreover, intact peritoneal cells collected 17 days after sensitization lost their ability to passively transfer sensitivity when incubated in balanced salt solution at 37 C for 30 min. The resulting incubation supernatant was capable of transferring sensitivity. These results demonstrated the necessity for careful control of procedures and handling of peritoneal cells in passive transfer studies using the guinea pig model.

Lawrence and Pappenheimer (1957) reported that sensitive human leukocytes, when incubated with specific antigen, released a soluble factor (transfer factor) into the

supernatant fluid which was capable of conferring dermal sensitivity to tuberculin in normal individuals. Guthrie et al. (1966) found that when peritoneal exudates from guinea pigs sensitized to DNFB were incubated with a conjugate of dinitrophenol and guinea pig serum the supernatant fluid from cells collected 13 days after sensitization was capable of transferring sensitivity to normal animals. However, cells collected at 17 days post-sensitization failed to release transfer factor into the supernatant following exposure to antigen. Lawrence (1969) has thoroughly studied human transfer factor. It has been characterized as a dialyzable, low-molecular weight (<10,000) moeity that is not antigenic and is not an immunoglobulin or a protein. It is insensitive to DNase and RNase. Lawrence has suggested that human transfer factor may be a polynucleotide or polypeptide molecule which may function either to convey immunological information and/or act as a derepressor of a small number of normal lymphocytes.

Recently, Dupuy et al. (1969, 1970) reported passive transfer of delayed hypersensitivity to PPD and to a conjugate of dinitrophenol-bovine serum albumin in guinea pigs by repeated injection of plasma from sensitized, x-irradiated donors into normal recipients. Transfer of sensitivity was also achieved by incubating normal spleen cells with plasma or serum from sensitized, x-irradiated donors prior to injection into normal guinea pigs. Chase (1970) was unable to confirm these findings and attributed the reactions to humoral

antibody rather than delayed hypersensitivity. Pabst and Dupuy (1970) transferred experimental allergic encephalomyelitis (EAE) to 25% of normal recipients which had been injected with plasma from x-irradiated donors with EAE.

The role of the small lymphocyte as the immunocompetent cell associated with delayed-type reactions has been established. Wesslen (1952) passively transferred tuberculin hypersensitivity in the guinea pig with viable lymphocytes from the thoracic duct. Waksman et al. (1961) studied the histology of tuberculin and contact sensitivity reactions in sensitive guinea pigs pre-treated with specific rabbit anti-guinea pig lymphocyte serum. This treatment effectively lowered the level of circulating lymphocytes and suppressed the cutaneous tuberculin reaction. Slavin and Garvin (1964) reported successful transfer of sensitivity to dinitrochlorobenze and tuberculin in rabbits and guinea pigs with purified lymphocyte suspensions, but not with neutrophils. In 1967, Turk and Polak reported similar findings in that local passive cutaneous transfer of tuberculin hypersensitivity was consistently obtainable only with purified lymphocyte suspensions prepared from sensitive guinea pig peritoneal exudates. Occasional transfer of Arthus-like skin reactivity was obtained with macrophages from the same source. Aranson et al. (1962) reported that neonatal thymectomy of rats suppressed subsequent delayed-type cellular hypersensitivity and the number of small, circulating lymphocytes, although plasma

cell and immunoglobulin levels were normal. Cooper et al. (1965, 1966), in delineating the ontogeny of the immune response in the chicken, found that thymectomized and sublethally irradiated newly hatched chicks were incapable of developing immunological responses associated with delayed-type cellular immunity. Morphologically, these animals were depleted of small lymphocytes of the circulation and the white pulp type of development in the spleen. Further support for the role of the lymphocyte in cellular immune phenomena is provided by current in vitro correlates of delayed hypersensitivity (Lawrence, 1968 and 1969) which are dependent on interaction of specific antigen with the sensitive lymphocyte.

Immunosuppression of delayed hypersensitivity by chemical agents has been given much attention recently with the advent of tissue and organ transplantation in man (Gabrielson and Good, 1967). The role of gluco-corticoids as anti-inflammatory and immuno-suppressive agents has been clearly established both experimentally and clinically. Long and Favour (1950) noted the transient ability of adrenocorticotropic hormone (ACTH) and cortisone to alter delayed-type tuberculin skin sensitivity in human patients on steroid therapy. Tuberculin reactions were obliterated in 13 of 27 patients with known sensitivity. The remainder of the patients showed significant suppression of induration and erythema of the dermal response. In 1951, Vollmer reported the

suppression of tuberculin skin reactions in 9 known sensitive human volunteers when cortisone was injected along with the skin test antigen. Mirick (1951) noted that therapeutic doses of cortisone did not diminish the humoral antibody response in humans. Neither did it cause more than a transitory lymphopenia. Large doses of cortisone administered to patients with lymphatic leukemia resulted in prolonged lymphopenia. In studies in experimental animals, Harris and Harris (1950) reported that cortisone treatment of BCG-infected guinea pigs and rabbits suppressed dermal reactivity and systemic shock to Old Tuberculin. Cortisone treatment, however, had no effect on Arthus reactivity and humoral antibody production in rabbits, nor did it protect guinea pigs against anaphylaxis when given prior to shocking challenge with antigen.

The suppression of delayed-type skin reactivity may not necessarily be specific for an immune mechanism, however, since adrenal steroids block inflammatory responses to a variety of non-specific stimuli. In 1951, Osgood and Favour, found that treatment of normal guinea pigs with ACTH suppressed the inflammatory response produced by intracutaneous injection of oil of turpentine as well as tuberculin skin reactivity in sensitive animals. ACTH produced marked eosinophilia and lymphopenia in these animals. Gell and Hinde (1951), in a similar study, analyzed the histological response to tuberculin and the response to a non-specific

irritant (brain phospholipid). In both types of lesions the mononuclear component was markedly reduced by cortisone. The polymorphonuclear leukocyte component was less effected. Raffel (1961) suggested that one of the primary difficulties in interpreting the effect of immunosuppressive agents in experiments carried out in vivo is that the agent might well interfere with the inflammatory response nonspecifically, aside from whether or not the animal had responded immunologically. Germuth (1956) reviewed the role of adrenocortical steroids in infection, immunity, and hypersensitivity. He stressed that cortisone has a direct effect on the capillary tone. That is, decreased capillary permeability results in depression of the exudation of fluid and cells from the vessels. Ebert (1952) observed the effect of cortisone on experimental tuberculosis using the rabbit ear chamber. It was noted that cortisone reduced the intensity of the inflammatory process of sensitized animals by maintaining the vascular tone and thus reducing diapedesis of leukocytes and damage to the vessels involved. Increased susceptibility to infection following cortisone therapy appears to be secondary to the suppression of the protective inflammatory response. Moreover, one of the most striking effects of cortisone administration is a reduction in the number of circulating lymphocytes and the involution of lymph node tissue, including the thymus, spleen, and lymph nodes. Lymphocytopenia is usually transient and lymphocyte destruction by cortisone has not been

demonstrated convincingly. Cummings and Hudgins (1952) found that long term administration of cortisone to tuberculin-sensitive guinea pigs suppressed skin reactivity but did not alter the ability of their cells to passively transfer delayed hypersensitivity. However, cortisone administration to recipient animals which had received sensitive donor cells suppressed the dermal reaction to tuberculin. These results suggested that cortisone suppressed the dermal response to tuberculin nonspecifically, without altering immune competency. Jeter and Seebohm (1952) were unable to suppress the induction of contact dermal sensitivity to DNCB in guinea pigs with high doses of cortisone or ACTH. In further studies, Seebohm et al. (1954) were unsuccessful in suppressing the passive transfer of delayed hypersensitivity to DNCB by treating donor animals with cortisone or ACTH. However, recipient animals treated with ACTH, but not those given cortisone, displayed depressed dermal reactions following passive sensitization. Shewell and Long (1956) studied the effect of cortisone acetate on body weight, gamma globulin levels, and production of circulating anti-toxin in various species. They noted that man, monkey, and the guinea pig are relatively resistant to cortisone as compared to rabbits, rats, and mice. In 1957, Long suggested that the effect of adrenal steroids varied greatly in different species based on their innate potential to synthesize various steroid derivatives endogenously. For example, the major steroids

elaborated by the adrenal cortex of the rat, mouse, and rabbit are corticosterones, but these species produce very little cortisone or hydrocortisone. Thus, exogeneous administration of these agents leads to dramatic effects on their immune capability. In contrast, in those species (man, monkey and guinea pigs) in which cortisone is normally manufactured there is a relatively small effect following exogenous administration of the agents. Furness (1959) studied the effect of hydrocortisone succinate on the macrophages of various species of animals *in vitro*. Guinea pig macrophages were found to be 10 to 100 times more resistant to the effect of steroid, as measured by morphological changes and their ability to adhere to glass, than were those from the rat and mouse. Cortisone and its analogues have been shown to inhibit thymus-dependent experimental autoimmune disease. Development of experimental allergic encephalomyelitis in the guinea pig was suppressed by cortisone in studies by Moyer *et al.* (1950) and Ferraro and Roizin (1953). The latter workers noted that the disease developed after treatment was discontinued. Cortisone administration has also been reported to suppress adjuvant arthritis in rats (Pearson and Wood, 1959).

Adrenal steroids have been used most successfully in suppression of transplantation immunity. Billingham *et al.* (1951) and Morgan (1951) observed independently that treatment with cortisone prolonged skin allograft survival significantly in rabbits. In guinea pigs, Sparrow (1953) reported

that cortisone acetate was effective in doubling the survival of skin allografts when large doses were injected subcutaneously. In a similar study, Weisman et al. (1951) reported that intramuscular administration of high doses of cortisone or ACTH was ineffective in prolonging homologous skin grafts in guinea pigs. Thus, it appears that cortisone is most effective in prolonging homografts when given in depot form at the time of grafting. The role of cortisone in thymus-dependent immunity was supported by the studies of Udall (1955). He observed that treatment of newborn rats with cortisone significantly reduced the number of thymocytes in the thymus and suggested that cortisone may prevent the maturation of the precursors of lymphocytes in the thymus.

Results of in vitro studies on the influence of adrenal steroids on delayed hypersensitivity are confusing. Leahy and Morgan (1952) reported that cortisone inhibited the cytotoxic action of PPD on macrophages from splenic explants of tuberculous guinea pigs. However, preincubation of the cells for 24 hr with a high concentration of cortisone (100 mcg/ml) prior to the addition of PPD was found to be essential. Glasgow et al. (1958) demonstrated a protective effect of cortisone against specific antigenic damage in cultures of spleen from guinea pigs sensitive to mumps viral antigen. In contrast, Holden et al. (1953) were unable to detect any protective effect of cortisone on damage by PPD to splenic explants from sensitive guinea pigs, mice, and rabbits or buffy

coats from tuberculin-sensitive rabbits. However, cortisone was added in small concentrations (0.5 mcg/ml) simultaneously with antigen in these studies. Heilman (1945), using a different *in vitro* method, was also unable to demonstrate cortisone protection against the cytotoxic effects of tuberculin on sensitive cells. Johnson and Sherago (1964) reported that intraperitoneal cortisone treatment of tuberculin-sensitive guinea pigs significantly decreased *in vitro* sensitivity of peripheral blood leukocytes as measured by inhibition of migration of buffy coat leukocytes by tuberculin when the number of mononuclear cells was significantly lowered.

The specific mechanism by which adrenal steroids suppress delayed hypersensitivity at the cellular level remains controversial. There is, however, some indirect evidence, aside from the immunologically non-specific pharmacologic effects of cortisone on cell mediated immunity, that these agents probably act directly on the macrophage and the lymphocyte. Cortisone has been shown to inhibit the release of lysosomal enzymes from isolated rabbit liver lysosomal granules by ultraviolet irradiation (Weissman and Dingle, 1961) and by endotoxin (Weissman and Thomas, 1962). The authors postulated that the mechanism of action of cortisone on the macrophage was through stabilization of the lysosomal membranes. In support of this concept, Lurie *et al.* (1952) noted that macrophages from cortisone-treated rabbits readily engulfed bacteria, but that the organisms tended to accumulate

in the phagocytic cells and were not as well digested as in control animals. In a similar study, Hsu (1969) examined the effect of *in vitro* infection with virulent tubercle bacilli on macrophages from cortisone-treated and untreated guinea pigs. No difference was noted in the rates of bacillary multiplication within these two populations of macrophages. Cytologic studies, however, revealed that the intracellular infection was significantly more toxic to the host cells derived from the cortisone-treated animals. One of the most striking effects of cortisone and ACTH administration is a reduction in the number of circulating lymphocytes and involution of lymphoid tissue, including the thymus, spleen, and lymph nodes (Dougherty, 1952). However, lysis of lymphocytes due to exposure to cortisone has never been demonstrated convincingly (Germuth, 1956). In a histological study, Craig (1952) found that cortisone treatment of rabbits prior to antigen administration suppressed stimulation and mitosis of lymph node lymphocytes. Hinz *et al*. (1970) observed that specific antigenic stimulation of lymphocytes from tuberculin-sensitive patients correlated quantitatively with the tuberculin skin reactivity of the cell donor but failed to correlate with levels of serum antibody. Moreover, one tuberculin-positive patient given high doses of prednisone as a therapeutic regimen displayed significantly decreased stimulation of his lymphocytes by PPD, as measured by indirect assays for blast transformation by incorporation of tritiated thymidine.

Dermal reactivity to tuberculin remained positive in this patient during the period of *in vitro* suppression. Upon reduction of steroid dosage and subsequent cessation of therapy his lymphocyte response *in vitro* returned to pre-treatment levels. These findings suggest that the specific role of cortisone on the lymphocyte may be in part due to inhibition of lymphocyte proliferation.

The recent development of *in vitro* models for studying delayed hypersensitivity at the cellular level has provided reproducible, quantitative methods which may eventually shed light on the molecular basis of this phenomenon. Early attempts to develop *in vitro* systems were plagued by a lack of reproducibility. However, Rich and Lewis (1932) developed an *in vitro* system which clearly demonstrated both the inhibition of migration and the cytotoxic effects of tuberculin on mononuclear cells from splenic explants of tuberculous guinea pigs. Their findings stimulated a great deal of further *in vitro* work by various workers using similar methods. This subject has been reviewed extensively by Heilman (1963).

More recently, several *in vitro* methods have been described which appear to correlate with some phase of delayed-type cellular immunity *in vivo*. All of these *in vitro* correlates of delayed hypersensitivity are mediated by a group of soluble factors generated by antigen-activated lymphocytes from sensitive animals (Lawrence, 1968). The interaction between the immunocompetent lymphocyte and specific

antigen can result in a variety of biological phenomena, the form of which depends on the environment in which the cellular response to antigen is assayed. Four such in vitro phenomena include: (1) production and transfer of delayed hypersensitivity by means of viable lymphoid cells or their subcellular fractions containing transfer factor (Lawrence, 1969), (2) transformation of sensitized lymphocytes by specific antigen (Robbins, 1964; Oppenheim, 1968; and Dutton, 1967), (3) cytopathic or cytotoxic effects of sensitized lymphocytes on target cell populations, the surfaces which bear the specific antigen (Perlmann and Holm, 1969 and Rosenau, 1968), or target cell destruction not dependent on histocompatibility differences (Ruddle and Waksman, 1967, 1968; and Waksman, 1968), and (4) inhibition of macrophage migration (David, 1968 and Bloom and Bennett, 1968). These non-antibody mediators of cellular immunity generated by lymphocyte activation have recently been categorized as "Lymphokines" by Dumonde et al. (1969).

One of the most useful and thoroughly studied in vitro models of cellular immunity in recent years has been the inhibition of macrophage migration from capillary tubes. George and Vaughan (1962) developed an in vitro method which consisted of culturing peritoneal exudate cells from guinea pigs exhibiting delayed hypersensitivity in capillary tubes. Migration of the sensitive cells out of the tubes in fan-like patterns onto glass coverslips enclosed in Mackaness

chambers was inhibited when specific antigen, incorporated in tissue culture medium, was added to the chambers. Migration of normal cells in the presence of antigen and sensitive peritoneal cells without antigen was not inhibited. Cells from animals sensitized to egg albumin in complete Freund's adjuvant (CFA) were inhibited in vitro only when delayed skin reactions were demonstrable in the animals. David et al. (1964a) established that the specific inhibition of macrophage migration correlated with delayed-type hypersensitivity and not humoral antibody production. Peritoneal exudate cells from guinea pigs with delayed-type hypersensitivity to tuberculin, and either ovalbumin or diphtheria toxoid as antigen-antibody complexes in CFA, were specifically inhibited in vitro only by the corresponding antigen used for sensitization. Moreover, cells obtained from animals producing only precipitating antibody without delayed hypersensitivity or normal cells incubated with serum from tuberculin-sensitive animals were not inhibited in vitro. Inhibition of macrophage migration occurred when heat-inactivated serum was incorporated into the tissue culture medium, suggesting that the system was not complement dependent. These workers were unable to demonstrate passive sensitization of normal cells incubated in serum containing a high titer of humoral antibody to various protein antigens. Bloom and Bennett (1966) also reported that peritoneal cells from guinea pigs that had been immunized with protein antigens adsorbed to alumina gel

(in order to produce high titers of hemagglutinating antibody but no detectable delayed hypersensitivity) were not inhibited *in vitro* by the inducing antigen. Further evidence has established the correlation of this *in vitro* model with delayed cellular immunity. David and Paterson (1965) reported that peritoneal cells obtained from guinea pigs with experimental allergic encephalomyelitis (EAE) were inhibited specifically *in vitro* by antigens prepared from nervous tissue. In a similar study, Rauch *et al.* (1969) found that peritoneal macrophages obtained from guinea pigs 7 days following treatment with an EAE-inducing dose of purified basic protein from bovine spinal cord in CFA were inhibited by the same antigen *in vitro*. Al-Askari *et al.* (1965) used the George and Vaughan *in vitro* method to detect homograft sensitivity. They found that peritoneal cells from inbred mice with homograft sensitivity were inhibited *in vitro* when mixed with viable peritoneal cells from the strain of mice that had donated the sensitizing skin homografts. *In vitro* inhibition of migration was not demonstrable, however, when cells from one inbred strain of mice were mixed with cells from a genetically different strain.

Benacerraf and Gel (1959) demonstrated that the carrier protein determined the specificity of delayed-type reactivity *in vivo* in animals immunized with hapten-protein conjugates, while the immediate-type response was predominantly hapten-specific. David *et al.* (1964b) confirmed the

importance of delayed-type carrier protein specificity using the *in vitro* capillary tube system. Peritoneal cells from guinea pigs immunized with DNP-protein conjugates were specifically inhibited *in vitro* only by the homologous antigen and not by hapten conjugated to a heterologous carrier protein. In contrast, serum antibodies from the same animals reacted with the hapten regardless of the carrier protein.

Lipsmeyer and Kantor (1969) demonstrated *in vitro* desensitization of peritoneal cells from guinea pigs with delayed hypersensitivity to hapten-protein conjugates by pre-exposing the cells to the sensitizing antigen prior to *in vitro* assay for inhibition of macrophage migration. When cells from animals sensitized to multiple antigens (i.e., bovine gamma globulin, PPD, and picryl-guinea pig albumin) were pre-exposed to the latter antigen, there was a loss of responsiveness to all three antigenic specificities *in vitro*.

Recent immunochemical studies utilizing highly defined antigens have revealed the unique stereo-specificity required for elicitation of delayed or immediate hypersensitivity. Antigens which consisted of DNP conjugated to oligopeptides of L-lysine were found to be immunogenic for guinea pigs if the hapten oligopeptide had 7 or more lysyl residues (Yaron and Schlossman, 1966). *In vivo* delayed-type skin responses were demonstrable only to ∝DNP-oligopeptide conjugates consisting of 7 or more lysyl residues, while those containing 3 to 6 lysyl residues (non-immunogenic) could

readily elicit immediate-type reactions *in vivo*, but not delayed type responses (Schlossman *et al.* 1966). David (1968) confirmed these *in vivo* findings using the *in vitro* macrophage migration inhibition technique. Peritoneal cells from guinea pigs immunized with αDNP oligolysines with an average of 18 residues were inhibited *in vitro* in the presence of αDNP oligopeptides with 8 or more lysyl residues, but were not inhibited with those composed of 3, 4, or 6 lysines. These studies demonstrated that only αDNP oligopeptides, which are immunogenic and can elicit delayed hypersensitivity *in vivo*, are capable of specifically inhibiting cell migration *in vitro*.

Several attempts have been made by various workers to compare the time required for induction of delayed-type skin reactivity with that required for development of *in vitro* cellular sensitivity. Leu and Patnode (1968) reported that delayed-type skin reactivity in guinea pigs rendered sensitive to PPD was uniformly positive at 1 week, while significant *in vitro* sensitivity could not be detected until 3 weeks post-sensitization. Ferraresi *et al.* (1969), in a similar study in guinea pigs sensitized with diphtheria toxoid (DT) and human gamma globulin (HGG) incorporated in CFA, found that skin reactivity developed earlier and more rapidly than did *in vitro* sensitivity. Ricci *et al.* (1969) reported that *in vitro* sensitivity of peritoneal cells from tuberculin-sensitive guinea pigs was, in general, not significant until

3 weeks following sensitization. David (1964a) reported that, although inhibition of migration could not be directly correlated with the diameter of PPD skin reactions, there was a close parallelism. He suggested that skin reactivity may not necessarily parallel the sensitivity of other tissues. In contrast, Dumonde (1967) stated that, in unpublished work, in vitro sensitivity of guinea pig peritoneal cells could be detected on the sixth day after immunization and correlated with the occurrence of delayed skin reactivity, but not with the size of the skin reaction. Recently, Marcus (1970) reported that, under carefully controlled experimental conditions, macrophage inhibition was demonstrable simultaneously with the onset of tuberculin skin sensitivity. The conditions that appeared to be crucial in this experiment included: (1) intradermal injection of inbred guinea pigs in multiple sites with minimal doses of sensitizing antigen (i.e., 12.5 to 25 mcg. of heat killed mycobacteria), (2) use of homologous guinea pig serum, carefully selected to be non-toxic for cells, and (3) avoid chilling of peritoneal exudate cells. However, it was not apparent from this study whether any one, or all, of these factors were prerequisite to the early induction and/or detection of in vitro sensitivity.

The observation that small numbers of sensitive cells can effect large numbers of normal cells in eliciting delayed-type hypersensitivity reactions has been well established in vivo (Najarian and Feldman, 1961, 1963, and McCluskey et al.,

1963). David et al. (1964c) noted that when as few as 2.5% sensitive peritoneal cells were mixed with normal peritoneal cells inhibition of the whole cell population by antigen could be detected in vitro. Furthermore, they found that the sensitive cells must be viable to exert their effect on normal cells. In further studies (David, 1966 and 1967), attempts were made to determine the cell type (lymphocyte or macrophage) responsible for conferring the effect on normal cells. When cell preparations containing 95% lymph node or spleen lymphocytes obtained from guinea pigs sensitive to PPD or to a hapten-protein conjugate were mixed with normal peritoneal macrophages, the resulting population was inhibited from migrating by specific antigen. Since the sensitive lymph node cells alone or normal peritoneal cells alone were not inhibited by antigen in vitro, it was concluded that two different cell types were required for the inhibition of migration. Bloom and Bennett (1966) described similar experiments using lymphocytes and macrophages separated from peritoneal exudates. They reported that migration of normal peritoneal exudate cells was inhibited by PPD only when a few sensitive peritoneal lymphocytes were present in the mixture. Moreover, peritoneal macrophages from sensitive exudates rendered free of lymphocytes were no longer inhibited from migrating by specific antigen. It was concluded that, in this system, the lymphocyte possessed the immunological information, while the macrophage was simply the indicator cell which

migrated. The fact that so few lymphocytes could affect the migration of normal macrophages led to a search for a soluble material elaborated by lymphocytes upon interaction with specific antigen. Bloom and Bennett (1966), using purified populations of sensitized peritoneal lymphocytes, and David (1967), utilizing sensitized lymph node lymphocytes, reported that incubation of these cells in the presence of specific antigen resulted in the elaboration of a soluble factor into the surrounding medium. When the cell-free supernatants were used as the medium for normal peritoneal cells, inhibition of migration occurred. Bartfeld and Kelly (1968) reported similar findings using peripheral blood lymphocytes from tuberculin-sensitive guinea pigs. Therefore, it became clear that sensitized lymphocytes, upon interaction with antigen, elaborate a soluble migration inhibitory factor (MIF) which can inhibit migration of normal macrophages.

The mechanism of action of MIF remains unclear. Early in vitro observations (Rich and Lewis, 1932, and Kapral and Stinebring, 1958) suggested that tuberculin was both inhibitory and cytotoxic for macrophages from sensitized animals. Recently, Salvin et al. (1969) observed microscopically a specific aggregation of sensitized lymphocytes with normal macrophages in the presence of antigen. However, it should be emphasized that, in contrast to other in vitro systems, the capillary tube method is performed under short term tissue culture conditions (24-48 hr incubation) with relatively low,

non-cytotoxic concentrations of antigens. Bloom and Bennett (1966) noted that the macrophages remained viable even though their migration was inhibited. Bloom and Bennett (1968) suggested two possible mechanisms for MIF-mediated macrophage inhibition. Either MIF has specificity and requires combination with specific antigen, or it acts as a non-specific pharmacologic agent on the macrophage. Using an indirect approach, Bennett and Bloom (1967) reported that MIF-containing supernatants, resulting from short term exposure (i.e., "pulsing") of sensitive lymphocytes to PPD followed by washing and overnight incubation of the same cells in antigen-free medium, resulted in some inhibition of migration when they were added to normal macrophages in vitro. However, if excess antigen was added to the same supernatants, the degree of inhibition was markedly enhanced. The authors concluded that the potentiation of MIF by added antigen suggested that MIF may have specificity, although the possibility existed that the potentiation was due to the non-specific cytotoxic effects of PPD on the macrophage. Svejcar et al. (1968) confirmed these findings using the rabbit as an experimental animal. Amos and Lachmann (1970) reported that the presence of antigen was required for MIF to inhibit macrophage migration. These workers found that MIF-containing supernatants from lymphoid cells cultured with an insoluble antigen-polymer complex (PPD coupled to polyaminostyrene), and subsequently cleared of antigen by centrifugation, were largely

devoid of inhibitory activity. Activity was restored by adding antigen back during incubation with normal macrophages. MIF thus appeared to be antigen-specific and, to this extent, antibody-like. The authors postulated that MIF acts as a cytophilic factor for macrophages which are then held together by antigen. In contrast, Dumonde et al. (1969) separated specific protein antigen, gamma globulin and MIF from cell-free incubation supernatants by means of gel filtration (Sephadex G-100) and salt precipitation. MIF activity still remained, in the absence of antigen, gamma globulin, and immune complexes. The authors concluded that antigen was not necessary for the inhibitory effect of MIF on the macrophage once it is produced by sensitive lymphocyte-antigen interaction. Concanavalin A has been reported to suppress delayed hypersensitivity to tuberculin in vivo (Leon and Schwartz, 1969). In in vitro studies, (Schwartz et al., 1970) concanavalin A was found to induce the non-specific release of MIF from non-immune lymphoid cells obtained from guinea pigs, rabbits, and man. These results support the concept that MIF is not specific and that antigen is not required for MIF-mediated macrophage inhibition once MIF has been released by sensitive lymphocyte-specific antigen interaction.

Numerous attempts have been made to better characterize the mechanism and kinetics of MIF synthesis, as well as its physical, chemical, and macromolecular properties. Studies using metabolic inhibitors of protein synthesis

indicated that puromycin (David et al., 1964d) and mitomycin-C (Bloom and Bennett, 1966) prevent inhibition of migration by antigen. It appeared, therefore, that MIF production was associated with active protein synthesis. Bennett and Bloom (1967) reported that MIF could be detected in supernatants as early as 6 hr after exposure of sensitized lymphocytes to antigen. In addition, MIF was elaborated continuously for 5 days in long term lymphocyte cultures. In attempts to determine the effect of proteolytic enzymes on the in vitro system, David et al. (1964d) found that when sensitive peritoneal exudate cells were incubated with trypsin or chymotrypsin they were no longer inhibited by antigen. Sensitive cells recovered from the effects of trypsinization, however, if they were cultured in suspension for 24 hr prior to in vitro assay. Similar treatment of sensitive cells with RNase and DNase had no effect. In a more definitive study, Pochyly (1967) found that trypsinization of sensitive lymph node lymphocytes blocked their ability, when mixed with normal exudate cells, to elicit in vitro inhibition of macrophage migration. Incubation of normal peritoneal exudates with trypsin had no effect on the reaction when untreated sensitive lymphocytes and antigen were added to the system. Bloom and Bennett (1968a) confirmed these results using purified guinea pig peritoneal and lymph node lymphocytes. These results suggested that trypsinization may have removed a specific receptor site on the sensitized lymphocyte which was resynthesized when trypsin

activity was blocked. However, Remold and David (1970) recently found that chymotrypsin and neuraminidase treatment abolished inhibitory activity of MIF-containing cell free supernatants. These results suggested that soluble MIF was susceptible to enzymatic degradation.

In attempts to fractionate the active component(s) of inhibitory supernatants by gel filtration on Sephadex G-200 (David, 1968; Bloom and Bennett, 1968; and Dumonde et al. (1969), inhibitory activity has been consistently associated with the third peak obtained, i.e. that containing albumin. These results suggest that MIF has a molecular weight of approximately 70,000. MIF is non-dialyzable (Bloom and Bennett, 1966), heat-stable at 56 C for 30 min (David, 1966), cryolabile at 4 C and -20 C (Bartfeld and Kelly, 1968), and lyophilizable and stable at -70 C after lyophilization (Bennett and Bloom, 1967).

A role of a cytophilic antibody in delayed hypersensitivity has been postulated (Karush and Eisen, 1962). Although most workers have failed to demonstrate any correlation between MIF-mediated macrophage migration inhibition and humoral antibody, there is currently some convincing evidence that there may exist a separate macrophage inhibition mechanism which is dependent on an antibody cytophilic for macrophages. Boyden (1964) demonstrated an antibody in guinea pig anti-sheep erythrocyte sera which was selectively bound to peritoneal macrophages, but not to lymphocytes or granulocytes.

This cytophilic antibody was shown to confer to macrophages the ability to bind sheep red cells on their surface (rosette formation). Production of cytophilic antibody was induced only by incorporation of the antigen in complete Freund's adjuvant (CFA) and not with incomplete Freund's adjuvant, which indirectly suggested a correlation with delayed-type hypersensitivity. Amos et al. (1967) reported that conventional γ-2 guinea pig antibody cytophilic for peritoneal macrophages mediated inhibition of normal macrophage migration in the presence of specific antigen. Bloom and Bennett (1966) also reported that normal macrophages could be inhibited in vitro by antigen and specific antiserum from guinea pigs immunized with bovine serum albumin, ovalbumin, or CFA. In the tuberculin-sensitive animals, repeated PPD skin tests were performed prior to the collection of immune serum until strong Arthus reactions were obtained. Krejci et al. (1968) found that serum collected from hypersensitive guinea pigs previously challenged intravenously with antigen conferred in vitro sensitivity on normal macrophages. Recently, Spitler and Fudenberg (1970) reported that intravenous injection of immune serum from guinea pigs sensitized with CFA into normal recipients conferred MIF-mediated sensitivity on their peritoneal exudates assayed in vitro 3 days later. Benacerraf (1968), using the "rosette" technique of Boyden and guinea pig alveolar cells as a source of macrophages, identified the guinea pig cytophilic antibody by zone electrophoresis and

other criteria as γ-2, and not γ-1 immunoglobulin. Pepsin digestion destroyed its ability to bind to macrophages. Therefore, the cytophilic binding site was apparently associated with the Fc fragment of the molecule. However, the author concluded that cytophilic antibodies of this type, added passively to macrophages, did not elicit inhibition of macrophage migration in the presence of added antigen. Nelson and Mildenhall (1968) found no correlation between cytophilic antibody titer and delayed-type dermal response to sheep erythrocytes or human serum albumin at 1-3 weeks following sensitization. Heise et al. (1968) clearly demonstrated in vitro inhibition of migration of alveolar macrophages obtained from BCG-sensitized guinea pigs to be due to a cytophilic antibody which was not MIF. The cytophilic antibody activity was transferable passively to normal alveolar macrophages by heat elution from sensitive alveolar and peritoneal macrophages or by immune serum. Further studies revealed that MIF-mediated activity was demonstrable in supernatants when sensitive lymph node lymphocytes from the same animals were incubated with antigen. Similar incubation of alveolar macrophages from sensitive animals resulted in no activity in most experiments. Finally, the cytophilic antibody was characterized by gel filtration, salt precipitation, and immunochemical methods as an IgG immunoglobulin, probably of the γ-2 type, which was distinct from MIF.

Thor (1967, 1968) reported that delayed-type immune competency was transferable to normal cells *in vitro* via RNA extracts of human lymph nodes from tuberculin-sensitive and histoplasmin-sensitive individuals or patients with active tuberculosis using the macrophage inhibition technique. When the cell-free extracts containing RNA from sensitive donors were incubated with normal human lymphoid cells, *in vitro* inhibition of migration occurred in the presence of specific antigen. The active fraction was inactivated by treatment with RNase, but not by trypsin or DNase. Jureziz *et al.* (1968) reported transfer of MIF-like activity with RNA extracts by the same methods using the guinea pig as the experimental animal. The RNA extracts used in these experiments were not transfer factor, but were similar to the material found by Fishman (1961), Adler *et al.* (1966), and Cohen (1967) to convert non-immune cells into antibody-forming cells in experimental animals. Thor and Schlossman (1969) reported that specific antigen-induced MIF-activity was detected in culture supernatants from normal lymphoid cells passively treated with RNA extracts from guinea pigs sensitive to DNP-oligolysine antigens.

At present, a considerable amount of confusion exists as to the relationship, if any, between MIF and the various other *in vitro* correlates of delayed hypersensitivity. Bennett and Bloom (1967) reported that "blast-cell" transformation is not a prerequisite to MIF production, since the

latter is elaborated into culture supernatants prior to the time that transformation occurs. This experiment did not, however, disprove the possibility that MIF-producing lymphocytes are the same sensitive cells that subsequently undergo transformation. Spitler and Lawrence (1969) compared the *in vitro* biologic activity of substances produced during stimulation of human peripheral blood lymphocytes by specific antigen. They found that the degree of MIF activity produced was proportional to the degree of lymphocyte transformation. Moreover, MIF-containing supernatants enhanced transformation of normal lymphocytes. Maclaurin (1969) noted that phytohemagglutinin (PHA) induced the formation of intercellular cytoplasmic bridges between macrophage monolayers in tissue culture. The same effect was induced by stimulating sensitive macrophages with tuberculin. Fireman *et al.* (1967) reported similar inducement of transformation when dialyzable transfer factor was added to normal human lymphocytes in the presence of antigen *in vitro*. Paque *et al.* (1969) reported that when non-sensitive human lymphocytes were incubated with specific antigen and lymphoid cell lysates obtained from histoplasmin, coccidiodin, or tuberculin skin test positive human donors, the non-sensitive cells were sensitized and released a substance which specifically inhibited the migration of guinea pig macrophages. Dumonde *et al.* (1969) demonstrated that cell free supernatants resulting from sensitive lymphocyte-antigen interaction were capable of eliciting

delayed-type dermal responses *in vivo*, as well as *in vitro* activities including MIF, mitogenic factor, and a factor cytotoxic for target cells. Bennett and Bloom (1968a and 1968b) reported that intradermal injection into normal guinea pigs of MIF-containing cell-free supernatants produced a cellular response typical of delayed hypersensitivity, although the time required for development was accelerated (maximal at 8-12 hr and disappeared by 30 hr). Krejci *et al.* (1969) confirmed these results. Ward and David (1969) described a leukotactic factor in supernatants from sensitive lymphocytes incubated with antigen which was not dissociable from MIF activity by physical methods.

The immunologic specificity of acquired cellular resistance to infection has not been established (Mackaness, 1968). In recent *in vitro* studies, however, Mackaness (1969) presented evidence that lymphoid cells from mice infected with *Listeria monocytogenes* confer immunologically specific protection upon normal animals, as determined by activation of peritoneal macrophages. Mooney and Waksman (1970) demonstrated the activation of rabbit macrophages by supernatants of antigen-stimulated, sensitive lymphocytes which contained MIF activity. They suggested that a similar specific activation may be responsible for cell-mediated immunity.

The objectives of this research were fourfold: (1) Develop a modified *in vitro* method for studying delayed hypersensitivity to tuberculin in the guinea pig; (2) Study and

correlate the time required for induction of delayed-type skin reactivity and *in vitro* macrophage migration inhibition; (3) Examine the sensitivity of guinea pig alveolar macrophages to tuberculin *in vitro*; and (4) Study the effect of cortisone administration on the induction and expression of tuberculin skin reactivity and *in vitro* cellular sensitivity in guinea pigs.

CHAPTER II

METHODS AND MATERIALS

Experimental Animals

Male, albino, Hartley strain guinea pigs (Cavia porcellus), weighing approximately 300-400 g, were used in these experiments. All animals were obtained from the same commercial source[1] and were maintained on a diet of commercial pellets which was supplemented weekly with fresh cabbage leaves. The animals were housed no more than 5 per cage and were held for observation at least 2 weeks prior to experimental use.

Organisms and Cultural Conditions

Mycobacterium tuberculosis, (strain H37Ra) was grown on a modified Proskauer and Beck synthetic medium (Youmans and Karlson, 1947) for 4 to 6 weeks at 37 C in Roux flasks containing 150 ml of medium. Whole cells were separated from the culture medium by suction filtration through a Buchner funnel containing Whatman No. 1 filter paper. The moist cells

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[1] Camm Research Institute, Incorporated, 414 Black Oak Ridge Road, Wayne, New Jersey.

were pooled, washed three times in pyrogen-free 0.85% NaCl,[2] and killed by heating in a hot water bath at 60 C for 1 hr (Redmond and Bowman, 1955). The heat-killed organisms were dried in a hot air oven at 110 C for 1 hr, ground to a fine powder in a sterile mortar, and stored at 4 C in a sealed Petri dish. A preparation of heat-killed Mycobacterium tuberculosis, strain H37Rv, grown on Proskauer and Beck medium and prepared in a similar manner, was kindly supplied through the courtesy of Dr. B. W. Janicki, Veterans Administration Hospital, Washington, D.C. The latter antigen was used in most of the experiments.

Preparation of Sensitizing Antigens

Sensitizing antigens were prepared by homogenizing a mixture of heat-killed mycobacterial cells (strain H37Rv), 10 ml Freund's incomplete adjuvant,[3] and 5 ml pyrogen-free 0.85% NaCl for 4 min on a Sorval Omnimixer.[4] The H37Ra antigen preparation contained 5 mg organisms per ml. Another group of sensitizing antigens was prepared with the H37Rv strain to yield final concentrations of 0.5, 1.0, 2.5, 5.0, and 10 mg organisms per ml. All of the antigens were freshly prepared just prior to sensitization of experimental animals.

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[2]Cutter Laboratories, Berkeley, California.

[3]Difco Laboratories, Detroit, Michigan.

[4]Ivan Sorvall, Inc., Norwalk, Connecticut.

Sensitization of Guinea Pigs

Preliminary Sensitization Studies

In order to determine the optimal method to render normal guinea pigs hypersensitive to tuberculin, the following procedures were evaluated:

1) Subcutaneous injection in the nuchal area (nape of the neck) of 1 ml (5 mg) of H37Rv antigen.

2) Intramuscular injection in the groin of 1 ml (5 mg) of H37Rv antigen.

3) Intramuscular injection in the groin and subcutaneous injection in the nuchal area with 1 ml/site (10 mg total) of H37Rv antigen.

Sensitization of Animals for *In Vitro* Studies

To obtain a source of sensitive peritoneal exudate cells for *in vitro* macrophage migration studies, normal animals were injected subcutaneously in the nuchal area with 1 ml of antigen containing 0.5, 1.0, 2.5, 5.0, or 10.0 mg of heat-killed H37Rv organisms.

Sensitization of Animals for Alveolar Macrophage Studies

For most *in vitro* studies of guinea pig alveolar macrophages, animals were sensitized subcutaneously with 5 mg H37Rv antigen as described previously. In addition, a group of normal guinea pigs was sensitized according to the method of Heise *et al*. (1968). Each animal was injected

subcutaneously on the dorsal side with a total of 2.4 mg H37Rv antigen by injecting 0.1 ml (0.4 mg) into 6 different sites. A third method of sensitization involved the intravenous injection of 0.75 or 1.25 mg of heat-killed H37Rv cells in pyrogen-free 0.85% NaCl to which 3 drops of Tween 80[5] had been added. In a similar experiment, 2 groups of normal guinea pigs were injected either intravenously or intracardially with 0.5 ml (2.5 mg) of H37Rv cells in pyrogen-free 0.85% NaCl to which 3 drops of Tween 80 had been added. A final group of animals was injected intrapleurally into the lung with 0.5 mg of H37Rv antigen.

Skin Tests

In preliminary studies, sensitized animals were skin tested by the intradermal injection of 0.1 ml (5 mcg) Purified Protein Derivative, PPD, (Merck Sharp and Dohme)[6] diluted in the commercial phosphate buffer supplied by the manufacturer. In order to avoid non-specific skin reactivity attributed to certain batches of the commercial diluent, PPD was subsequently diluted in pyrogen-free 0.85% NaCl. PPD was injected into the shaved dorsal skin. The injection sites were varied in experiments where multiple skin tests were performed on the same animal. The diameter of induration and the presence

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[5]Atlas Chemical Industries, Inc., Wilmington, Delaware.

[6]Merck Sharp and Dohme, West Point, Pennsylvania.

of erythema and/or central necrosis were recorded 24 hr after injection of PPD. Reactions measuring 15 mm or more of induration were considered to be significant as an index of sensitization.

Histological Studies

Skin test sites were obtained by biopsy from normal and tuberculin-sensitive guinea pigs 24 hr after injection of the skin test antigen. Tissues were placed in an automatic tissue processor (Auto-technicon)[7] for dehydration through 70, 80, 90, 95, and 100% ethanol, clearing in absolute ethanol-xylol in a ratio of 1:1, followed by pure xylol, and imbedding in paraffin using standard Tissue-Tek[8] imbedding equipment. Tissue sections were cut on a rotary microtome at a thickness of 7 microns. Sections were stained with Harris's hematoxylin-eosin stain according to the methods outlined in the Manual of Histologic and Special Staining Techniques (1960).

Collection of Guinea Pig Peritoneal Exudate Cells

Peritoneal exudate cells were obtained from normal and tuberculin-sensitive guinea pigs 72 hr after intraperitoneal injection of 30 ml sterile light paraffin oil[9] (viscosity

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[7]The Technicon Company, Chauncey, New York.

[8]Lab-Tek Instruments Company, Westmont, Illinois.

[9]Fisher Scientific Company, Fairlawn, New Jersey.

125/135). In certain experiments animals were injected in the same manner with 2.5% Starch Gel[10] in pyrogen-free 0.85% NaCl. Food was withheld from all animals 24 hr prior to cell collection. The animals were sacrificed by intracardial bleeding, followed by injection of an air embolus and/or cervical dislocation. Exudate cells were collected by aseptic technique. A ventral midline incision was made in the peritoneal wall and the viscera and peritoneal cavities were washed with approximately 120 ml Hanks balanced salt solution (HBSS) containing 0.4 units heparin/ml (sodium heparin Lipoheparin).[11] The peritoneal washings were centrifuged at 150 x g for 15 min and the supernates were discarded. The packed cells were resuspended and pooled in 5 ml Eagles minimal essential medium (EMEM) with Earls balanced salt solution[12] containing 15% normal homologous commercial guinea pig serum[13] (EMEM-S). Normal guinea pig serum was decomplemented by heating at 56 C for 30 min prior to incorporation into EMEM. The cell suspension was centrifuged at 64 x g (920 rpm) for 5 min in 13 ml graduated screwcap centrifuge tubes and the packed cell volumes were recorded. Total leukocyte counts

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[10]Mann Research Laboratories, Division of Becton, Dickinson and Company, New York, New York.

[11]Riker Laboratories, Northridge, California.

[12]Hyland Laboratories, Los Angeles, California.

[13]Colorado Serum Company, Denver, Colorado.

were made using the Unopette[14] diluting method, followed by standard hemocytometer counts. The cells were resuspended in EMEM-S to a final concentration of approximately 1.5-1.8 x 10^7 cells/ml. For most experiments a 10% packed cell volume, which resulted from centrifugation at 64 x g for 5 min, was diluted in 90% EMEM-S to give a final cell concentration of approximately 1.5-1.8 x 10^7 cells/ml. Cell suspensions were kept in ice throughout the experiment. Differential counts were performed on Wright's-stained dried smears of the cell suspensions. Peritoneal exudates consisted mainly of mononuclear cells with approximately 10-30% lymphocytes, 70-90% macrophages, and 0-10% polymorphonuclear leukocytes.

Collection of Guinea Pig Alveolar Macrophages

For the purpose of obtaining sufficient quantities of alveolar macrophages relatively free of erythrocyte contamination, two methods were evaluated.

In Situ Method

The in situ method of Maxwell et al. (1964) was used in preliminary studies. The animals were sacrificed by intracardial bleeding and cervical dislocation. The thoracic cavity was opened and the trachea was dissected away from surrounding connective tissue. A hemostat was attached to the tracheal wall and the trachea was transected above this point.

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[14]Becton, Dickinson Company, Rutherford, New Jersey.

The tapered end of a small glass tube which was connected to the tip of a 3-way stopcock (Luer lock)[15] by means of polyethylene tubing was inserted into the lumen of the trachea. Two 25 ml Luer lock syringes were attached to the stopcock and supported by a ring stand and clamps. The upper reservoir syringe was filled with HBSS (non-heparinized). After adjusting the stopcock valve, 10-15 ml of HBSS was carefully injected into the lungs. The stopcock valve was then adjusted to allow the cell suspension to enter the receiving syringe. The above procedure was repeated, using 5-10 ml volumes, until a total of 120 ml of washing fluid was obtained.

Method of Myrvik

For most experiments, guinea pig lung macrophages were collected according to a method modified from that of Myrvik et al. (1961). Prior to sacrifice by intracardial bleeding, the animals were injected intraperitoneally with 0.5 ml epinephrine[16] (adrenalin chloride, 1:1,000) and 5 ml (125 mg) of Nembutal[17] (pentobarbital, sodium) in 0.85% NaCl. The thoracic cavity was opened and the trachea dissected free and transected as described previously. The anterior portion of the trachea was clamped off by means of a hemostat and the trachea-lungs-heart complex was removed as a unit. The heart

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[15]Becton, Dickinson Company, Rutherford, New Jersey.

[16]Parke, Davis and Company, Detroit, Michigan.

[17]J. T. Baker Chemical Company, Phillipsburg, New Jersey.

was carefully dissected away from the lungs. In order to eliminate residual blood contamination, the lungs-trachea complex was rinsed repeatedly in 0.85% NaCl and blotted with sterile gauze pads. Sterile HBSS (10 ml) was carefully injected into the lungs via the trachea by means of a syringe and needle. The tapered end of a glass tube connected to a 3 inch polyethylene tube was inserted into the lumen of the trachea and the washing fluid was drained into a sterile tube by gravity, with gentle massage of the lungs. The lungs were perfused repeatedly in the same manner until 80-120 ml of washing fluid had been collected.

The macrophage-containing washing fluids obtained by either method of collection were centrifuged at 64 x g (920 rpm) for 15 min. The cell pellets were resuspended in 5-10 ml HBSS and centrifuged at 64 x g for 5 min. The cell pellets were then resuspended in 5 ml EMEM-S and centrifuged at 64 x g for 5 min prior to adjustment of cell concentrations to approximately 1 x 10^7 cells/ml of EMEM-S. Differential leukocyte counts were performed on Wright's stained dried smears. Lung washings consistently contained 95-98% alveolar macrophages with an occasional lymphocyte or polymorphonuclear leukocyte. The total cell yields obtained by the <u>in situ</u> method varied from none to 7 x 10^6 cell/lung, while the modified Myrvik method resulted in dependable yields of 1.5-3 x 10^7 cells/lung.

Preparation of Purified Lymphocyte Suspensions

Attempts were made to prepare purified lymphocyte suspensions from guinea pig peritoneal exudates by the method of Bloom and Bennett (1966). Washed, oil-induced peritoneal exudate cells from normal or sensitive animals were pooled in EMEM-S to a final concentration of approximately 5×10^6 cells/ml. The cell suspensions were pipetted, in 5 ml amounts, into standard, 100 mm plastic disposable Petri dishes and incubated at 37 C for 45 min to allow adsorption of the macrophages to the bottom surface of the plates. After incubation, the plates were agitated gently to remove the unattached cells. The supernates which contained lymphocytes and unadsorbed macrophages were aspirated from each Petri dish and pooled prior to centrifugation at 150 x g for 5 min. The cell pellets were resuspended in EMEM-S, distributed again in sterile Petri dishes in 5 ml amounts, and the adsorption procedure was repeated. The resulting adsorbed cell suspension consisted of approximately 85% lymphocytes and the cell recovery constituted 14% of the original volume.

Passive Transfer Experiments

Passive transfer of tuberculin hypersensitivity was attempted with viable and sonic-disrupted cells. Donor cells were obtained from pooled, oil-induced peritoneal exudates obtained from animals sensitized intramuscularly with 5 mg H37Rv or H37Ra antigen. One-half of each pooled cell suspension was

sonicated in a Branson Sonifier (Model S-75)[18] at a peak setting of 8 (14 amp) for 3 min. The cell suspension was kept cool in ice during the sonication process to prevent overheating. All donor guinea pigs were skin tested with 5 mcg PPD 24 hr prior to sacrifice. Normal guinea pigs that had not been previously skin tested were used as recipients. The recipient animals were injected intraperitoneally with 2.3-2.6 x 10^9 cells or the equivalent of sonic lysate. The recipient animals were skin tested with 5 mcg PPD 3 and 6 days after transfer. In a preliminary experiment cells were obtained from donors with relatively weak tuberculin skin reactivity (i.e., mean induration of 12 mm). This group had been sensitized with 5 mg H37Rv antigen 10-12 months previously. In a second experiment 20 donor animals were divided into 2 groups of 10 animals each, based on the type of sensitizing antigen used. Group 1--sensitized with H37Rv antigen, strong skin reactivity (mean induration, 22 mm); and Group 2--sensitized with H37Ra antigen, weak skin reactivity (mean induration, 15 mm). All donor animals were sensitized 5 weeks prior to cell collection. In addition, cells and sonic lysates from normal guinea pigs served as controls.

In Vitro Macrophage Migration Method

A method modified from that of George and Vaughan (1962) was used as a means of assaying delayed

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[18]Heat Systems Company, Melville, New York.

hypersensitivity to tuberculin in vitro. The method described by George and Vaughan, and used extensively by several other workers, involves the use of Mackaness Chambers for incubating cell-containing capillary tubes in the presence of antigen incorporated in tissue culture medium. In the present studies, a short-style, Leighton-type tissue culture tube[19] was substituted for the Mackaness Chamber (Leu and Patnode, 1968). Small capillary tubes,[20] 1.3-1.5 mm in diameter and 75 mm long, were filled with the cell suspension, sealed at one end with sterile plasticene and centrifuged at 64 x g for 5 min. The tubes were then cut at the cell-fluid interface with a diamond point glass etcher. The portion of the tube containing the cells was attached to a glass coverslip by means of a small amount of silicone lubricant[21] and placed in a short-style, Leighton-type tissue culture tube. Peritoneal or alveolar cells from each donor animal, whether normal or sensitive, were prepared so that at least 2 Leighton tubes were filled with 1.0 ml of EMEM-S without antigen and at least 2 tubes were filled with EMEM-S to which appropriate concentrations of PPD had been added. After the medium was added, each Leighton tube was stoppered tightly with a siliconized rubber stopper, incubated at 37 C, and examined at 18 hr. Quantitation was performed by projecting the image of the

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[19]Bellco Glass, Incorporated, Vineland, New Jersey.

[20]Scientific Products Company, Dallas, Texas.

[21]Dow Corning Company, Midland, Michigan.

area of migration onto drawing paper by means of a Bausch and Lomb microprojector.[22] The outer edge of the projected image was outlined and the area of migration was measured by means of a planimeter.[23] The following formula was used to express the degree of migration:

$$\text{Per Cent Migration With Antigen} = \frac{\text{Mean Area of Migration With Antigen}}{\text{Mean Area of Migration Without Antigen}} \times 100$$

In preliminary experiments, inhibition of migration was determined qualitatively by examining the preparations with an inverted light microscope.

Two types of PPD were used for in vitro experiments. Several batches of a commercial PPD preparation,[24] which was also used as a skin test antigen, were evaluated. The preparations were supplied as 40 mcg tablets which contained purified cresols (0.06 mg) as a preservative. A second PPD preparation, free of preservatives and excipients, was kindly supplied in powder form by Dr. M. W. Fisher, Parke Davis Company, Research Division, Detroit, Michigan.

Effect of Incubation Time on Macrophage Migration

Studies were performed to determine the optimal incubation time for recording macrophage migration in the in

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[22]Bausch and Lomb, Incorporated, Rochester, New York.

[23]Keuffel and Esser Company, New York, New York.

[24]Merck Sharp and Dohme, West Point, Pennsylvania.

vitro system. Two types of experiments were conducted. In the first experiment, capillary tubes containing oil-induced peritoneal exudate cells from a sensitive donor were prepared by the techniques described previously. To one-half of the Leighton tubes was added 1.0 ml EMEM-S (without antigen). One ml of EMEM-S containing 5 mcg PPD was added to each of the remaining tubes. The tubes were then incubated at 37 C and the area of migration of cells in each tube was recorded after 4, 8, 18, and 24 hr. In the second experiment, peritoneal exudate cells obtained from 4 sensitive animals were pooled and 4 identical sets of capillary tubes were prepared. One-half of the Leighton tubes in each set were filled with medium containing 5 mcg PPD/ml, while the remaining tubes served as controls. All of the tubes were incubated at 37 C and one set was examined at each time interval (4, 8, 18, and 24 hr). By this procedure it was possible to avoid sloughing of cells from the coverslips due to repeated handling of the tubes.

Endpoint Titration of PPD

It became evident early in this work that commercial PPD preparations were highly toxic for normal cells. These findings necessitated the development of an endpoint titration method as a standardization procedure for each lot of commercial PPD used. This was accomplished by determining the degree of migration of both normal and tuberculin-sensitive cells in the presence of various concentrations of each lot of PPD. The endpoint was defined as that

concentration of PPD that would significantly inhibit sensitive cell migration but would be least inhibitory for normal cell migration.

Studies on the Time Required for Induction of Tuberculin Hypersensitivity in Guinea Pigs

Studies were performed on the development of the delayed response to mycobacterial antigen and attempts were made to correlate *in vitro* cellular sensitivity with tuberculin skin reactivity. In a preliminary experiment, two groups of normal guinea pigs were sensitized intramuscularly with 5 mg H37Rv antigen. Representative animals from one group were sacrificed, without skin testing, at weekly intervals for 5 weeks and their peritoneal cells were tested *in vitro* for sensitivity to 10 mcg/ml of PPD. Animals in the second group were skin tested with 5 mcg PPD at weekly intervals for the same time period. A second experiment was performed in the same manner in which normal animals were sensitized by subcutaneous injection of 5 mg H37Rv antigen. Peritoneal cells from representative animals were tested *in vitro* at weekly intervals for 5 weeks for sensitivity to 5 mcg PPD. The effect of different dosages of sensitizing antigen on the induction of sensitivity was studied in similar experiments. Groups of normal animals were sensitized with 0.5, 1.0, 5.0, or 10.0 mg of H37Rv antigen and *in vitro* assays were performed on peritoneal cells from representative members of each group of animals at weekly intervals for 3 weeks. A portion of the

animals from each group was reserved for repeated weekly skin testing.

In Vitro Studies on the Sensitivity of Alveolar Macrophages

In preliminary studies alveolar cells obtained from PPD-sensitive animals (sensitized subcutaneously with 5 mg H37Rv antigen) were collected and studied *in vitro* for their sensitivity to PPD by the methods described previously.

In a subsequent experiment, the cellular sensitivity of alveolar and peritoneal cells from the same sensitive donors were compared. In order to accomplish this, 2 normal and 2 sensitive (sensitized subcutaneously with 5 mg H37Rv antigen) guinea pigs were injected with sterile paraffin oil by methods described previously. The animals were sacrificed 72 hr later and both the alveolar and peritoneal cells were collected and tested individually *in vitro* using 5 or 15 mcg PPD by the methods described previously.

Intravenous and Intracardial Challenge of Sensitive Guinea Pigs with Mycobacterial Antigens

In order to determine the effect of intravenous and intracardial challenge with PPD on the *in vitro* sensitivity of alveolar macrophages obtained from tuberculin-sensitive guinea pigs the following experiment was performed: Tuberculin-sensitive and normal guinea pigs were injected either intravenously or intracardially with 25 mcg or 1 mg PPD (without preservatives, obtained from National Institutes

of Health, Bethesda, Maryland) in pyrogen-free 0.85% NaCl. Twenty-four hours later the animals were sacrificed and their alveolar cells were collected and studied for sensitivity to PPD *in vitro*.

Transfer of MIF-Mediated Sensitivity *In Vitro* By Mixing Sensitive and Normal Cells

In order to transfer MIF-mediated sensitivity *in vitro*, peritoneal cell suspensions from tuberculin-sensitive and nonsensitized donors were mixed in various proportions according to the method described by David *et al*. (1964c). By this method, 5, 10, 20, 40 or 50% sensitive cells were mixed with 95, 90, 80, 60, and 50% normal cells, respectively. The cell mixtures were then tested *in vitro* for their sensitivity to PPD. In addition, the normal and sensitive cells were tested individually. In several experiments, attempts were made to transfer MIF-mediated sensitivity *in vitro* from tuberculin-sensitive peritoneal cells to normal alveolar macrophages by the same methods. In one experiment, purified peritoneal lymphocytes, obtained by the Petri Dish Adsorption Method, were mixed with an equal number of normal alveolar macrophages prior to *in vitro* testing in the presence of appropriate concentrations of PPD. In another experiment, peritoneal and alveolar cells obtained from the same sensitive donors were mixed in various proportions prior to *in vitro* assay.

Effect of Serum from Normal and Sensitive Guinea Pigs on Macrophage Migration

The effect of serum from normal and tuberculin-sensitive guinea pigs on the *in vitro* reactivity to PPD of normal and sensitive cells was studied next. Tissue culture medium (EMEM) was prepared containing either 15% pooled normal guinea pig serum or pooled serum from tuberculin-sensitive donors which had been sensitized 3 weeks previously. All sera were heat-inactivated at 56 C for 30 min. Appropriate concentrations of PPD were added to a portion of each medium. Leighton tubes were filled with 1.0 ml of the appropriate medium, with or without PPD. Peritoneal or alveolar cells obtained from normal or sensitive animals were suspended in the medium containing either normal or sensitive serum to a final concentration of approximately 1.5×10^7 cells/ml. Finally, the cells were placed in capillary tubes and assayed for *in vitro* sensitivity to tuberculin as described previously. In a similar experiment, the effect of serum collected at 1, 2, and 3 weeks post-sensitization on the *in vitro* sensitivity of alveolar macrophages to tuberculin was determined.

Effect of Cortisone on In Vitro Sensitivity and Skin Reactivity to Tuberculin

Intraperitoneal Injection of Cortisone

Normal and tuberculin-sensitive guinea pigs were injected intraperitoneally with 30 ml of sterile paraffin oil 72 hr prior to cell collection. Twenty-four hours prior to sacrifice, animals from each group were injected

intraperitoneally with 5 ml of a suspension containing 0, 25, 50, or 100 mg of cortisone acetate[25] (25 mg/cc) diluted in pyrogen-free 0.85% NaCl. The cells were harvested as described previously and tested for their in vitro sensitivity to 5 and 15 mcg PPD/ml.

In Vitro Treatment of Peritoneal Cells with Cortisone

Pooled normal and tuberculin-sensitive peritoneal cells were divided into 3 equal aliquots and suspended in EMEM-S which contained either 0, 100, or 200 mcg cortisone acetate/ml. The cell suspensions were then incubated on a roller drum[26] at 37 C for 45 min. After incubation, the cells were washed once in 5 ml EMEM-S and resuspended in the same medium. Finally, the cells were tested in vitro for sensitivity to 10 mcg PPD/ml. In a second experiment, peritoneal cells from 3 sensitive guinea pigs (mean skin reactivity of 20 mm induration) were divided into 3 equal aliquots and incubated with cortisone by the methods used in the previous experiment. After incubation, the cortisone-treated cells were washed once in 5 ml of EMEM-S and resuspended in EMEM-S containing 0, 100, or 200 mcg/ml of cortisone corresponding to the concentrations the same cells had been incubated with previously. The cells were then tested in vitro for sensitivity to 5 and 10 mcg PPD/ml.

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[25]Merck Sharp and Dohme, West Point, Pennsylvania.

[26]Cole Parmer Company, Chicago, Illinois.

Effect of Intramuscular Cortisone Treatment on the Induction and Expression of Delayed Hypersensitivity to Tuberculin

The effect of the intramuscular injection of tuberculin-sensitive animals with cortisone on the expression of skin reactivity and *in vitro* sensitivity to tuberculin was studied. Groups of normal guinea pigs were sensitized with 5 mg of H37Rv antigen and sacrificed 21 days later. Five days prior to sacrifice, one group was injected intramuscularly with 10 mg cortisone daily for 5 days (total dosage, 50 mg). A second group was injected in the same manner with 20 mg of cortisone daily (total dosage, 100 mg). Control groups consisted of normal animals injected with cortisone. Skin tests were administered 24 hr prior to initiation of cortisone therapy and again 24 hr prior to sacrifice. Oil-induced peritoneal exudate cells from each donor were assayed for *in vitro* sensitivity to 15 mcg PPD/ml.

In order to study the effect of cortisone on the induction of tuberculin hypersensitivity, 4 groups of 5 normal guinea pigs were treated as follows: Group 1 was injected subcutaneously in the nuchal area with a mixture of 25 mg cortisone and 5 mg of the H37Rv antigen which had been homogenized in a Sorval omnimixer;[27] Group 2 was injected intramuscularly in the groin with 20 mg cortisone acetate daily for 5 days prior to sensitization and for 5 days post-sensitization

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[27]Ivan Sorvall, Inc., Norwalk, Connecticut.

(total dosage, 200 mg); Group 3 was injected intramuscularly with 20 mg of cortisone daily for 5 days prior to sensitization and for 20 days post-sensitization (total dosage, 500 mg); and Group 4 constituted the non-cortisone treated tuberculin-sensitive controls. Cortisone-treated normal animals corresponding to the first 3 groups were not sensitized and comprised the cortisone-treated, non-sensitized controls. The remainder of the animals were sensitized by the subcutaneous injection of 5 mg of H37Rv antigen in the nuchal area. Each animal was skin tested at weekly intervals for 4 weeks. The final skin tests were performed 24 hr prior to sacrifice of the animals. Skin test sites were biopsied from the sacrificed animals and saved for histological studies. Approximately 21 days following sensitization, peritoneal exudates were harvested from donor animals which had been injected intraperitoneally with 30 ml sterile paraffin oil 72 hr previously. The cells from each donor were studied for their <u>in vitro</u> sensitivity to 15 mcg PPD/ml.

Statistical Analyses

Statistical analyses were made using a correlation coefficient or a one-tailed Student T Test (Dunn, 1966). Differences between means were considered to be significant if $P \leq 0.05$.

CHAPTER III

RESULTS

The initial phase of this investigation involved the determination of the optimal methods for rendering normal guinea pigs hypersensitive to mycobacterial antigens. For the purpose of determining the best route of sensitization, 2 groups of 12 normal guinea pigs were injected either intramuscularly or subcutaneously with 5 mg of H37Rv antigen. To determine whether injection by dual routes might enhance sensitization, a third group of 12 animals was injected both intramuscularly and subcutaneously with 5 mg of the antigen (total dosage=10 mg). Results of weekly skin tests of the animals in each group for 4 weeks following sensitization indicated that the intramuscular route was the least effective of the 3 routes tested (Table 1). This conclusion was based on the size of the skin reactions, as well as the central necrosis, displayed in the animals 3-4 weeks post-sensitization. Dual sensitization by both routes seemed to offer no particular advantage over the subcutaneous route alone.

The effect of various doses of sensitizing antigen on the progressive development of tuberculin skin reactivity was

TABLE 1

EFFECT OF ROUTE OF INJECTION ON THE DEVELOPMENT OF TUBERCULIN SKIN REACTIVITY IN GUINEA PIGS

No. of Weeks Post-Sensitization	Mean Skin Test Reactions at 24 Hr. (mm Induration)		
	Route of Sensitization (Dose)		
	Intramuscular (5 mg)	Subcutaneous (5 mg)	Intramuscular and Subcutaneous (10 mg)
1	9	15	15
2	14	18	18
3	16	20 CN[a]	20 CN
4	18	23 CN	23 CN

[a]Central necrosis.

studied next. Four groups of normal guinea pigs were injected subcutaneously with 0.5, 1.0, 5.0, or 10.0 mg of H37Rv antigen. Skin tests were performed at weekly intervals for 3 weeks following sensitization. Skin reactivity increased progressively in each group throughout the study (Table 2). At the first week following sensitization, the animals injected with 5 and 10 mg of antigen displayed stronger skin reactivity than those sensitized with the lower doses. However, no detectable differences were noted in the various groups at 2 and 3 weeks post-sensitization. The apparent uniformity of the response was further supported by the simultaneous development of central necrosis in the skin test sites in all groups of animals at 3 weeks post-sensitization. Most of the animals also developed a necrotic breakdown of the tissue at the site of injection of sensitizing antigen at approximately 3-4 weeks after sensitization. This response appeared at a time when skin reactivity was maximal.

Prior to studying delayed hypersensitivity to tuberculin *in vitro* it seemed desirable to attempt passive transfer experiments using the same methods of sensitization and inducement and collection of peritoneal exudates that would be used later for *in vitro* studies. To accomplish this, a preliminary experiment was performed in which sensitive donors with relatively weak skin reactivity (i.e., mean induration of 12 mm) were used. The results of passive transfer of viable and sonically disrupted cells to normal recipients were

TABLE 2

EFFECT OF SUBCUTANEOUS SENSITIZING DOSE OF H37Rv ANTIGEN ON THE DEVELOPMENT OF TUBERCULIN SKIN REACTIVITY IN GUINEA PIGS

Sensitizing Dose (mg)	No. of Animals	Mean Skin Test Reaction (mm Induration)		
		No. of Weeks Post-Sensitization		
		1	2	3
0.5	6	11	20	21 CN[a]
1.0	5	11	18	20 CN
5.0	6	15	20	21 CN
10.0	4	16	17	20 CN

[a]Central necrosis.

inconclusive in this experiment since the 24 hr skin test reaction of a normal control animal injected only with PPD was of the same size (approximately 5-7 mm induration) as those in the sensitive cell recipients. Results obtained in a second passive transfer experiment are presented in Table 3. Only intact viable cells from highly sensitive donors (i.e., mean induration of 22 mm) were capable of transferring tuberculin skin reactivity to normal recipients. Sonic-disrupted cells prepared from the same pooled cell suspension were incapable of transferring sensitivity. Neither intact or sonic-disrupted cells from donors with weaker skin reactivity (i.e., mean induration of 15 mm) nor viable cells from normal animals transferred sensitivity. It should be noted that all recipient animals in this experiment displayed transient skin reactions consisting of approximately 4-6 mm of erythema 24 hr after injection of PPD. These reactions disappeared by 48 hr with the exception of the recipients injected with viable cells from H37Rv-sensitized donors. The latter reactions were well indurated and remained so at the 48 hr reading.

The problem of transient skin reactions observed early in this study in normal guinea pigs that had been injected with 5 mcg PPD was considered to be the result of one of the following possibilities: (1) acquired or native low grade sensitivity of "normal" animals; (2) non-specific reactivity of normal animals to the skin test materials (PPD antigen and/or diluent); (3) non-specific trauma due to the injection

TABLE 3

PASSIVE TRANSFER OF DELAYED SKIN REACTIVITY TO TUBERCULIN BY MEANS OF VIABLE AND SONICALLY DISRUPTED PERITONEAL EXUDATE CELLS

Source of Donor Cells	Mean Skin Test Reactivity of Donors (mm Induration)	Mean Skin Reactivity of Recipient at 24 Hr (mm Induration)	
		Time of Skin Test After Cell Transfer	
		Day 3	Day 6
Sensitive (H37Rv):			
Intact Cells	22	15	10
Sonic Lysate		0	0
Sensitive (H37Ra):			
Intact Cells	15	0	0
Sonic Lysate		0	0
Normal:			
Intact Cells	0	0	0

of the skin test antigen. To study these possibilities, 34 newly acquired "normal" male albino guinea pigs were skin tested with 5 mcg PPD diluted in the commercial phosphate buffer provided by the manufacturer. All of these animals developed 3-5 mm areas of erythema after 24 hr which completely disappeared after 48 hr. To test for possible non-specific effects of the commercial diluent, 4 normal guinea pigs were shaved on both sides. Two of the animals were injected on one side with 5 mcg (0.1 ml) PPD prepared in commercial phosphate buffered diluent. Both animals were injected on the opposite side with 0.1 ml of the diluent alone. Two other normal animals received the same treatment with the exception that pyrogen-free 0.85% NaCl (PF-saline) was substituted for the phosphate buffer as the diluent. Results (Table 4) indicated that non-specific reactions occurred with PPD diluted in the commercial diluent as well as with the diluent alone. The use of PF-saline as a diluent eliminated the non-specific skin reactivity. To determine if non-specific skin reactivity was an isolated incident associated with a particular lot of the commercial diluent, 5 different lots of the commercial diluent were tested in the same manner. All of the diluents produced the same transient erythematous response. Again, a control animal injected with PF-saline showed no reaction. Throughout the remainder of this work non-specific skin reactivity was avoided by the use of PF-saline as a diluent for PPD.

TABLE 4

COMPARISON OF THE EFFECTS OF COMMERCIAL PHOSPHATE BUFFER AND PYROGEN-FREE SALINE AS DILUENTS FOR PPD ON NON-SPECIFIC SKIN REACTIVITY IN NORMAL GUINEA PIGS

Material Injected	Normal Animal No.	Skin Reactions (mm Induration) 24 Hr	48 Hr
PPD in Phosphate Buffer	1	4	2
	2	4	0
Phosphate Buffer	1	4	0
	2	4	0
PPD in Pyrogen-Free Saline	3	0	0
	4	0	0
Pyrogen-Free Saline	3	0	0
	4	0	0

Different incubation times have been used by various workers for recording the results of macrophage migration in the in vitro system. Most workers have recorded the results after incubation at 37 C for 24 and/or 48 hr. In order to determine the optimal incubation time for recording cell migrations in the in vitro system, 2 types of time studies were performed. In the first experiment (Table 5, Experiment A), migration of oil-induced peritoneal exudate cells from a single sensitive donor was followed by repeated readings of each tube after incubation at 37 C for 4, 8, 18, and 24 hr. Each value represents the mean of 8 different migration tubes. Results of a similar experiment are shown in Table 5, Experiment B. In this experiment, four identical sets of tubes were prepared with pooled sensitive peritoneal cells and each set was read at a single time interval. Results were similar in both experiments. Leukocyte migration increased progressively throughout the first 18 hr of the study. Maximal cell migration and inhibition of migration occurred after 18 hr incubation. Essentially no changes occurred after incubation for 24 hr.

In preliminary in vitro macrophage migration studies, concentrations of PPD (15, 20, and 30 mcg/ml) which had been used by other workers to detect in vitro sensitivity were found to inhibit the migration of normal cells and in some instances caused sloughing of macrophages from coverslips. The toxicity was thought to be due to the cresol derivatives

TABLE 5

EFFECT OF INCUBATION TIME ON THE DEVELOPMENT OF MAXIMAL CELL MIGRATION AND INHIBITION OF MIGRATION OF SENSITIVE PERITONEAL CELLS

Experiment	PPD Concentration (mcg/ml)	Mean Area of Migration (mm^2) Incubation Time (Hr) at 37C 4	8	18	24
A	0	132	200	339	339
	5	58	85	110	110
Percent Migration:		44	42	32	32
B	0	133	165	432	450
	5	70	70	108	134
Percent Migration:		53	42	25	30

used as preservatives in the commercial PPD preparation being used. For this reason, an endpoint titration method was developed to determine the least toxic concentration of PPD which would detect in vitro sensitivity. For this purpose, both normal and sensitive peritoneal cells, pooled from 2 donor animals, were tested in vitro in the presence of 0, 5, 10, 15, and 20 mcg/ml of a commercial PPD preparation. Results of this experiment are presented in Table 6. All concentrations of PPD inhibited the migration of both normal and sensitive cells to varying degrees. In general, the Mean Percent Migration decreased with increasing concentrations of PPD and the sensitive cells were inhibited more than were normal cells. Results of a similar titration experiment with lower concentrations of the same PPD preparation are presented in Table 7. These results were very similar to those seen in the previous experiment, although the pooled sensitive peritoneal cells used in this experiment were more sensitive to PPD. The migration of normal cells in the presence of 5 mcg PPD was very similar to that observed in the previous experiment. From these results, it appeared that 0.5 mcg of PPD was the optimal concentration for this particular lot. However, this was investigated further in a subsequent experiment wherein peritoneal cells from 3 highly sensitive guinea pigs were compared with cells from 3 moderately sensitive donors for their in vitro sensitivity to 0.5 and 5.0 mcg PPD. To accomplish this, peritoneal exudate cells were pooled from 3 highly

TABLE 6

IN VITRO ENDPOINT TITRATION OF HIGH CONCENTRATIONS OF A COMMERCIAL PPD PREPARATION CONTAINING PRESERVATIVES

Cell Type	Mean Percent Migration PPD Concentration (mcg/ml) 0	5	10	15	20
Normal	100	72	39	39	29
Sensitive	100	42	12	14	7

TABLE 7

IN VITRO ENDPOINT TITRATION OF LOW CONCENTRATIONS OF A COMMERCIAL PPD PREPARATION CONTAINING PRESERVATIVES

Cell Type	Mean Percent Migration PPD Concentration (mcg/ml) 0	0.5	1.0	2.5	5.0
Normal	100	91	78	75	68
Sensitive	100	41	37	23	18

sensitive donors (mean skin reactivity = 31 mm induration, with central necrosis) which had been dually sensitized both subcutaneously and intramuscularly with 5 mg H37Rv antigen 8 weeks previously. Cells were obtained from 3 moderately sensitive donors (mean skin reactivity = 20 mm induration) which had been sensitized 3 weeks previously by subcutaneous injection of 5 mg H37Rv antigen. Cells from the highly sensitive donors were inhibited *in vitro* by both 0.5 and 5.0 mcg concentrations of PPD while those from the less sensitive animals were inhibited by only the 5.0 mcg concentration (Table 8). Since 5 mcg of PPD was effective in detecting low grades of sensitivity *in vitro*, with minimal toxicity for normal cells, this concentration was used for subsequent experiments.

The results of a series of experiments in which cells from normal and sensitive animals were tested *in vitro* in the presence of 5 mcg of PPD containing preservatives are presented in Table 9. Typical patterns are shown in Figure 1. The cells from sensitive animals were markedly inhibited by 5 mcg of PPD/ml in comparison with those from normal animals. There was a statistically significant correlation between the inhibition of migration and the diameter of the skin reactions in this group of animals.

It had been postulated that the non-specific *in vitro* toxicity of commercial PPD preparations was due to the cresol derivatives which had been added as preservatives. The preservatives apparently had no detectable effect on skin tests,

TABLE 8

EFFECT OF PPD CONCENTRATION ON THE DETECTION OF
IN VITRO SENSITIVITY OF PERITONEAL CELLS
FROM SENSITIVE GUINEA PIGS

Cell Type	Mean Skin Reactivity (mm Induration)	Mean Percent Migration PPD Concentration (mcg/ml) 0.5	5.0
Normal	0	83	71
Highly Sensitive	31 CN[a]	43	19
Moderately Sensitive	20	76	32

[a]Central necrosis.

TABLE 9

PERCENT MIGRATION OF PERITONEAL CELLS FROM NORMAL AND TUBERCULIN-SENSITIVE GUINEA PIGS IN THE PRESENCE OF COMMERCIAL PPD CONTAINING PRESERVATIVES

Tuberculin-Sensitive			Normal	
Animal No.	Mean Skin Test (mm Induration)	Mean Percent Migration 5 mcg PPD	Animal No.	Mean Percent Migration 5 mcg PPD
S-1	32 CN[a]	10	N-1	72
S-2	16	42	N-2	68
S-3	16	46	N-3	84
S-4	20 CN	18	N-4	84
S-5	28 CN	6	N-5	71
S-6	26 CN	26	N-6	90
S-7	18	46	N-7	70
S-8	20 CN	32	N-8	113
S-9	16	35	N-9	101
S-10	20	38	N-10	75
S-11	18	37	N-11	77
S-12	20	32	N-12	74
S-13	20	30	N-13	76
S-14	18	41		
S-15	20 CN	43	Mean:	80
S-16	22 CN	12		
S-17	22 CN	11		
S-18	22 CN	20		
S-19	25 CN	11		
Mean:	21	28		

[a]Central necrosis.

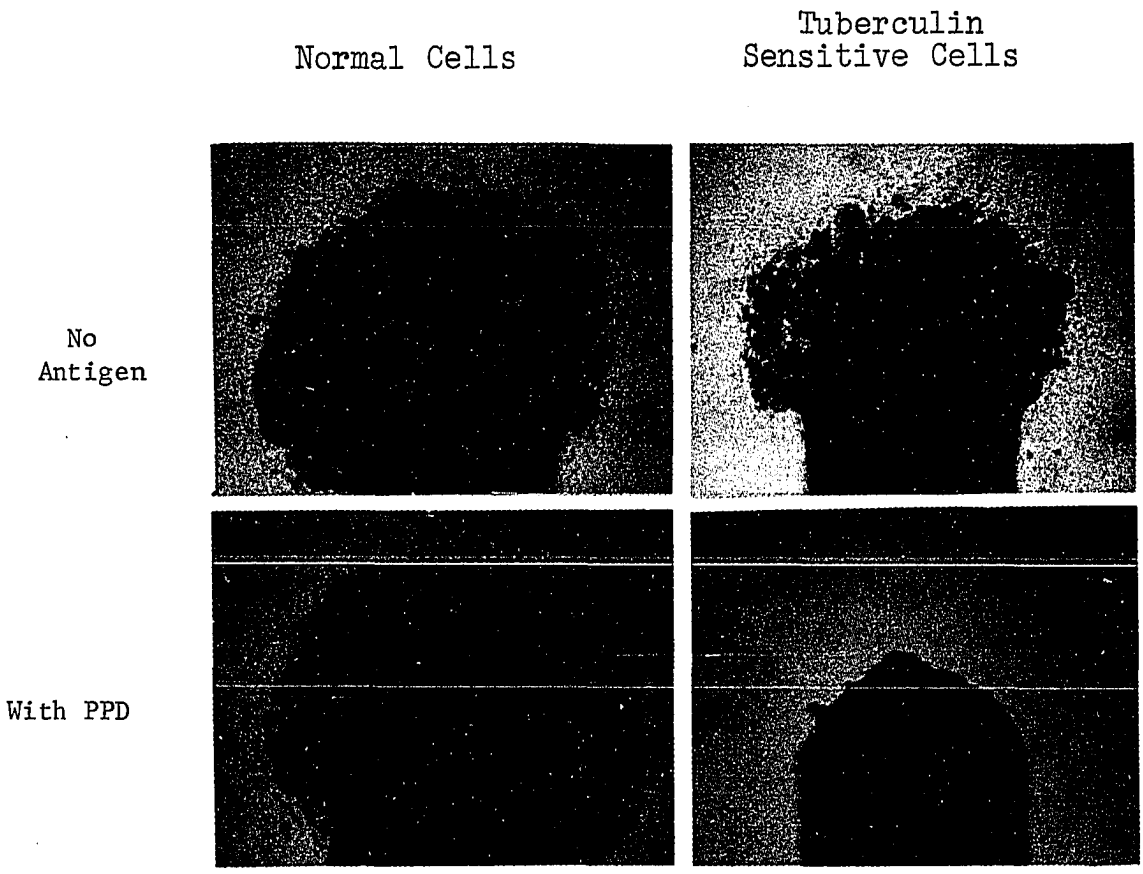

Figure 1.--Migration of peritoneal cells from normal and tuberculin-sensitive guinea pigs in the presence or absence of PPD.

since no reactions were seen when PPD was injected into the skin of normal guinea pigs. In order to explore this phenomenon further, a PPD preparation was obtained which contained no preservatives or additives. In these experiments, peritoneal cells from several normal and sensitive donors were tested individually in vitro with various PPD concentrations. Sensitive cells were obtained from donors that had been injected subcutaneously with 5 mg H37Rv antigen 3-4 weeks previously. Results of endpoint titration experiments using the PPD preparation without preservatives are summarized in Table 10. Non-specific inhibition of normal peritoneal cells was not observed in these experiments. However, there was a slight stimulation of migration of normal cells in the presence of all PPD concentrations tested. This was not a consistent finding, however, since in later experiments occasional inhibition of normal cell migration by high concentrations of PPD without preservatives was observed. Sensitive peritoneal cells were significantly inhibited by all concentrations of PPD tested. Although as little as 1.0 mcg of PPD was effective in detecting in vitro sensitivity, the results suggested that 5 mcg was the endpoint concentration for this particular PPD preparation. It should be noted that the endpoint concentrations for PPD preparations with and without preservatives were identical.

In order to compare the relative effectiveness of the PPD preparations, a series of experiments was done in which

TABLE 10

IN VITRO ENDPOINT TITRATION OF A PPD PREPARATION CONTAINING NO PRESERVATIVES

Cell Type	Animal No.	Mean Percent Migration PPD Concentration (mcg/ml) 1.0	2.5	5.0	10.0	15.0	20.0
Normal							
	N-1	127	119	126	126	104	--[a]
	N-2	143	124	157	119	102	--
	N-3	--	--	108	112	103	116
Sensitive							
	S-1	39	26	20	25	22	--
	S-2	26	30	31	30	30	--
	S-3	--	--	21	17	18	17
	S-4	--	--	29	28	21	20
	S-5	--	--	29	32	25	31
	S-6	--	--	10	10	10	13

[a]Not tested.

normal and sensitive peritoneal cells were tested *in vitro* in the presence of 5 mcg PPD with preservatives and 15 mcg PPD without preservatives. The results of this experiment, which are presented in Table 11, indicated that 5 mcg of PPD with preservatives was equivalent to 15 mcg of PPD without preservatives in inhibiting both normal and sensitive cell migration.

It seemed pertinent to determine the optimal time interval following sensitization for the detection of *in vitro* sensitivity to PPD. Also, it seemed desirable to determine whether there was any correlation between the development of tuberculin skin reactivity and *in vitro* cellular sensitivity. In a preliminary qualitative experiment, 16 normal guinea pigs were sensitized by intramuscular injection of 5 mg H37Rv antigen. Half of the animals were reserved for skin testing, while peritoneal cells from 2 representative animals of the remaining group were studied *in vitro* at weekly intervals by methods described previously. The results indicated that *in vitro* sensitivity was not demonstrable until 3 weeks post-sensitization whereas significant skin reactivity could be detected at one week. In a similar experiment, animals sensitized by the subcutaneous route were followed for a 5 week period. Appreciable skin reactivity was noted as early as 1-2 weeks post-sensitization, and it increased throughout the study (Table 12). However, significant cellular sensitivity could not be detected until 3 weeks post-sensitization. It

TABLE 11

COMPARISON OF THE EFFECTS OF PPD WITH AND WITHOUT PRESERVATIVES ON THE IN VITRO MIGRATION OF NORMAL AND TUBERCULIN SENSITIVE PERITONEAL CELLS

Cell Type	Animal No.	Mean Percent Migration: PPD With Preservatives (5 mcg/ml)	Mean Percent Migration: PPD Without Preservatives (15 mcg/ml)
Normal	N-1	76	75
	N-2	77	75
	N-3	76	75
	N-4	102	113
	Mean:	83	84
Sensitive	S-1	19	20
	S-2	14	12
	S-3	9	6
	S-4	29	22
	S-5	10	12
	S-6	15	19
	S-7	4	10
	S-8	20	15
	S-9	51	50
	S-10	7	11
	S-11	7	10
	S-12	13	16
	S-13	18	18
	Mean:	17	17

TABLE 12

DEVELOPMENT OF SKIN REACTIVITY AND IN VITRO CELLULAR SENSITIVITY IN GUINEA PIGS SENSITIZED BY THE SUBCUTANEOUS ROUTE

Cell Type	Weeks Post-Sensitization	Mean Skin Reactions (mm Induration)	Mean Percent Migration (5 mcg PPD/ml)
Sensitive	1	14 (5)[a]	83 (3)
	2	18 (5)	73 (3)
	3	28 (5) CN[b]	40[c] (3)
	4	28 (5) CN	42[c] (3)
	5	30 (5) CN	33[c] (2)
Normal			76 (4)

[a]Number of animals.

[b]Central necrosis.

[c]P = 0.05.

appeared from these results that *in vitro* cellular sensitivity to PPD developed more slowly than did skin reactivity.

It seemed possible that the slower development of *in vitro* cellular sensitivity, as compared with skin reactivity, might be due to one or more of several factors: (1) The concentration of PPD (containing preservatives) used for the detection of *in vitro* cellular sensitivity may have been too low to detect minimal levels of sensitivity during the induction phase; (2) excess sensitizing antigen (i.e., 5 mg H37Rv antigen) might have preferentially blocked the expression of *in vitro* cellular sensitivity by some unknown mechanism, which could presumably be similar to immune tolerance; (3) subtle differences may exist in the basic mechanisms of MIF-mediated sensitivity *in vitro* and dermal sensitivity which might explain the discrepancy in their time of development; and (4) either the number or distribution of immunocompetent lymphocytes in the sensitized animal during the early stages of induction may be such that the expression of skin reactivity precedes *in vitro* cellular sensitivity.

In order to test the possibility that excess amounts of sensitizing antigen had delayed the expression of *in vitro* sensitivity during the induction period, an experiment was designed to examine the effect of low and high doses of antigen on the development of *in vitro* sensitivity. For this purpose 3 groups of normal guinea pigs were sensitized with 1.0, 5.0, or 10.0 mg H37Rv antigen by the methods described

previously. Peritoneal cells from 2 selected non-skin tested donors from each group were tested at weekly intervals *in vitro* in the presence of 5 mcg PPD without preservatives. The remaining sensitive animals in each group were reserved for skin testing. The results of this experiment are presented in Table 13. The immune response of the animals in this experiment was not as uniform as it had been in previous studies. *In vitro* sensitivity was not detected until 2 weeks post-sensitization in 1 of 2 animals sensitized with 1.0 mg of antigen. Cells from 1 of 2 animals sensitized with 5 mg of mycobacterial antigen were inhibited at 1 week post-sensitization, but no *in vitro* sensitivity could be detected in animals from the same group after 2 weeks. Peritoneal cells obtained from 2 donors sensitized with 10 mg of antigen were first inhibited at 2 weeks post-sensitization. Cells from all 3 groups of animals were inhibited *in vitro* 3 weeks following sensitization. No definite pattern was observed in the induction of *in vitro* sensitivity with varying amounts of sensitizing antigen. It should be noted at this point that the relative uniformity of the immune response observed in previous experiments may have been fortuitous since Ferraresi, *et al*. (1969), in a similar study, reported variation in the time required for the induction of *in vitro* sensitivity to tuberculin in individual animals in a group of guinea pigs sensitized with mycobacterial antigen. This phenomenon was studied further in the next experiment in which a larger

TABLE 13

THE EFFECT OF SENSITIZING ANTIGEN DOSE ON THE DEVELOPMENT OF SKIN REACTIVITY AND *IN VITRO* CELLULAR SENSITIVITY

Dose of H37Rv Antigen (mg)	Test System	Day after Sensitization 7	14	21
1.0	Skin Reaction	4/4[a]	4/4	4/4
	In Vitro Inhibition	0/2[b]	1/2	2/2
5.0	Skin Reaction	4/4	4/4	4/4
	In Vitro Inhibition	1/2	0/2	2/2
10.0	Skin Reaction	4/4	4/4	4/4
	In Vitro Inhibition	0/2	2/2	2/2

[a]Number of animals with more than 12 mm induration/total.

[b]Number of animals with less than 70% *in vitro* migration/total.

number of animals was used. Two groups of 16 animals were sensitized subcutaneously with either 0.5 or 5.0 mg of H37Rv antigen. Peritoneal cells were collected at weekly intervals from 4 representative non-skin tested donors of each group and tested *in vitro* using 15 mcg/ml of PPD without preservatives by the methods described previously. The uniform early and progressive development of dermal sensitivity in both groups during the 3 week experimental period was again confirmed (Table 14). *In vitro* sensitivity first appeared at a time when skin reactivity was nearly maximal (i.e., 2 weeks after sensitization).

In a preliminary study of alveolar macrophages, lung washings containing 90-95% alveolar macrophages obtained from 7 donors with known dermal sensitivity (i.e., 20 mm induration) were studied *in vitro* for their cellular sensitivity to PPD by the macrophage migration method. The results indicated that alveolar cells from sensitive donors were not inhibited significantly by 5 or 20 mcg/ml of PPD (Table 15). Typical migration patterns are shown in Figure 2.

In the next experiment, the *in vitro* sensitivity of peritoneal cells and alveolar cells obtained from the same donors was compared. For this purpose, both peritoneal and alveolar cells were collected from 2 normal and 2 sensitive donors and tested *in vitro* by the methods described previously. The results of this experiment are presented in Table 16. Peritoneal cells from the sensitive animals were

TABLE 14

EFFECT OF HIGH OR LOW SENSITIZING ANTIGEN DOSAGE ON THE DEVELOPMENT OF *IN VITRO* CELLULAR SENSITIVITY

Dose H37Rv Antigen (mg)	Weeks Post-Sensitization	No. of Animals	Mean Skin Reaction (mm)	Mean Percent Migration[a]
0.5	1	4	10	98
	2	4	20	39
	3	4	22 CN[b]	31
5.0	1	4	12	96
	2	4	20	48
	3	4	24 CN	34
0	1	4	--[c]	102
	2	2	--	102
	3	2	--	102

[a] 15 mcg PPD/ml.

[b] Central necrosis.

[c] Not done.

TABLE 15

ABSENCE OF IN VITRO SENSITIVITY OF ALVEOLAR CELLS FROM GUINEA PIGS OF KNOWN SKIN REACTIVITY

Cell Type	Animal No.	PPD Concentration (mcg/ml)		Percent Migration
Sensitive	S-1	5		95
	S-2	5		89
	S-3	5		112
	S-4	5		106
	S-5	20		106
	S-6	20		82
	S-7	20		84
			Mean:	96
Normal	N-1	5		117
	N-2	5		116
	N-3	20		116
	N-4	20		85
			Mean:	109

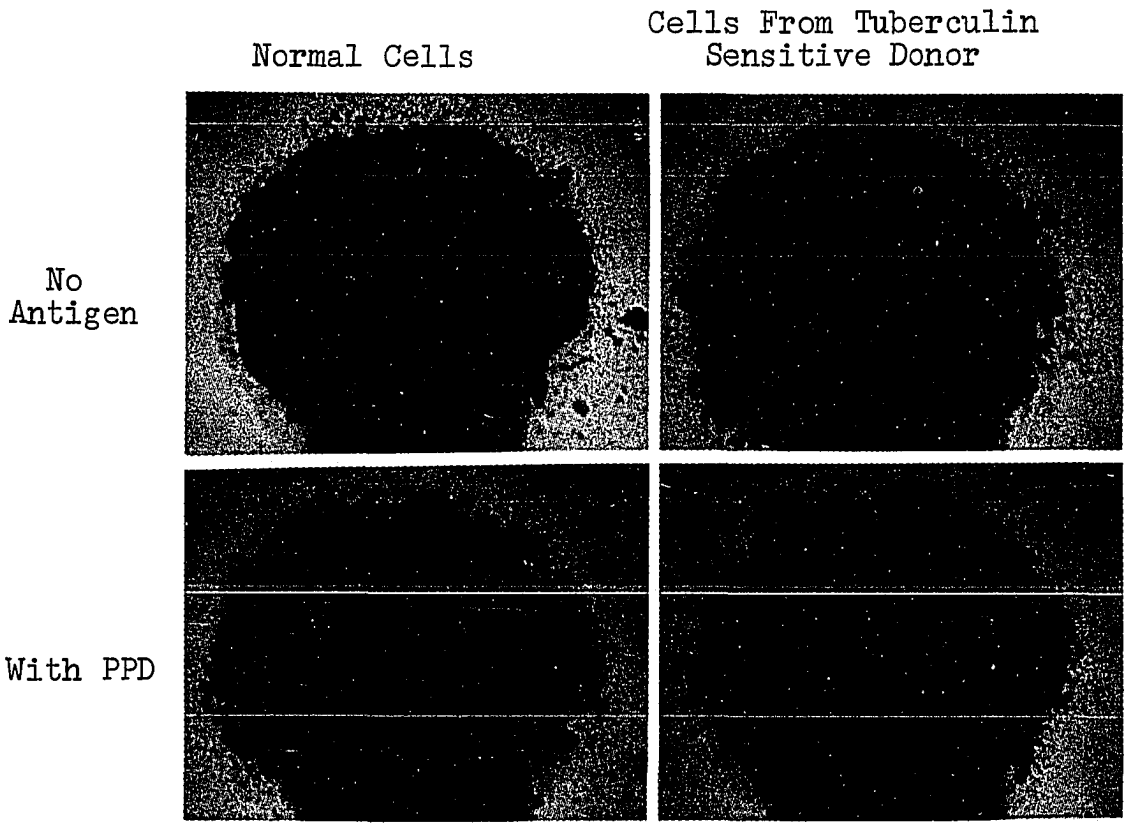

Figure 2. Migration of alveolar cells from normal and tuberculin-sensitive guinea pigs in the presence or absence of PPD.

TABLE 16

COMPARISON OF IN VITRO SENSITIVITY OF PERITONEAL AND ALVEOLAR CELLS OBTAINED FROM THE SAME DONORS

Cell Type	Skin Reactivity (mm Induration)	Cell Type	Percent Migration[a]
Sensitive	24 CN[b]	Alveolar	84
		Peritoneal	32
Normal		Alveolar	90
		Peritoneal	98
Sensitive	20 CN	Alveolar	106
		Peritoneal	51
Normal		Alveolar	112
		Peritoneal	102

[a] 5 mcg PPD/ml.

[b] Central necrosis.

significantly inhibited by PPD, while alveolar cells from the same animals were not. The migration of peritoneal and alveolar cells from the normal animals was not inhibited by PPD.

The failure to demonstrate alveolar cell sensitivity in animals injected by the subcutaneous route led to the investigation of a different method of sensitization. Heise et al. (1968) reported the induction of alveolar cell sensitivity to PPD in the guinea pig by a specific method of sensitization. These workers attributed the cellular sensitivity as being due to a cell-bound antibody which was cytophilic for the alveolar macrophage, rather than to Migration Inhibitory Factor. An experiment was performed in which 4 normal animals were sensitized with H37Rv antigen by the method of Heise et al. (1968), as described previously. Alveolar cells were collected from the 4 sensitized guinea pigs and from 4 normal animals 4 weeks after sensitization and tested in vitro for sensitivity to 15 mcg PPD. Oil-induced peritoneal exudates were collected from the sensitive alveolar cell donors and studied in vitro by the same method. Skin tests which were performed 24 hr prior to cell collection revealed that the mean skin reactivity of the sensitive animals was 24 mm induration, with central necrosis (Table 17). Although skin reactivity was pronounced in all of the sensitive animals, only minimal inhibition of alveolar cell migration was detected in vitro. Peritoneal cells obtained from 2 of the donor animals were inhibited significantly in the presence of

TABLE 17

EFFECT OF SENSITIZATION BY THE METHOD OF HEISE ET AL. (1968) ON IN VITRO SENSITIVITY OF GUINEA PIG ALVEOLAR MACROPHAGES

Cell Type	No. of Animals	Mean Skin Reactions (mm Induration)	Mean Percent Migration[a]
Sensitive		24 CN[b]	
Alveolar	4		76
Peritoneal	2		30
Normal			
Alveolar	4		94
Peritoneal	2		98

[a]15 mcg PPD/ml.

[b]Central necrosis.

PPD. These results suggested that pronounced alveolar cell sensitivity could not be demonstrated by the techniques used, even though skin reactivity and peritoneal cell sensitivity were clearly demonstrated.

Differential leukocyte counts on Wright's stained smears of all cell suspensions obtained from guinea pig lung washings revealed 90-95% alveolar macrophages. Virtually no lymphocytes were present. It seemed possible that the lack of alveolar cell sensitivity in the present study might be due to the absence of sufficient quantities of immunocompetent lymphocytes. For this reason attempts were made to induce the infiltration of immunocompetent cells (i.e., lymphocytes) into the guinea pig lungs by several different methods.

It seemed possible that the failure to induce alveolar cell sensitivity to PPD in earlier experiments may have been due to the route of sensitization used. Subcutaneous injection of antigen presumably did not promote direct exposure of lung tissue to the mycobacterial antigen. This assumption was supported by the observation that the gross appearance of lungs from sensitive animals was indistinguishable from that of normal animals in being free of inflammation or granulomatous lesions. In addition, there were no inflammatory cells in the lung washings from these animals. These observations suggested that the lung tissue must come in direct contact with the sensitizing antigen before cellular sensitivity can develop. To accomplish this, two groups

of normal animals were injected intravenously in the foreleg with 1.5 mg or 2.5 mg of mycobacterial antigen by the methods described previously. A third group of animals was injected intracardially with 2.5 mg of the same antigen preparation. A final group of animals was injected by the intrapleural route directly into the lobe of the lung with CFA containing 2.5 mg of heat-killed H37Rv cells. Alveolar cells were collected 4 weeks post-sensitization and tested in vitro for sensitivity to 15 mcg PPD by the methods described previously. Due to technical difficulties, alveolar cells were not obtained from some of the sensitive donors. The results presented in Table 18 indicate that intravenous and intrapleural injection of antigen were ineffective in inducing alveolar cell sensitivity, although dermal sensitivity was demonstrable in certain of these animals. Alveolar cells from 3 animals sensitized by the intracardial route were inhibited slightly by PPD in vitro. Differential counts revealed that all of the lung cell suspensions from both sensitized and normal donors contained 90-95% alveolar macrophages and 5-10% lymphocytes. The gross appearance of the lungs of the animals injected with mycobacterial antigen directly into the lungs was definitely altered. Extensive adhesions were observed in the chest cavity. Nodules of fibrosis covered the outer surface. Extensive fibrosis was evident throughout the lung tissue and the lobes were firm rather than spongy in consistency. Based on the gross pathological changes, it was surprising that the

TABLE 18

EFFECT OF SENSITIZATION BY THE INTRACARDIAL, INTRAVENOUS, OR INTRAPLEURAL ROUTES ON THE IN VITRO SENSITIVITY OF GUINEA PIG ALVEOLAR MACROPHAGES

Route (Dose H37Rv Antigen)	No. of Animals	Mean Skin Reactivity (mm Induration)	Mean Percent Migration[a]
Intravenous			
1.5 mg	2	--[b]	95
2.5 mg	3	16 CN[c]	90
Intracardial			
2.5 mg	3	18 CN	72[d]
Intrapleural			
2.5 mg	3	--	86
Normal	5	--	94

[a] 15 mcg PPD/ml.

[b] Not done.

[c] Central necrosis.

[d] P=.05.

cells collected from the alveolar spaces of these lungs showed no increase in the number of inflammatory cells. Minor changes in gross appearance were noted in the lungs of animals sensitized by the intravenous and intracardial routes. In general, this consisted of no more than a few solid nodules in one or more lobes of the lung. These results suggested that the alveolar cells were obtained from the remaining healthy areas of the lung in these animals. Since the areas of pathology in the lungs were completely walled off by fibrosis, inflammatory cells probably could not be obtained by the methods of collection used.

The failure to demonstrate high levels of alveolar cell sensitivity in the earlier experiments led to attempts to induce the release of Migration Inhibitory Factor (MIF) *in vivo* by intracardiac challenge of sensitive animals. It seemed possible that systemic challenge of the animals with PPD might induce the release of MIF in sufficient quantities to passively sensitize alveolar macrophages *in vivo*. In the first experiment, sensitive (subcutaneous injection of 5 mg H37Rv antigen) and normal animals were injected intracardially with 25 mcg of PPD 24 hr prior to *in vitro* assay of their alveolar macrophages by the methods described previously. In a second experiment, 2 groups of sensitive animals which had been injected either intravenously or intracardially with 2.5 mg of mycobacterial antigen in 0.85% NaCl were injected intracardially with 1 mg PPD prior to *in vitro* studies on

their alveolar cells. The skin reactions of all the animals measured 15 mm induration or greater. Results of both experiments are presented in Table 19. Under the conditions of these experiments, the challenge of sensitive animals with PPD had no effect on in vitro sensitivity of their alveolar macrophages.

It seemed possible that previous attempts to induce MIF-mediated sensitivity in the alveolar cell population of the guinea pig lung were unsuccessful due to the presence of inadequate numbers of immunocompetent lymphocytes. Therefore, attempts were made to demonstrate alveolar cell sensitivity by mixing the cells with peritoneal cell suspensions from sensitive animals which were known to contain sufficient quantities of immunocompetent cells. In the first experiments, the in vitro migration of various peritoneal cell mixtures from normal and tuberculin-sensitive animals was tested in the presence of PPD. Cell mixtures containing 10, 40, and 50% sensitive and 90, 60, and 50% normal cells, respectively, were inhibited in vitro in the presence of PPD (Table 20). The degree of inhibition of migration increased with the increase in the number of sensitive cells in the cell mixtures.

In the next experiment, peritoneal exudate cells and alveolar cells from the same sensitive donors were collected, mixed in various proportions, and tested in vitro against 5 mcg/ml PPD by the methods described previously. Results of this experiment are presented in Table 21. None of the 100%

TABLE 19

EFFECT OF INTRACARDIAL PPD CHALLENGE OF SENSITIVE GUINEA PIGS 24 HOURS PRIOR TO CELL COLLECTION ON THE IN VITRO SENSITIVITY OF THEIR ALVEOLAR CELLS

Cell Type	No. of Animals	Dosage (mcg)	Mean Percent Migration[a]
Sensitive	2		100
		25	
Normal	2		96
Sensitive	2		96
Sensitive	2	1000	89
Normal	2		86

[a]20 mcg PPD/ml.

TABLE 20

IN VITRO MIGRATION OF MIXTURES OF NORMAL AND SENSITIVE PERITONEAL CELLS IN THE PRESENCE OF PPD

Experiment	Percent Peritoneal Cell Mixture		Mean Percent Migration[a]
	Sensitive	Normal	
A	0	100	88
	100	0	10
	10	90	29
B	0	100	113
	100	0	43
	10	90	65
	40	60	56
	50	50	47

[a] 5 mcg PPD/ml.

TABLE 21

IN VITRO MIGRATION OF MIXTURES OF PERITONEAL AND ALVEOLAR CELLS OBTAINED FROM THE SAME TUBERCULIN SENSITIVE GUINEA PIGS

Animal No.	Percent Cell Mixture		Mean Percent Migration[a]
	Peritoneal	Alveolar	
1	0	100	101
	100	0	23[b]
	10	90	111
	20	80	94
2	0	100	102
	100	0	17[b]
	10	90	83
	20	80	70[b]
3	0	100	103
	100	0	52[b]
	20	80	99
	50	50	88
4	0	100	100
	100	0	36[b]
	20	80	103

[a] 15 mcg PPD/ml.

[b] P=0.05.

alveolar cell suspensions was inhibited by PPD, while all of the 100% peritoneal cell suspensions were inhibited. Cell mixtures from 3 of the 4 donors (animals no. 1, 3, and 4) were not inhibited. However, in one instance, a cell mixture from a sensitive animal (animal no. 2), which consisted of 20% peritoneal and 80% alveolar cells was apparently inhibited significantly. It should be noted that the in vitro sensitivity of the peritoneal cells from this animal was greater than that displayed by the other guinea pigs used in this experiment. These results suggested that peritoneal cells from only highly sensitive donors were capable of influencing the migration of alveolar cells. Further, only low levels of sensitivity were detected in alveolar cell mixtures as compared to corresponding mixtures of peritoneal cells.

In order to compare the sensitivity of cell mixtures containing different cell types, an experiment was performed in which peritoneal cells from 2 highly sensitive guinea pigs were pooled and mixed in various proportions with either alveolar cells from the same donor animals or with peritoneal cells pooled from 2 normal animals. The cell mixtures were then tested in vitro by the methods described previously. The results of this experiment (Table 22) revealed that mixtures of sensitive and normal peritoneal cells were much more sensitive to PPD than were corresponding mixtures of the same sensitive peritoneal cells and sensitive alveolar cells. Significant inhibition of migration occurred with all of the

TABLE 22

COMPARISON OF THE IN VITRO MIGRATION OF SENSITIVE PERITONEAL CELLS MIXED WITH EITHER NORMAL PERITONEAL OR SENSITIVE ALVEOLAR CELLS

Percent Cell Mixtures			
Sensitive Peritoneal	Normal Peritoneal	Sensitive Alveolar	Mean Percent Migration[a]
0	0	100	102
0	100	0	96
100	0	0	12[b]
5	0	95	87
10	0	90	82
20	0	80	70[b]
5	95	0	65[b]
10	90	0	53[b]
20	80	0	37[b]

[a] 15 mcg PPD/ml.

[b] P=0.05.

sensitive-normal peritoneal cell mixtures tested. Only the mixture of 20% sensitive peritoneal and 80% sensitive alveolar cells was inhibited significantly, although the other alveolar cell mixtures appeared to be inhibited slightly. As observed previously, the degree of inhibition was dependent on the concentration of sensitive cells in the cell mixtures.

Based on the previous experiments, it appeared that it might be possible to enhance the low levels of sensitivity of peritoneal-alveolar cell mixtures by increasing the number of immunocompetent lymphocytes in the cell mixture. An experiment was performed in which a purified sensitive peritoneal lymphocyte suspension, pooled from 4 sensitive animals and obtained by the Petri dish adsorption method described previously, was mixed with an equal quantity of alveolar cells, obtained from the same donors, prior to in vitro assay. In this experiment, a 50% mixture of sensitive lymphocytes and sensitive alveolar macrophages was inhibited to a degree which was comparable to that seen with the sensitive cell control (Table 23). These results, although preliminary in nature, confirmed that alveolar cells are capable of expressing cellular sensitivity to PPD when the number of immunocompetent cells in the mixture is high.

Most workers have reported that humoral antibody in the form of serum obtained from sensitive animals, has no detectable effect on MIF-mediated in vitro sensitivity of peritoneal cells. However, Heise et al. (1968) reported that

TABLE 23

IN VITRO MIGRATION OF A MIXTURE OF POOLED PURIFIED PERITONEAL LYMPHOCYTES AND ALVEOLAR MACROPHAGES OBTAINED FROM THE SAME SENSITIVE GUINEA PIGS

Percent Cell Mixture		
Peritoneal	Alveolar	Mean Percent Migration[a]
0	100	95
100[b]	0	30
50[c]	50	46

[a]5 mcg PPD/ml.

[b]Pooled sensitive peritoneal cells prior to adsorption.

[c]Peritoneal lymphocyte suspension after adsorption.

immune sera from BCG-sensitized guinea pigs enhanced the inhibition of migration of alveolar cells from both normal and BCG-sensitive animals *in vitro*. In addition, it seemed possible that the low levels of sensitivity of alveolar cells from animals sensitized by various methods in previous experiments might be enhanced by immune serum. In order to test this possibility an experiment was performed in which normal and sensitive peritoneal or alveolar cells were tested *in vitro* in tissue culture medium which contained 15% pooled normal or immune serum. Immune serum had little effect on the migration of normal or sensitive peritoneal cells (Table 24). *In vitro* sensitivity of alveolar cells from 2 animals sensitized by the method of Heise *et al*. (1968) was enhanced by the addition of pooled immune serum to the culture medium. The migration of normal alveolar cells was again stimulated by the immune serum, as was the case in the previous experiment with normal peritoneal cells.

In the previous experiments, sera from animals that had been sensitized 3 weeks previously were pooled and used as a source of immune serum. In the next experiment sera from animals sensitized subcutaneously with 5 mg H37Rv antigen were collected at 3 weekly intervals following sensitization and examined for their effect on the *in vitro* sensitivity of normal and sensitive alveolar cells. Alveolar cells were collected and pooled from 2 normal and 2 sensitive animals (sensitized by subcutaneous injection of 5 mg H37Rv antigen). *In*

TABLE 24

EFFECT OF IMMUNE SERUM ON THE IN VITRO SENSITIVITY OF PERITONEAL AND ALVEOLAR CELLS FROM NORMAL OR SENSITIVE GUINEA PIGS

		Mean Percent Migration[a]	
		Type of Serum[b]	
Cell Type	Cell Source	Normal	Immune
Peritoneal	Normal	90	104
	Sensitive	36 16	33 11
Alveolar	Normal	99 87	117 90
	Sensitive	63 75	43 64

[a] 15 mcg PPD/ml.

[b] Fifteen percent normal or immune serum in EMEM.

vitro tests were performed in media containing either 15% normal or 15% sensitive guinea pig serum collected at 1, 2, or 3 weeks post-sensitization. From the results of this experiment (Table 25), it was apparent that immune serum collected at 1 and 2 weeks post-sensitization had no effect on the migration of normal or sensitive alveolar cells. However, the immune serum collected at 3 weeks post-sensitization clearly enhanced the *in vitro* sensitivity of alveolar cells from sensitive animals. A minimal effect on normal alveolar cell migration was observed with the same serum. These results suggested that alveolar cells from sensitive animals are much more responsive to the effects of a humoral factor, which occurs in immune serum collected 3 weeks after sensitization, than are alveolar cells from normal animals. Moreover, alveolar cells differ from peritoneal cells in that immune serum appears to have no effect on the *in vitro* sensitivity of the latter.

In order to determine the effect of intraperitoneal injection of cortisone on MIF-mediated cellular sensitivity of oil-induced peritoneal cells, 2 experiments were performed. In the first experiment, 2 sensitive animals were each injected intraperitoneally with either 0.85% NaCl or 25, 50, or 100 mg cortisone acetate 24 hr prior to collection of cells. *In vitro* assay of their peritoneal cells was accomplished by the methods described previously. The results of this experiment are presented in Table 26. No significant differences

TABLE 25

EFFECT OF IMMUNE SERA COLLECTED AT VARIOUS TIMES AFTER SENSITIZATION ON THE IN VITRO SENSITIVITY OF NORMAL AND SENSITIVE ALVEOLAR CELLS

Alveolar Cell Donor	Mean Percent Migration[a]			
	Weeks Post-Sensitization			
	0	1	2	3
Normal	97	99	95	82
Sensitive	89	84	94	47

[a] 15 mcg PPD/ml; 15% serum in EMEM.

TABLE 26

EFFECT OF INTRAPERITONEAL INJECTION OF CORTISONE ACETATE ON THE IN VITRO SENSITIVITY OF PERITONEAL CELLS

Cell Type	No. of Animals	Intraperitoneal Injection	Mean Percent Migration[a]
Normal	1	None	91
Sensitive	2	Saline	18
	2	25 mg cortisone	28
	2	50 mg cortisone	10
	2	100 mg cortisone	58[b]

[a] 15 mcg/ml. PPD.

[b] P=0.05.

were detected in the *in vitro* sensitivity of peritoneal cells from saline injected controls and those treated with 25 and 50 mg of cortisone. However, 100 mg of cortisone partially suppressed *in vitro* sensitivity when compared to sensitive controls. In a second experiment, cells from 4 sensitive animals which had been injected intraperitoneally with 100 mg of cortisone prior to assay again demonstrated partial suppression of *in vitro* sensitivity to PPD (Table 27). Intraperitoneal injection of cortisone also reduced the total yields of exudate cells as compared to saline-treated controls, although no affect on differential cell counts was noted. These results suggested that peritoneal cells exposed *in vivo* to high concentrations of cortisone were affected in their *in vitro* sensitivity to PPD.

Experiments were also performed to determine the effect of *in vitro* cortisone treatment of peritoneal cells on macrophage migration and MIF-mediated sensitivity to PPD. For this purpose, pooled normal and sensitive peritoneal cells were incubated with 0, 100, or 200 mcg/ml concentrations of cortisone prior to *in vitro* assay. The results of this experiment are presented in Table 28. Treatment of normal and sensitive cells with cortisone resulted in stimulation of macrophage migration in the absence of PPD. This was evident by the values obtained for the mean areas of migration of the cortisone-treated cells in the absence of PPD as compared to the untreated controls. The values for the mean area of

TABLE 27

EFFECT OF INTRAPERITONEAL INJECTION OF CORTISONE ACETATE ON THE *IN VITRO* SENSITIVITY OF OIL INDUCED PERITONEAL CELLS TO PPD

Cortisone Treatment (mg)	No. of Animals	Mean Percent Migration[a]
None	2	32
100	4	67[b]

[a] 5 mcg PPD/ml.

[b] P=0.05.

TABLE 28

EFFECT OF INCUBATION OF PERITONEAL CELLS WITH CORTISONE ACETATE ON SUBSEQUENT *IN VITRO* SENSITIVITY

Cell Type	Cortisone Treatment (mcg/ml)	Mean Area Migration (mm^2) PPD Concentration (mcg/ml) 0	10	Mean Percent Migration
Normal	0	275	174	63
	100	416	119	28
	200	689	118	17
Sensitive	0	577	272	47
	100	704	217	31
	200	812	257	44

migration of normal cells migrating with PPD were very similar. The stimulatory effect on migration noted in the absence of PPD was overcome in the presence of antigen. As a result of the stimulatory effect of cortisone on the migration of macrophages in the absence of antigen, the Mean Percent Migration values obtained were highly variable. Thus cortisone treatment definitely stimulated macrophage migration, but evaluation of MIF-mediated sensitivity was not possible.

A second experiment was performed using the same methods, with the exception that after incubation with cortisone the cells were resuspended in tissue culture medium containing the same concentrations of cortisone which had been used for incubation. Results of this experiment indicated that prolonged cortisone treatment of sensitive peritoneal cells inhibited the migration of macrophages in the absence of PPD (Table 29). Treatment with 200 mcg cortisone apparently inhibited the migration of sensitive macrophages nonspecifically either in the presence or absence of PPD, as compared to non-treated controls. The migration of sensitive peritoneal cells which had been treated with 100 mcg of cortisone were not inhibited to the same degree in the presence of 5 and 10 mcg PPD as compared to untreated controls. The Mean Percent Migration values were interpreted as indicating that the 100 mcg concentration of cortisone was effective in suppressing the expression of MIF-mediated sensitivity to PPD.

TABLE 29

EFFECT OF PRETREATMENT AND SUBSEQUENT INCUBATION OF PERITONEAL CELLS WITH CORTISONE ACETATE ON THEIR IN VITRO SENSITIVITY

Cell Type	Cortisone Treatment (mcg/ml)	Mean Area Migration (mm^2)			Mean Percent Migration	
		0[a]	5	10	5	10
Normal	None	359	357	253	99	70
Sensitive	None	351	143	83	41	24
	100	277	229	171	83[b]	62[b]
	200	145	46	54	32	37

[a]PPD concentration (mcg/ml).

[b]P=0.05.

The effect of cortisone treatment on the skin reactivity and *in vitro* sensitivity to PPD of fully sensitive guinea pigs was studied next. The effect of cortisone treatment of tuberculin-sensitive guinea pigs with 5 daily intramuscular injections of 10 mg or 20 mg of cortisone acetate (total dosage of 50 mg and 100 mg, respectively) on the Mean Total Cell Yields and the Percent Mononuclear Cells of oil-induced peritoneal exudates is presented in Table 30. The Mean Total Cell Yield of exudates collected from sensitive animals treated with 100 mg of cortisone acetate were depressed nearly three-fold when compared to untreated controls. However, cortisone-treated normal animals were not similarly depressed in their cell yields. Treatment of sensitive or normal animals with 50 mg of cortisone appeared to have no effect on the percent mononuclear leukocytes. However, the percentage of mononuclear cells was depressed in both groups treated with 100 mg cortisone. The effect of cortisone treatment on delayed skin reactivity and *in vitro* sensitivity to tuberculin is presented in Table 31. The mean skin reactivity of the untreated sensitive group was the same on day 0 and on day 5. The reactions of all of these animals were well indurated and displayed central necrosis. Both groups of sensitive animals treated with cortisone demonstrated depressed skin reactivity after 5 days of cortisone injections. Although the skin test sites were moderately indurated, no central necrosis was observed in the

TABLE 30

EFFECT OF INTRAMUSCULAR INJECTION OF TUBERCULIN SENSITIVE GUINEA PIGS WITH CORTISONE ON TOTAL CELL YIELDS AND PER CENT MONONUCLEAR CELLS OF PERITONEAL EXUDATES

Group	No. Animals	Mean Total Cell Yield (ml)	Mean Percent Mononuclear Cells
Sensitive:			
Untreated	5	0.21	86
Cortisone 50 mg	5	0.15	96
Cortisone 100 mg	5	0.08	50[a]
Normal:			
Untreated	2	0.19	84
Cortisone 50 mg	1	0.13	94
Cortisone 100 mg	2	0.12	45[a]

[a] $P=0.05$.

TABLE 31

EFFECT OF INTRAMUSCULAR INJECTION OF TUBERCULIN SENSITIVE GUINEA PIGS WITH CORTISONE ON DELAYED SKIN REACTIVITY AND IN VITRO SENSITIVITY TO TUBERCULIN

Group	No. Animals	Mean Skin Reactivity (mm) Day 0	Day 5	Mean Percent Migration[a]
Sensitive:				
Untreated	5	21 CN[b]	21 CN	25[c]
Cortisone 50 mg	5	20 CN	15	34[c]
Cortisone 100 mg	5	21 CN	15	39[c]
Normal:				
Untreated	2	--[d]	--	92
Cortisone 50 mg	1	--	--	96
Cortisone 100 mg	2	--	--	92

[a] 15 mcg PPD/ml.

[b] Central necrosis.

[c] P=0.05.

[d] Not done.

cortisone-treated groups. Peritoneal exudate cells obtained from the same cortisone-treated and untreated sensitive donors were significantly inhibited by PPD in vitro as compared with normal controls. A slight increase in the Mean Percent Migration was noted in the cortisone-treated sensitive animals as compared to untreated controls. The differences, however, were not statistically significant.

The effect of intramuscular cortisone treatment on the induction of delayed hypersensitivity in normal guinea pigs was also studied. The effect of cortisone treatment on the Mean Total Cell Yields and the Percent Mononuclear Cells of peritoneal exudates from each group are presented in Table 32. A depression in both the total cell yields and the percent mononuclear cells, as compared with untreated controls, was noted in the groups treated with 500 mg of cortisone. The reduction in the number of mononuclear cells included a decrease in both lymphocytes and macrophages. Peritoneal exudates from the other cortisone-treated groups were not effected either qualitatively or quantitatively. The effects of cortisone treatment on the induction of dermal reactivity and in vitro cellular sensitivity to PPD are shown in Table 33. Although all animals developed progressively increasing dermal sensitivity throughout the 3 week period, the skin reactivity but not MIF production of the animals treated with less than 500 mg of cortisone was depressed as compared to untreated controls. Central necrosis was displayed by

TABLE 32

EFFECT OF INTRAMUSCULAR INJECTION OF TUBERCULIN SENSITIVE GUINEA PIGS WITH CORTISONE ACETATE DURING THE INDUCTION PHASE ON TOTAL CELL YIELDS AND PER CENT MONONUCLEAR CELLS OF PERITONEAL EXUDATES

Group	No. Animals	Mean Total Cell Yield (ml)	Mean Percent Mononuclear Cells
Sensitive:			
Untreated	5	0.13	99
Cortisone 25 mg	5	0.12	95
Cortisone 200 mg	5	0.10	98
Cortisone 500 mg	5	0.07[a]	72[a]
Normal:			
Untreated	4	0.15	99
Cortisone 25 mg	2	0.12	97
Cortisone 200 mg	2	0.14	98
Cortisone 500 mg	2	0.09[a]	75[a]

[a] $P=0.05$.

TABLE 33

EFFECT OF INTRAMUSCULAR CORTISONE TREATMENT ON THE INDUCTION OF DELAYED SKIN REACTIVITY AND *IN VITRO* SENSITIVITY TO TUBERCULIN

Group	No. Animals	Mean Skin Reactivity (mm) No. of Days Post-Sensitization 7	14	21	28	Mean Percent Migration[a]
Sensitive:						
Untreated	5	7	16	18 CN[b]	20 CN	33
Cortisone 25 mg	5	5	12	15	15	29
Cortisone 200 mg	5	2	11	14	15	30
Cortisone 500 mg	5	4	10	12 E[c]	11 E	83[d]
Normal:						
Untreated	4	-	--	--	--	104
Cortisone 25 mg	2	-	--	--	--	92
Cortisone 200 mg	2	-	--	--	--	94
Cortisone 500 mg	2	-	--	--	--	89

[a] 15 mcg/ml PPD.

[b] Central necrosis.

[c] Erythema without induration.

[d] P=0.05.

animals in the untreated control group at 2 and 3 weeks following sensitization. Central necrosis was not observed in any of the cortisone-treated animals. This critical observation is best interpreted as evidence that 25 and 200 mg of cortisone did not suppress induction of delayed hypersensitivity. The group of animals treated with 500 mg cortisone displayed pronounced suppression of skin reactivity and MIF-mediated sensitivity. The skin reactions in these animals were not indurated and only erythematous areas were measurable. Histological sections of biopsied skin test sites from this group of animals revealed a significant depression in the mononuclear cell infiltration as compared to untreated sensitive animals. Results of *in vitro* studies revealed that peritoneal cells from the group of animals treated with 500 mg cortisone were not inhibited significantly *in vitro* in the presence of 15 mcg PPD as compared with untreated normal and sensitive controls. Although skin reactivity was depressed in the groups treated with 25 and 200 mg of cortisone, no suppression of their peritoneal cell sensitivity *in vitro* was observed. In order to confirm these results, treatment with 500 mg of cortisone over a 25 day period was repeated in a second group of animals. Results of this experiment confirmed the previous findings. Both skin reactivity and *in vitro* cellular sensitivity were suppressed as compared to untreated controls (Table 34).

TABLE 34

EFFECT OF LONG TERM CORTISONE TREATMENT ON THE INDUCTION OF DELAYED SKIN REACTIVITY AND *IN VITRO* SENSITIVITY TO TUBERCULIN

Group	No. Animals	Mean Skin Reactivity 7	14	21	Mean Percent Migration[a]
Sensitive:					
Untreated	5	14	18	21 CN[b]	40
Cortisone 500 mg	5	12	12	16 E[c]	74[d]
Normal:					
Untreated	2	--	--	--	90
Cortisone 500 mg	2	--	--	--	89

[a] 15 mcg/ml PPD.

[b] Central necrosis.

[c] Erythema without induration.

[d] P=0.05.

Thus, it would appear that low doses of cortisone suppress non-specific components of the inflammatory response associated with delayed hypersensitivity. Higher doses (500 mg) suppress, in addition, the formation of cells capable of releasing MIF in response to specific antigenic challenge.

CHAPTER IV

DISCUSSION

Methods for rendering guinea pigs sensitive to tuberculin are highly variable. Such factors as source, species, and strain of mycobacterial antigen; dose of immunogen and route of injection; and type and method of preparation of complete Freund's adjuvant (CFA) appear to differ with each worker, according to reports in the literature. The lack of standardized procedures is due, in part, to the apparent ease with which the guinea pig can be sensitized to mycobacterial antigen, regardless of the method used. Leskowitz and Waksman (1960) reported, however, that the toe-pad and intradermal routes were most effective for production of delayed hypersensitivity in rabbits to bovine serum albumin incorporated in CFA. The intramuscular, subcutaneous, and intraperitoneal routes were less effective and the intravenous route was totally ineffective. Various methods have been used for the sensitization of guinea pig donors of cells for <u>in vitro</u> macrophage migration studies. In the present study, subcutaneous injection of 5 mg of H37Rv in FA was found to be as effective as sensitization by dual routes (subcutaneously and

intramuscularly) in rendering normal guinea pigs sensitive to PPD, as measured by the progressive development of dermal sensitivity. The intramuscular route was less effective. It should be noted that the animals used in this study failed to display detectable signs of Arthus reactivity, as determined by the time required for the development of maximal skin reactions and by the examination of sections of biopsied skin test sites. However, since the two phenomena have been shown to be rather difficult to distinguish, when expressed simultaneously in the same animal, it is possible that both were present to some degree in these animals.

The effect of the dose of immunogen may be a critical factor in inducing the delayed type response. Salvin (1958) found that very small doses of protein antigen produced a transient state of pure delayed hypersensitivity, in the absence of detectable antibody, which persisted for only 2-3 days. Uhr et al. (1957) reported that pure delayed hypersensitivity occurred 5-12 days after injection of normal guinea pigs with small amounts of antigen-antibody complexes precipitated in antibody excess and incorporated in CFA. Thus it would appear that guinea pigs injected with microgram amounts of protein antigen are more likely to develop a pure state of delayed hypersensitivity, while milligram amounts induce vigorous and protracted antibody formation (Gell and Benacerraf, 1961). Raffel and Newell (1958) performed experiments using relatively high doses of protein antigen (0.5-1.0 mg

ovalbumin) in an attempt to prove that the reactions seen in the earlier studies were different from classic delayed hypersensitivity of the tuberculin type. The latter is known to be of long duration, while the former has been of a transient nature in most studies. Leskowitz and Waksman (1960), however, using high immunizing doses of protein antigen (2.5 and 25 mg BSA) in rabbits, reported the production of both delayed hypersensitivity and humoral antibody to varying degrees. It should be noted that the use of high sensitizing doses of heat-killed mycobacterial cells has become customary, most probably due to the desire by most workers to induce high levels of delayed sensitivity in experimental animals, regardless of the simultaneous development of humoral antibody. The amount of undenatured tuberculoprotein remaining after heat-killing of mycobacterial cells raises a question as to whether the actual quantity of immunogenic material injected is in excess, even when it is used in milligram amounts. Recent studies by Larson et al. (1969) and Marcus (1970), however, indicated that microgram quantities of mycobacterial antigen were capable of producing delayed hypersensitivity to tuber-sensitivity to tuberculin in guinea pigs. Efforts in the present study to determine the effect of relatively high doses of heat-killed mycobacterial cells (0.5-10.0 mg) on the induction of tuberculin skin reactivity revealed that greater reactivity was produced with the higher sensitizing doses (5.0 or 10 mg) at 1 week after sensitization. However, no

differences were detected in the reactivity of animals sensitized with low and high doses at 2 and 3 weeks following sensitization. These results suggested that the quantities of sensitizing antigen tested were all in excess of that required for sensitization.

Although a direct relationship between in vivo passive transfer of reactivity to tuberculin and the in vitro correlates of delayed hypersensitivity remains to be shown, it is apparent that they are all elicited by immunocompetent lymphoid cells. Chase (1965) emphasized the importance of the degree of sensitivity of donors of viable sensitive lymphoid cells, as well as the quantity of cells used, as prerequisite to successful passive transfer of delayed-type hypersensitivity in the guinea pig. In the present study, peritoneal cells from weakly sensitive donors failed to transfer tuberculin sensitivity in vivo, while those from highly sensitive animals did. Cell lysates prepared by sonic disruption of samples of the same cell suspension failed to confer dermal sensitivity to PPD to normal recipients. These results agree with those of Chase (1953). It was assumed that the cells to be used in subsequent in vitro studies were also capable of transferring delayed-type hypersensitivity in vivo. It should be noted that the less sensitive donors in this experiment were sensitized with 5 mg of heat-killed mycobacteria (strain H37Ra) while the highly sensitive animals were immunized with an

equal quantity of H37Rv antigen which had been prepared by similar methods. These results confirm the variations in potency of various antigen preparations.

The transient, non-specific skin reactions observed following PPD skin testing of normal recipients in passive transfer experiments *in vivo* were found to be due to the commercial phosphate buffer diluent rather than the tuberculin itself. Substitution of pyrogen-free saline for the commercial buffer as a diluent eliminated this problem. These reactions may have been a result of the unique sensitivity of the guinea pig skin to some component of the commercial diluent, since there appear to be no reports of similar non-specific reactions in humans skin tested with the same materials. It should be noted that David *et al.* (1964a) prescribed the use of a pyrogen-free saline to dilute PPD for skin testing guinea pigs. The non-specific reactions noted in the present study were predominantly erythematous, with little induration. Stetson (1959) noted that the skin reaction resulting from the intradermal injection of bacterial endotoxin was very similar to the tuberculin reaction in timing and in gross and histological appearance. Although the non-specific erythema seen in the present study did not mimic the tuberculin reaction, it seems possible that minute quantities of endotoxin contaminants could elicit such a response. A more probable cause for the non-specific skin reactions might have been the

presence of preservatives (0.5% phenol) in the commercial diluent. This possibility arose again later when PPD containing preservatives was found to be highly toxic for normal guinea pig peritoneal cells in vitro.

The in vitro model introduced by George and Vaughan (1962) for studying delayed hypersensitivity prescribed the use of Mackaness-type incubation chambers for the capillary tube migration system. It seemed desirable to evaluate the possibility of utilizing an incubation chamber which would be less complicated and expensive than the Mackaness chamber. For this purpose, a short-style Leighton type tissue culture tube was evaluated (Leu and Patnode 1968). This modification was adapted successfully to the in vitro system and seemed to offer definite advantages in ease of handling, ready availability, and economy.

Preliminary studies designed to evaluate various aspects of the in vitro capillary tube method revealed that the optimal time for recording the inhibition of macrophage migration was 18-24 hr at 37 C. Essentially no changes in migration occurred after 24 hr incubation. David (1968), using Mackaness-type chambers, reported that inhibition of migration from capillary tubes could be observed in a few hours, although the tests were read after 18-24 hr. It appears that MIF-mediated sensitivity is fully expressed within the 18 hr period of incubation, regardless of the chamber used.

Most workers have used relatively high concentrations of PPD (15-50 mcg/ml) for detection of *in vitro* sensitivity of peritoneal cells. David *et al.* (1964a) reported that, occasionally, cells from normal animals were inhibited in the presence of 15 or 30 mcg/ml concentrations of PPD. In the present investigation 15, 20, and 30 mcg/ml concentrations of a commercial PPD preparation were found to be inhibitory and cytotoxic for normal guinea pig peritoneal macrophages. Sensitive cells were inhibited to a greater extent than normal cells with the same concentrations of PPD. However, an endpoint titration method was devised to determine the concentration of PPD which would inhibit migration of sensitive cells with minimal inhibition of normal cells. It was found, in an initial experiment, that as little as 0.5 mcg/ml of PPD inhibited cells from highly sensitive animals. However, this concentration did not inhibit cells from less sensitive animals. Therefore, 5 mcg/ml was chosen as the endpoint concentration. The cytotoxicity of the commercial PPD was apparently due to the presence of purified cresols (0.06 mg/40 mcg PPD tablet) in the commercial preparation, since in subsequent experiments a batch of PPD which contained no preservatives was found to be non-toxic for normal cells. Waksman and Matoltsy (1958) reported that antigens containing germicides as preservatives were non-specifically toxic for peritoneal cell cultures *in vitro*. Heilman *et al.* (1960) observed similar cytotoxic effects of antigens containing

preservatives. Bloom and Bennett (1966) described the use of PPD, free of additives, for in vitro macrophage migration studies.

David (1968) reported that the degree of macrophage migration inhibition was related to the dose of antigen in the incubation chambers. He demonstrated a dose response curve in which increasing concentrations of bovine gamma globulin antigen (BGG) resulted in increasing inhibition of macrophages from BGG-sensitive animals. Results of a similar experiment in the present study indicated that essentially no dose response was demonstrable with increasing concentrations of PPD (without preservatives). No major differences were noted in the degree of inhibition of cells from highly sensitive animals in the presence of 1.0-20 mcg/ml of PPD. Similarly, Ferraresi et al. (1969) were unable to demonstrate an in vitro dose response in guinea pigs sensitized to diphtheria toxoid except in animals with moderate skin reactivity.

These results suggested that the antigen concentrations used in the present in vitro studies were in excess and that a dose response may be demonstrable with lower concentrations of PPD. It became evident from these results that the response to increasing doses of PPD containing preservatives, observed in previous experiments, was due to the non-specific cytotoxic effects of the preservatives rather than the specific inhibition by antigen. In a subsequent experiment, 5 mcg/ml of PPD containing preservatives was found to be no

more toxic for normal macrophages than was 15 mcg/ml of PPD without preservatives. Moreover, inhibition of migration of sensitive cells in the presence of these two antigen preparations was found to be nearly identical. It was concluded that the 5 mcg/ml concentration of PPD containing preservatives was equivalent to 5-20 mcg/ml concentrations of the PPD preparation free of preservative.

David et al. (1964a) suggested that in vitro inhibition of migration of peritoneal macrophages from tuberculin-sensitive guinea pigs could not be directly correlated with the diameter of the skin reaction in the same animal, although there was, in general, a close parallelism. In the present study, there was a statistically significant correlation between the diameters of the tuberculin skin reactions and the degree of inhibition of peritoneal macrophage migration in vitro. In a few cases, cells from animals with pronounced skin reactivity were not as markedly inhibited as were cells from animals with equally high skin sensitivity. Contrary to the findings of David et al. (1964a), however, there were no instances in which cells from animals with minimal skin reactivity were markedly inhibited in vitro. It should be noted that these workers utilized guinea pigs that had been sensitized 2-9 months prior to testing, whereas the animals used in the present study had been sensitized only 3-6 weeks previously. Another possible explanation for the disparity in results could be the difference in concentrations of PPD used

for skin testing and *in vitro* assays. David *et al.* (1964a) used 10 mcg of PPD for skin testing and 15 mcg/ml of PPD for *in vitro* tests, while in the present study 5 mcg of PPD was used for both *in vivo* and *in vitro* testing. It seems possible that a higher degree of correlation between skin reactivity and *in vitro* sensitivity may exist only during the earlier stages of immunization. Ferraresi *et al.* (1969) noted less correlation between delayed-type skin reactivity and *in vitro* sensitivity when sensitivity was declining (i.e., 3-4 months after sensitization) than earlier in the sensitization process. The use of the same concentration of PPD for both *in vivo* and *in vitro* testing may also increase the possibility of detecting equivalent levels of sensitivity.

Although there is strong presumptive evidence that MIF-mediated *in vitro* sensitivity is an expression of delayed hypersensitivity, no attempts had been made to correlate quantitatively the induction and expression of dermal reactivity and *in vitro* MIF-mediated sensitivity to PPD. Results of preliminary studies suggested that detectable skin reactivity preceded *in vitro* cellular sensitivity to PPD (Leu and Patnode, 1968). Significant inhibition of cell migration was not detected until skin reactivity became maximal (3-5 weeks post-sensitization). Histological studies of biopsied skin test sites from these animals revealed that the reactions were characteristic of delayed-type hypersensitivity. However, it seemed possible that low levels of MIF-mediated

sensitivity were not detected early in the sensitization process due to the inability to use higher concentrations of PPD (containing cytotoxic preservatives) for *in vitro* assays. In subsequent studies, using higher concentrations of a non-toxic PPD preparation, significant inhibition of migration was detected with peritoneal cells from some individual sensitive animals as early as 1-2 weeks after sensitization. As was found in the initial experiments, however, significant cellular sensitivity to PPD was detected in most animals only when pronounced skin reactivity was evident in a parallel group of animals reserved for skin testing. Other workers have reported similar results. Ricci *et al.* (1969) noted that *in vitro* cellular sensitivity to PPD in most animals was significant only at 3 weeks post-sensitization, concurrent with the appearance of intense tuberculin skin reactivity. In a similar study, Ferraresi *et al.* (1969) confirmed that skin reactivity developed earlier and more rapidly than did *in vitro* sensitivity. They also noted that, once sensitivity was well established in guinea pigs sensitized with diphtheria toxoid or human gamma globulin in CFA, *in vivo* and *in vitro* reactivities became more closely correlated quantitatively than early in the induction phase of sensitization.

It seemed possible that an excess dose of sensitizing antigen could preferentially block the development of *in vitro* cellular sensitivity by some mechanism similar to immune tolerance. Based on this premise one would have to

presume that subtle differences exist between the basic mechanisms involved in skin reactivity and *in vitro* sensitivity. In further studies, however, no significant differences were detected in the time of development of *in vitro* cellular sensitivity in animals sensitized with 0.5, 5.0, or 10 mg H37Rv antigen. Based on a recent report by Marcus (1970), it became evident that the dose of sensitizing antigen may play an important role in the early induction of MIF-mediated sensitivity simultaneously with tuberculin skin reactivity. In carefully controlled studies, it was demonstrated that inbred guinea pigs sensitized by multiple intradermal injections of minimal doses (total of 12.5 or 25 mcg) of dead mycobacteria developed concurrent skin reactivity and *in vitro* sensitivity as early as 6-7 days following sensitization. The methods of sensitization employed appeared to be similar to those used to induce Jones-Mote type sensitivity (Crowle 1962). However, since the sensitivity was not transient, it is probable that a state of classical delayed hypersensitivity was established. The use of inbred strains of guinea pigs may also have been a contributing factor to the early and uniform development of *in vitro* sensitivity. In other studies, including the present work, outbred strains of guinea pigs were used. All workers have reported occasional, early detection of *in vitro* sensitivity in individual animals within a sensitized group. Therefore, it appears possible, that early uniform expression of both *in vivo* and *in vitro* sensitivity might be enhanced by

the use of inbred strains of animals selected for their high susceptibility to sensitization. The reason for the delay in the expression of *in vitro* sensitivity in studies performed using excess sensitizing antigen remains to be seen. One possible explanation is that there may be an uneven distribution of immunocompetent lymphoid cells during the early stages of sensitization. For example, more immunocompetent lymphocytes may be available from the peripheral blood for eliciting skin reactivity (Kosunen *et al.*, 1963) than from the local lymph nodes, which have been shown to be the primary source of small lymphocytes in guinea pig peritoneal exudates (Turk and Polak, 1967). This concept is supported by the *in vitro* studies of Carpenter (1963) who reported that macrophage sensitivity could be detected in explants of tuberculin-sensitive guinea pig lung and focal lymph nodes (adjacent to sites of injection of immunizing antigen), 5 days after sensitization, when delayed skin reactions were first demonstrable. However, macrophages from explants of distant lymph nodes and spleen were not inhibited until 2-4 weeks after immunization.

Since the alveolar macrophage is the predominant leukocyte type found in the alveolar spaces of the lung, it may be the primary line of defense against pulmonary infections with the tubercle bacillus in man. Tsuji *et al.* (1964) reported successful passive transfer of tuberculin sensitivity in rabbits using either viable cells or cell lysates of

alveolar macrophages. Dunn (1966) reported similar positive findings in local passive transfer experiments when alveolar macrophages from tuberculin-sensitive guinea pigs were mixed with antigen prior to intradermal injection into normal recipients. The methods of Myrvik et al. (1961) and Maxwell et al. (1964) were utilized to collect guinea pig alveolar macrophages in this study. These workers had reported yields of almost pure alveolar macrophages which contained virtually no lymphocytes, in the rabbit and guinea pig, respectively. Similar cell yields were obtained from the guinea pig lung using these methods in the present study. Preliminary studies revealed that alveolar macrophages obtained from tuberculin-sensitive guinea pigs, which displayed pronounced skin sensitivity and in vitro peritoneal exudate cell sensitivity to PPD, were not inhibited in vitro. It was concluded from microscopic observations of stained smears that the inability to demonstrate MIF-mediated sensitivity was due to the virtual absence of immunocompetent lymphocytes in alveolar cell suspensions (Bloom and Bennett, 1966). Subsequent attempts to induce MIF-mediated sensitivity in vivo by using various methods of sensitization were largely unsuccessful. Heise et al. (1968) demonstrated in vitro sensitivity of alveolar macrophages from BCG-sensitive guinea pigs. The macrophage inhibition was clearly demonstrated to be mediated by a cytophilic antibody (probably IgG of γ-2 type) and not by MIF. Bartfeld et al. (1969) reported that they had demonstrated in

vitro sensitivity of alveolar cells obtained from guinea pigs sensitized with CFA. They reported that their guinea pig lung washings contained 20% lymphocytes. However, in further studies (Bartfeld and Atoynaton, 1969) it was found that the inhibiting activity was due to a cytophilic factor other than MIF. In the present work, attempts to induce alveolar cell sensitivity by the methods of Heise et al. (1968) failed to produce convincing results. Only slight inhibition of alveolar macrophage migration was detected in vitro, while pronounced dermal and peritoneal cell sensitivity was demonstrable in the same donor animals. It should be noted that, although the same procedures were used, H37Rv antigen was used instead of BCG as the sensitizing antigen in this study. It seems possible that subtle differences in antigen preparations might account for the disparity in these results. The number and variability of potential immunogenic components of these different strains of mycobacteria has been demonstrated by Parlett and Youmans (1956).

Cohn and Weiner (1963) noted that the intravenous injection of rabbits with BCG suspended in Tween 80 resulted in infiltration of mononuclear cells into the alveolar spaces of the lung. In the present work, similar attempts to sensitize animals by the intravenous, intracardial, and intrapleural routes, were made with the hope of inducing the infiltration of immunocompetent lymphocytes into the lung tissue. Results of these experiments were surprising since MIF-mediated

alveolar cell sensitivity was not demonstrated in most of these animals in spite of pronounced granulomatous responses in their lung tissue. Even in the presence of gross pathology, lung washings from these animals contained virtually no lymphocytes. It was concluded that the fibrotic tissue response to mycobacterial antigen had effectively walled off the effected areas, so that only cells in the alveolar spaces from the remaining healthy areas of the lung were collected by the washing procedures used. Low levels of *in vitro* sensitivity were detected in the animals injected intracardially with mycobacterial antigen. Differential counts revealed only 5-10% lymphocytes in alveolar cell suspensions from these animals. The degree of alveolar macrophage inhibition was similar to that obtained with animals sensitized by the method of Heisé *et al*. (1968). Carpenter (1963) observed *in vitro* inhibition of macrophage migration from lung explants of tuberculin-sensitive guinea pigs. Since explants of lung tissue contain both lymphocytes and macrophages, it would appear that these results emphasized the necessity for the presence of immunocompetent lymphoid cells to elicit MIF-mediated alveolar macrophage inhibition. In studies utilizing chromosome markers to trace their migration, Pinkett *et al*. (1966) reported that most alveolar macrophages are of bone marrow (mesodermal) origin. Recently, Bowden *et al*. (1969), utilizing cells labelled with ^{3}H-thymidine in radiation chimeras, reported that the progeny of a stem cell in the

bone marrow was transported via the blood to the lung interstitium where it undergoes a division-maturation. Differentiation of the stem cells into mature alveolar macrophages occurs in the alveoli. It would appear from these results that the alveolar macrophage is not thymus dependent (Williams and Waksman, 1969).

Attempts to demonstrate MIF-mediated alveolar cell sensitivity by mixing sensitive and normal cell suspensions in various proportions prior to in vitro assay revealed that the alveolar macrophage was less susceptible to antigen specific inhibition by this method than was the peritoneal macrophage. Mixtures of sensitive and normal peritoneal cells were markedly inhibited in the presence of PPD. Generally, the degree of inhibition increased with higher concentrations of sensitive cells in the cell mixtures. Furthermore, the degree of sensitivity conferred on normal peritoneal cells appeared to be more pronounced when they were mixed with cells from highly sensitive donors. However, when alveolar and peritoneal cell mixtures from the same sensitive donors were tested for their in vitro sensitivity to PPD, no significant macrophage inhibition was detected, with one exception. In this instance only minimal inhibition was observed. In a subsequent study it was found that mixtures of sensitive and normal peritoneal cells were inhibited to a greater extent by PPD in vitro than were corresponding mixtures of the same sensitive peritoneal cells and alveolar cells from the same donors. Only when a

purified, sensitive lymphocyte suspension (separated from peritoneal exudates) was mixed with equal numbers of alveolar macrophages was pronounced inhibition of migration detected. It was apparent from these studies that the alveolar macrophage was less susceptible to the effects of MIF-mediated sensitivity in vitro than its peritoneal counterpart. Pollock et al. (1970) reported that inhibition of migration was more difficult to demonstrate with alveolar cells than with peritoneal cells when they were mixed with sensitive lymphocytes. They attributed this difference to the fact that alveolar macrophages migrated to a greater extent than did peritoneal macrophages. Gesner and Howard (1967) have pointed out distinct biochemical differences between these two macrophage types in that the alveolar macrophage utilizes both oxidative phosphorylation and aerobic metabolism as an energy source, while the peritoneal macrophage depends solely on anaerobic glycolysis as a source of energy. The alveolar cell is generally more active metabolically in producing much higher levels of lysosomal enzymes than is the peritoneal cell. Based on these observations, it seems possible that the difficulties encountered, in the present study, in demonstrating pronounced in vitro cellular sensitivity of the alveolar macrophage might be due to the inherent metabolic properties of this cell type. If MIF acts as a non-specific pharmacologic effector molecule in inhibiting the macrophage (Dumonde et al., 1969), it is possible that the unique metabolic

properties of the alveolar macrophage make it less susceptible to this type of mechanism. Alternatively, if macrophage inhibition is dependent on an antigen specific MIF effector molecule which is cytophilic for the macrophage (Amos and Lachman, 1970), the disparity in the expression of inhibition of alveolar and peritoneal macrophages could be due to differences in the nature of receptor sites on their cell surfaces.

It has been reported that immune serum may either enhance the in vitro sensitivity of sensitive cells (Carpenter, 1963) or passively sensitize normal cells to PPD (Bloom and Bennett, 1966 and Heise et al. 1968). In the present study, immune serum was found to have no effect on normal or sensitive peritoneal macrophage inhibition. However, in subsequent studies pooled serum from tuberculin-sensitive animals was found to markedly enhance the in vitro sensitivity of alveolar cells obtained from animals sensitized by the intradermal or subcutaneous routes. No similar enhancing effect was observed with alveolar cells from normal animals. Furthermore, no effect was seen with alveolar cells obtained from sensitive animals when the cells were incubated with immune serum collected at 1 and 2 weeks post-sensitization, while serum collected at 3 weeks after sensitization induced significant inhibition of migration. It appears that the sensitive alveolar cells may have had a low level of immunologic potential which was undetected in vitro until it was enhanced by the addition of the serum factor. Since the degree of alveolar

cell inhibition seen in the presence of immune serum was pronounced, as compared to the moderate lymphocyte-mediated inhibition observed in previous experiments, it seems possible that this inhibition was due to a cytophilic antibody similar to that described by Heise et al., 1968. In addition, only alveolar cells, and not peritoneal cells, from sensitive animals were effected by the serum factor.

The effect of cortisone on in vivo and in vitro sensitivity was examined in the present studies. Intraperitoneal injection of 100 mg (but not 25 or 50 mg) of cortisone acetate was found to partially suppress MIF-mediated sensitivity of peritoneal exudate cells. Johnson and Sherago (1964) reported partial suppression of in vitro sensitivity to PPD of peripheral blood leukocytes from guinea pigs injected with 25 mg cortisone by the intraperitoneal route. They also noted a concomitant lymphopenia. In the present study, no reduction in the number of mononuclear cells in peritoneal exudates was observed, although the total cell yields were reduced as compared to control animals.

Leahy and Morgan (1952) reported that prolonged cortisone treatment (100 mcg/ml) protected sensitive spleen explant macrophages from the cytotoxic effects of PPD in vitro. In the present study, short term incubation of normal or sensitive peritoneal cells with 100 or 200 mcg cortisone/ml prior to in vitro assay resulted in stimulation of macrophage migration in the absence of PPD. Samples of the same

cortisone-treated cells were inhibited in the presence of antigen and therefore no significant effect on *in vitro* sensitivity to PPD was demonstrated. Leahy and Morgan (1952) and Heilman (1969) reported that low concentrations of cortisone stimulated *in vitro* macrophage migration from spleen explants. In the present study, a subsequent experiment revealed that sensitive cells which had been preincubated with 100 mcg cortisone/ml, followed by continued *in vitro* treatment with the same concentration of cortisone incorporated into the tissue culture medium, were significantly suppressed in their MIF-mediated sensitivity to PPD as compared to untreated sensitive and normal control cells. Similar treatment of sensitive cells with 200 mcg cortisone/ml was found to be cytotoxic. These results suggest that prolonged incubation of sensitive cells with relatively high concentrations of cortisone acetate may suppress MIF-mediated sensitivity. However, the specific mechanism by which cortisone blocks the macrophage inhibition phenomenon remains to be determined. Based on the report of Hinz *et al*. (1970), lymphocyte transformation was suppressed in a patient on high doses of prednisone as a therapeutic regimen. Cortisone also has been shown to stimulate macrophage migration and protect against the cytotoxic effects of endotoxin (Heilman, 1969), presumably due to stabilization of lysosomal and perhaps other cell membranes (Weissman and Thomas, 1962). Therefore the suppression by cortisone of MIF-mediated sensitivity *in vitro*

may be due to effects on both the lymphocyte and the macrophage.

Most immunosuppressive agents are effective only when they are administered prior to the administration of immunogen (preinductive phase) or during the sensitization process (inductive phase). Generally, these agents are ineffective once the immune response is established (Gabrielsen and Good, 1967). In the present work, intramuscular administration of cortisone acetate to previously sensitized guinea pigs depressed tuberculin skin reactivity, total cell yields, and the percent mononuclear cells in peritoneal exudates, but failed to suppress in vitro sensitivity to PPD. These results do not negate the possibility that long term administration of cortisone to fully sensitive animals would result in immunosuppression. The relative ease with which parenteral administration of immunosuppressive agents suppresses delayed-type skin reactivity is well documented (Gabrielsen and Good, 1967, Gowans and McGregor, 1965). However, the possibility exists that this type of suppression is due only to the antiphlogistic and anti-inflammatory effects of these agents rather than their specific suppression of immunocompetency. The results of the present study support this concept. Based on the previous observation, prolonged incubation of sensitive cells in vitro with high concentrations of cortisone was required to block MIF-mediated sensitivity. Therefore, it seems logical to assume that prolonged administration of high,

doses of cortisone would be required to suppress immunocompetency in fully sensitive animals.

Studies on the effect of cortisone on the induction of tuberculin sensitivity revealed that only animals that had been treated continuously with high dosages (500 mg total) of cortisone, beginning prior to sensitization and continuing through the period of induction (i.e., approximately 3 weeks), were suppressed in both dermal reactivity and MIF-mediated sensitivity *in vitro*. Animals that were treated in the same manner with lower doses of cortisone (200 mg total) displayed depression of skin reactivity without suppression of *in vitro* cellular sensitivity. There have been numerous reports in the literature of failure to suppress *in vitro* sensitivity in the presence of depressed dermal reactivity following the administration of therapeutic doses of immunosuppressive agents. Cummings and Hudgins (1952) failed to block passive transfer of tuberculin hypersensitivity in the guinea pig by prolonged treatment (21 days) of sensitive donors with low doses (42 mg total) of cortisone acetate beginning at the time of sensitization. Depression of tuberculin skin reactivity was pronounced in the sensitive peritoneal cell donors. Lurie *et al.* (1949) reported that treatment of tuberculin-sensitive rabbits with estrogen suppressed dermal but not cellular sensitivity. Axelrod *et al.* (1963) noted that although pyridoxine deficiency depressed tuberculin skin sensitivity, both in the inductive and productive phases of BCG-sensitization in guinea

pigs, the ability of their peritoneal cells to transfer sensitivity passively was not compromised. Recently, Phillips and Zweiman (1970) reported that treatment of tuberculin-sensitive guinea pigs with 6-mercaptopurine suppressed skin reactivity to PPD, but had no effect on MIF-mediated sensitivity. These observations are in agreement with the present results and appear to support the concept that many immunosuppressives may act primarily as anti-inflammatory agents. It is interesting to note that immunosuppressive agents which are lympholytic or antimetabolites have been reported to be effective in suppressing delayed-type immune competency. For example, Cummings et al. (1955) noted that total body x-irradiation diminished tuberculin skin reactions and impaired the ability of sensitive leukocytes to transfer sensitivity passively. Bloom et al. (1964) reported that treatment of guinea pig peritoneal leukocytes in vitro with mitomycin C blocked their ability to transfer tuberculin skin reactivity to normal recipients. Results of the present study suggest that cortisone acetate may be relatively less potent in suppressing specific delayed-type immunocompetency than it is in depressing the non-specific inflammatory response.

CHAPTER V

SUMMARY

For *in vitro* studies of delayed hypersensitivity utilizing the inhibition of macrophage migration technique, the method of George and Vaughan was successfully modified by the adaptation of a short-style, Leighton-type tissue culture tube as an incubation chamber. This modification resulted in typical and reproducible cell migration and appeared to offer the advantages of simplicity, ease in handling, ready availability, and economy.

Experiments designed to determine the optimal route of sensitization of guinea pigs to tuberculin, through the use of heat-killed tubercule bacilli in Freund's incomplete adjuvant, revealed no significant differences between animals dually sensitized by the subcutaneous and intramuscular routes and those sensitized by either route alone. The dose of mycobacterial antigen used appeared to have no affect on the development of maximal tuberculin skin reactivity. *In vivo* passive transfer of sensitivity to tuberculin was accomplished with viable, but not sonic-disrupted, peritoneal cells from donors sensitized by the subcutaneous route.

Transient skin reactivity observed following injection of normal recipient animals with commercial PPD preparations was found to be due to a non-specific irritant in the commercial diluent. Substitution of pyrogen-free saline as a diluent eliminated this problem.

Commercial PPD preparations were also found to be cytotoxic for guinea pig peritoneal cells in vitro due to the presence of cresol preservatives. An endpoint titration method was devised for determining an effective, non-toxic concentration of PPD which would be suitable for in vitro macrophage migration studies. Subsequent in vitro studies, utilizing a PPD preparation without preservatives, eliminated the problem of non-specific macrophage inhibition.

Quantitative studies, designed to compare the time required for induction of in vivo and in vitro tuberculin hypersensitivity, revealed that the development of dermal sensitivity preceded detectable in vitro cellular sensitivity to PPD. Further studies showed that a few individual animals displayed low levels of early, in vitro sensitivity concomitantly with the development of skin reactivity. However, in most animals MIF-mediated sensitivity followed the development of skin reactivity and generally was demonstrable only at a time when the dermal response was maximal. No significant differences were seen in the induction of in vivo and in vitro sensitivity in animals sensitized with doses of mycobacterial antigen ranging from 0.5 mg to 10 mg.

Alveolar macrophages obtained from tuberculin skin test positive guinea pigs were not inhibited by PPD in vitro, even though peritoneal cells from the same donors were inhibited. Lack of alveolar cell sensitivity was attributed to the absence of immunocompetent lymphocytes which have been shown to be necessary for the production of Migration Inhibitory Factor (MIF). Attempts to induce alveolar cell sensitivity by intravenous, intracardial, intradermal, and intrapleural injection of mycobacterial antigen were generally unsuccessful. Intravenous challenge of sensitive animals with PPD also failed to confer sensitivity to alveolar macrophages passively in vivo. Sensitive peritoneal cells were shown to confer pronounced in vitro sensitivity on normal peritoneal cell populations. However, when similar mixtures of sensitive peritoneal and normal alveolar cells were tested, only slight inhibition of migration was detected. Only when purified lymphocytes, separated from sensitive peritoneal exudates, were mixed with alveolar cells was pronounced macrophage inhibition demonstrable.

The effect of immune serum on in vitro migration of normal and sensitive peritoneal and alveolar cells was also studied. Immune serum had no effect on the in vitro sensitivity of normal or sensitive peritoneal exudates or normal alveolar macrophages. However, alveolar macrophages from tuberculin-sensitive donors were significantly inhibited by PPD in vitro in the presence of pooled immune serum. The

serum factor was shown to be present in serum collected from animals at 3 weeks, but not at 1 or 2 weeks post-sensitization.

Partial suppression of *in vitro* sensitivity was seen in tuberculin-sensitive guinea pigs injected intraperitoneally with 100 mg cortisone acetate 24 hr prior to collection of peritoneal exudates. Preliminary studies on the effect of cortisone treatment of sensitive peritoneal cells *in vitro* revealed that continuous treatment with 100 mcg cortisone/ml effectively blocked MIF-mediated sensitivity to PPD.

Studies were performed to determine the effect of intramuscular cortisone administration on the induction and expression of tuberculin sensitivity in the guinea pig. The results revealed that partial suppression of dermal reactivity following short term intramuscular administration of high doses of cortisone may be due largely to a non-specific anti-inflammatory effect. It would appear that marked suppression of delayed hypersensitivity in the guinea pig, as measured by pronounced depression of skin reactivity, decreased numbers of mononuclear cells, and suppression of MIF-mediated *in vitro* sensitivity is attainable only by continuous, long term administration of high doses of cortisone. Short term treatment with cortisone, even during the induction phase of sensitization, produces, at most, only transient suppression of cellular sensitivity. These results do not negate the possibility that similar long term cortisone treatment of fully sensitive animals would result in marked immunosuppression.

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