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IN VITRO STUDIES OF TRANSPLANTATION IMMUNITY

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## ABSTRACT

The *in vitro* procedure of inhibition of migration of peritoneal exudate cells (PEC) was adapted for the study of transplantation immunity in mice. One adaptation involved the mixture of thymus cells from mice of the graft-donor strain with PEC induced in the recipients. Small numbers of the thymus cells served as potent cellular antigens and demonstrated the immunological specificity of the assay, as revealed by inhibition of migration of the cell mixture. Thymus cells from a third-party strain sharing donor transplantation antigens caused similar inhibitions. In another approach, the addition of lymph node cells (LNC) from graft-immunized mice to PEC induced in the donor strain caused the inhibition of migration of the cell mixture in an immunologically specific fashion. Experiments showed that low numbers of LNC (1 to 2% of the population) were able to inhibit antigenic PEC demonstrating the sensitivity of this test and its applicability for the analysis of transplantation reactions at a cellular level.

# IN VITRO STUDIES OF TRANSPLANTATION IMMUNITY

by

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# INTRODUCTION

## Cell-Mediated (Delayed-Type) Hypersensitivity

### i. Overview

Cell-mediated immune responses may be described as immunologic effector processes which cannot be transferred passively from an immune animal to a non-immune one with serum from the former, but can be adoptively transferred with lymphoid cells from the immunized animal. Considered among the most common responses are tuberculin-type delayed dermal hypersensitivity, contact hypersensitivity to simple chemical compounds, some autoimmune diseases, immunity to fungi and viruses, and transplantation immunity (Turk, 1967; WHO Report, 1969).

### ii. Transfer of cell-mediated immunity

A significant finding, which in part defines cellular immunity, was the discovery in 1942 by Landsteiner and Chase, of the ability of peritoneal cells (but not of serum) from sensitized guinea pigs to transfer contact hypersensitivity passively. Several years later this finding was confirmed with the passive transfer of tuberculin hypersensitivity (Chase, 1945). Lymphoid cells from other sources such as lymph node and spleen (Chase, 1945), as well as peripheral blood leukocytes (Stavitsky, 1948) were shown to be competent to transfer cellular immunity. Peritoneal exudate cells have been shown to be the population most capable of transferring delayed

hypersensitivity in the mouse (Asherson and Ptak, 1968). As discussed later, the vehicle of transfer of transplantation immunity also has been shown to be the lymphoid cell.

iii. Morphology of cell-mediated reactions

(a) Histology and cytology of delayed-type reactions

The histopathology of inflammatory reactions underlying diverse manifestations of cellular immunity has shown that similar cell types are involved (vide infra). In addition, the morphological changes occurring in lymphoid tissues during the induction of these states of immunity have in general common characteristics.

Following the intradermal injection of antigen into a guinea pig with delayed hypersensitivity to this antigen, there occurs an initial capillary dilatation and a perivascular entry of polymorphonuclear leukocytes. With time there is a steady increase in the proportion of mononuclear leukocytes, mostly lymphocytes and macrophages (Gell and Hinde, 1951; Goldberg et al. 1962). These cells are seen as perivascular infiltrates often several cells deep. The cellular infiltrate usually reaches a maximum at 24 hours following antigen injection, giving rise to a palpable induration, and begins to subside after 48 hours.

In cell-mediated reactions, distinct forms of tissue damage are found. These have been classified by Waksman (1962) as (1) the invasive-destructive lesion, observed in the rejection of tumors and skin, and lesions of auto-immune encephalomyelitis and graft-versus-host

reactions, where infiltrating lymphoid cells are associated with focal areas of destruction of antigen-containing parenchyma, (2) the vasculo-necrotic lesion of tuberculin reactions, where fibrinoid or necrotic changes in blood vessel walls and adjacent parenchyma are seen with the usual perivascular mononuclear cell infiltrate and an involvement of polymorphonuclear leukocytes, and (3) the massive necrosis reaction, often seen at sites of severe tuberculin reactions and which seems to be an ischemic infarction, probably contributing also to lesions of autoimmunity and transplant rejection, especially to the second-set rejection. The cellular infiltrate observed in lesions of delayed hypersensitivity, contact sensitivity, experimental allergic encephalomyelitis and thyroiditis is, in general, similar, i.e., a predominance of mononuclear leukocytes (Uhr, 1966).

The phenomenon of cell accumulation at sites of delayed-type hypersensitivity was studied in experiments of passive transfer of delayed hypersensitivity with lymphoid cells (labeled with tritiated thymidine) from sensitized animals. McCluskey et al. (1963) and Turk and Oort (1963) were unable to show a specific accumulation of labeled lymphoid cells in the reaction sites; they appeared in numbers consonant with the proportion they constituted of the total circulating cells of the recipient. Similar observations were made by Najarian and Feldman (1962) and McCluskey et al. (1963) in the study of skin graft rejection in mice, and in studies in rabbits by Prendergast (1964). These findings suggested that a mediator produced by a small number of sensitized cells had, by some process, involved large numbers

of non-sensitized lymphoid cells in the reaction. Prendergast concluded that most of the infiltrating cells were newly formed, i.e., after the immunization, and the results of Lubaroff and Waksman (1967) point to the bone marrow as the source of these cells. Many aspects of delayed-type hypersensitivity reactions have been confirmed in studies of transplantation immunity including the requirement of living lymphoid cells to transfer these states of immunity (vide infra), the appearance of similar cell types in the sites of lesions due to these reactions and the participation in the reactions of a majority of cells which had not been specifically sensitized.

(b) Cytology of transplantation reactions

On about the sixth day after orthotopic skin homotransplantation in non-sensitized mice, histological examination reveals that mononuclear cells, mainly lymphocytes and macrophages, infiltrate the graft tissue. If, however, the recipient has been sensitized with a previous graft, these cells accumulate between the graft and its bed, but do not infiltrate the graft tissue (Rapaport, 1958). Necrosis of the graft occurs ultimately in both cases.

A significant finding, relating in a direct manner the reactions of allograft immunity to those of delayed hypersensitivity is that a typical delayed skin reaction follows the intradermal injection of tissue extracts from a guinea pig into a recipient which had been presensitized with a skin graft from the first animal (Brent et al. 1958, 1962).

In summary, the morphological picture of the cells infiltrating antigenic sites and the histopathological events in transplantation and delayed-type reactions were shown to be similar. In addition, the observation that transplantation immunity could be elicited in the form of a typical delayed dermal reaction suggests that related mechanisms may be responsible for these two forms of cellular immunity.

(c) Induction of cellular immunity

A phenomenon common to the many forms of cell-mediated immunity is the transformation, during the inductive stages of cellular immunity, of large numbers of cells in the paracortex of regional lymph nodes into pyroninophilic lymphoblasts. This is seen after the first application of contact sensitizing chemicals (Turk and Stone, 1963), immunization with complete Freund's adjuvant to produce delayed hypersensitivity (Turk and Heather, 1965), and in regional lymph nodes draining the site of application of a foreign graft (Scothorne, 1957; Binet and Mathe, 1962). From the studies of Turk and Stone (1963) it appears that the ability of lymph node cells to transfer immunity is related to the number of blasts contained, and that these blasts eventually give rise to small lymphocytes.

The graft-versus-host reaction, shown to be a primary transplantation reaction to histocompatibility antigens (Billingham and Brent, 1959; Simonsen, 1962), is characterized by a massive blastoid transformation of immunocompetent cells followed by a rapid division of these cells into lymphocytes of progressively decreasing size

(Gowans and McGregor, 1965). It therefore appears that the same cellular events underlie both the primary response to transplantation antigens and the induction of classical delayed hypersensitivity.

iv. In vitro correlates of cell-mediated immunity

In vitro correlates of many in vivo immunological manifestations, e.g. antibody production (Mishell and Dutton, 1966), have been shown to be quantitative and often highly sensitive. In addition, analyses of such correlates of immune reactions have provided clues to the understanding of phenomena observed in vivo.

Three in vitro assays, i.e., the blast-cell transformation reaction, the technique of inhibition of peritoneal exudate cell migration, and the destruction of antigen-bearing 'target' cells by lymphoid cells, are considered to be correlates of cellular immunity.

For studies of blast-transformation lymphoid cells from an immunized animal are exposed to antigen in tissue culture. Beginning at 24 hours of incubation, metabolic changes, including DNA and RNA synthesis, are observed (Hirschhorn et al. 1963). While Oppenheim's adaptation of this technique in humans and guinea pigs (Oppenheim et al. 1967, 1968, 1969 ) appears to be correlated with delayed hypersensitivity and not with the antibody response, the results of Dutton and Bullman (1964) in rabbits do not follow this pattern since they showed that antigen stimulation of DNA synthesis was associated with the proliferative development of cells synthesizing humoral antibody. Nevertheless, it was shown that the carrier specificity of the humoral anamestic response (identical to that of delayed hypersensitivity reactions), is operative in this in vitro assay, i.e., a blastogenic

reaction occurred when the same hapten-protein conjugate used for immunization was the challenging antigen and no reaction was observed with the hapten conjugated to an unrelated carrier molecule. In contrast, it is known that in antibody-mediated reactions, such as the Arthus reaction, the specificity of the reaction is directed chiefly to the hapten and the reaction can be elicited even with the hapten coupled to different carrier molecules (Benacerraf and Gell, 1959; Silverstein and Gell, 1962).

The second in vitro assay, which correlates well with delayed hypersensitivity reactions, involves the inhibition of migration of peritoneal exudate cells (PEC). In this assay, cells from the peritoneal cavity of animals with delayed hypersensitivity to an antigen are packed into capillary tubes which are placed in a liquid tissue culture. The cells migrate out of the tubes and form fan-like patterns at the capillary mouth. When the PEC are derived from an immunized animal (having delayed hypersensitivity to an antigen), and the antigen is added to the tissue culture medium, the cells appear to become cohesive and fail to migrate out of the capillary tubes. This assay will be considered in further detail in a following section.

The third in vitro correlate of delayed hypersensitivity is primarily qualitative, but may offer additional insight into the effector mechanisms of cellular immunity. It is a cytotoxic assay, first described by Ruddle and Waksman (1967), and suggests a common mechanism for the diverse in vitro manifestations of cellular hypersensitivity and for the tissue damage accompanying severe tuberculin

reactions, lesions of autoimmunity and homograft rejection. The assay consists of a tissue culture procedure in which normal embryonic rat fibroblasts are cultured in the presence of lymphocytes obtained from rats having delayed hypersensitivity to various soluble antigens. These lymphocytes, in the presence of the specific antigen, destroy syngeneic or allogeneic fibroblasts during 72 hours of culture (Ruddle and Waksman, 1968a,b,c). This phenomenon was not correlated with Arthus reactivity but showed the carrier specificity of delayed hypersensitivity to hapten-protein conjugates. In a more recent study by Sin et al. (1971), using a similar assay system, it was shown that antigen need not be in the fluid phase of the tissue culture for the cytotoxic reaction to occur, but that it could be bound to the surface of the target cell, or that antigen could trigger lymphocytes into a cytotoxic state during a preincubation step. In both cases lymphocytes were cytotoxic to target cells when placed in antigen-free tissue culture medium. These results suggest a close relationship between this assay of delayed hypersensitivity and the phenomenon of target cell destruction (vide infra), which is considered to be an in vitro correlate of transplantation immunity. In both systems target cell destruction occurs; both systems have similar kinetics and lymphocyte:target cell ratios, are independent of exogenous complement, and are antigen specific.

In summary, these in vitro correlates of cellular immunity comply with the established criteria for cell-mediated reactions, such as carrier specificity and independence from antibody production, and

they suggest mechanisms which may be responsible for some of the *in vivo* manifestations of cellular immunity. Furthermore, as has been pointed out earlier for cell-mediated phenomena *in vivo*, there appears to be a common manifestation *in vitro* of transplantation immunity and delayed hypersensitivity, supporting the hypothesis that some mechanisms may be common to both forms of immunity.

## Transplantation Immunology

### i. Historical

Although substantial contributions concerning the fate of transplanted cells and tissues were made by early workers in the field of tumor transplantation (c.f. Medawar, 1958), it was Sir Peter Medawar and his co-workers who first studied in a systematic manner the fate of normal tissue transplants (1944, 1945). His early experiments performed with rabbits and followed later by the use of mice of different inbred strains, established the genetic basis of transplantation immunity. Most of the early work in the study of transplantation rejection concerned tumor and skin homografts.

In rabbits an allogeneic skin graft becomes entirely necrotic by about the ninth day, and a second-set graft, that is, a second graft from the same donor, is rejected more rapidly, on about the sixth day following grafting (Medawar, 1944, 1945). Histological examination of a graft undergoing first-set rejection reveals that by the fifth day after grafting an increasingly dense, mononuclear (predominantly lymphocytic) infiltration. However, some of the infiltrating cells may be seen on day two or three. The nature of the

mononuclear cell infiltrate varies with the species, but in general the cells comprise a variable proportion of lymphocytes and macrophages with some plasma cells (Weiner et al. 1964). These findings, which suggest that lymphoid cells may have a direct immunological effector role in the destruction of homografts, were first discussed with regard to tumor immunity in earlier years (Gorer, 1956).

ii. Role of antibody and of lymphoid cells

It was questioned whether the observed lymphoid cells alone were responsible for tissue rejection, or if antibody had an important cytopathic role as well. The work of Gorer et al. (1959) and of Jensen and Stetson (1961) showed that antibody to histocompatibility antigens in mice may be detectable from the third or fourth day after skin grafting, and cytotoxic anti-tumor antibodies could be demonstrated at the height of allograft rejection. The results of many attempts by different workers to transfer homograft immunity passively using serum from immunized animals has been reviewed (Stetson, 1963). While it appears that solid tissue grafts (except renal grafts) are not very vulnerable, certain dissociated cell grafts such as leukotic and hematopoietic grafts are damaged by the action of antibody. Thus, certain mouse neoplasms, especially those occurring as single cells, are sensitive to cytotoxic antibody (Gorer and Kaliss, 1959) and neonatal hybrid mice have been passively immunized with antiparental antisera to prevent runt disease induced by a graft of parental hemopoietic tissue (Russel, 1962).

However, there is abundant evidence indicating that antibody may not be responsible for many forms of transplant rejection. Thus, for example, Weaver et al. (1955) showed that allogeneic cells could survive for a long period in diffusion chambers, which were permeable to antibody. In addition, fetal lambs at 75 days of gestation which do not produce circulating gamma globulin, can reject allografts (Schinkel and Ferguson, 1953), and neonatally thymectomized birds which can produce normal antibody have impaired ability to reject skin homografts (Warner and Szenberg, 1964).

iii. Transfer of transplantation immunity

Further evidence supporting a cellular effector process for transplantation immunity is found in studies concerning the transfer of immunity by Gowans et al. (1961) and Billingham et al. (1963) which demonstrated that thoracic duct small lymphocytes were capable of abolishing tolerance and which implicate the small lymphocyte as the "agent and effector" of transplantation reactions. In addition, the lymphocyte transfer test of Brent and Medawar (1967) involving the intradermal injection of lymph node cells from a skin-grafted guinea pig, into an allogeneic recipient (which had served as the graft donor), gives rise to an indurated skin reaction (even when immune serum fails to do so), demonstrates that lymphoid cells may be the mediators of cellular immunity. (The above reaction is notably similar to the local passive transfer of delayed hypersensitivity first described by Metaxas and Metaxas-Buhler (1948) and refined by Blaskovec et al. (1965). In this procedure, the local intradermal injection of

lymphoid cells from a guinea pig having delayed hypersensitivity together with the specific antigen, into a normal recipient results in a typical delayed-type skin reaction. This illustrates again the common expression of transplantation and delayed-type responses). The most compelling evidence that transplantation immunity is due to cell-mediated effector processes is found in experiments of passive transfer of immunity. The work of Mitchinson (1953, 1954) and of Billingham et al. (1954) showed that immunity to allogeneic tumors or skin grafts could be transferred with cells from regional lymph nodes draining the site of graft application; however, they failed to transfer this immunity with serum having high levels of antibody (Billingham and Brent, 1956).

iv. Nature of the cellular infiltrate

Prendergast (1964) provided evidence concerning the accumulation of inflammatory cells at reaction sites of delayed hypersensitivity. He studied the localization of lymphoid cells which had become radiolabeled (due to administration of tritiated thymidine) in the regional lymph nodes subsequent to skin grafting. No specific accumulation of such labeled cells during the rejection of a second graft from the original donor was observed. He did however find an accumulation of large numbers of lightly labeled (newly formed) cells apparently not formed in the lymph nodes in response to the graft. These findings are in agreement with those of Najarian and Feldman (1962), who studied in a similar fashion the accumulation of radiolabeled cells in skin grafts of mice, and those of McCluskey et al. (1963) and Turk and Oort (1963) in their studies of delayed hypersensitivity. These experiments

suggest a very great ability of a few sensitized cells to influence large numbers of other lymphoid cells. It is unclear whether these 'new' cells (lymphocytes, macrophages and polymorphonuclear leukocytes) participate in cytopathic processes or serve some secondary function such as tissue repair. However, some experiments have shown that macrophages may have a direct role in the destruction of grafts. Macrophages obtained from the peritoneal exudate cells of tumor-grafted mice were able, when injected together with tumor cells into X-irradiated recipients, to supress tumor growth (Bennett, 1965). On a cell-to-cell basis they were about 1/16th as effective as peritoneal lymphocytes, but acted in synergy with them. Additional evidence of the possible role of macrophages in transplantation reactions is suggested by the in vitro work of Granger and Weiser (1964, 1966) who showed that partially purified macrophages from PEC of grafted mice were cytotoxic to target cells from the graft donor and that a cytophilic hemagglutinating antibody was the likely underlying agent of these macrophage activities.

As will be discussed (see iii. Inhibition of PEC migration), a mechanism for the attraction into and containment of macrophages at sites of cellular reactions appears to exist. Such a process could, explain in part, the large numbers of newly formed cells in these lesions and could add immune macrophages to the existing cytotoxic lymphocytes considered previously. Further consideration of the role of aggressor macrophages in transplantation reactions is presented in the Discussion.

It may be concluded that both transplantation and delayed hypersensitivity reactions are characterized by a large proportion of recruited cells, reinforcing the hypothesis that a common mechanism may be responsible for both reactions.

In summary, most transplantation reactions are considered to be manifestations of cell-mediated immunity, thus many aspects of the reactions of delayed hypersensitivity are reflected in those of transplantation immunity. Common to both forms of cell-mediated immunity are (1) the requirement of living lymphoid cells to transfer immunity and the inability of antibody to do so, (2) the cytological picture of lymphoid tissues during the induction of both types of immunity (i.e. the appearance of basophilic lymphoblasts), (3) the preponderance of mononuclear cells, lymphocytes and macrophages, which accumulate at the reaction sites, and (4) the low proportion of specifically sensitized cells among the infiltrating population.

## v. In vitro manifestations of transplantation reactions

### (a) The mixed leukocyte culture

The mixed leukocyte culture (MLC) and target cell destruction are two *in vitro* assays of transplantation reactions. The MLC is a tissue culture procedure in which lymphoid cells from two different individuals are mixed and cultured for several days. Changes in cell proliferation and morphology are observed when strong transplantation antigen differences are present between the two lymphoid cell donors. In 1963 Bain *et al.* described this proliferative reaction when leukocytes

from human donors were placed in culture. Subsequent studies in mice (Dutton, 1965) and in rats (Wilson, 1967) have shown that the reaction represents an immunologically specific response by immunocompetent lymphocytes to homologous cellular transplantation antigens. Lymphocyte transformation is defined by Oppenheim (1968) as "a morphological enlargement of small lymphocytes to larger lymphoblasts in vitro ... they resemble basophilic lymph node cells that appear in vivo after antigen stimulation".

The MLC is reminiscent of primary phases of other homograft reactions such as (a) the normal lymphocyte transfer reaction, which manifests itself as a delayed type of skin lesion, and involves the transformation of donor cells (Brent and Medawar, 1967), and (b) the cytology of the early phases of graft-versus-host reactions (Gowans and McGregor, 1965). The MLC is considered to reflect some of the afferent processes in transplantation immunity and supporting evidence for this view is that leukocytes from a 6-day-old MLC were able to confer accelerated graft rejection in rats (Gordon et al. 1967). Among the measured parameters of the MLC response are the observed increase in histone acetylation, followed by an increase in cellular protein, RNA and DNA synthesis culminating in mitosis (Oppenheim, 1968). As an extension of these findings one may cite the recent experiments of Ginsberg (1968) and Berke et al. (1969) who showed that rat lymphoid cells cultured on a mouse cell monolayer become transformed and then cytotoxic to the cells of the monolayer. Such experiments may reflect the entire cycle of an immunocompetent cell triggered by antigen,

becoming transformed and finally maturing through subsequent mitoses into an aggressor cell cytotoxic to antigenic cells.

(b) Target cell destruction

Lymphoid cells sensitized *in vivo* to transplantation antigens are capable to destroy *in vitro* cells bearing these antigens. This assay is termed 'target cell lysis' and is considered to be an *in vitro* manifestation of transplantation immunity. Most commonly, lymphoid cells from animals immunized by a grafting procedure are added to cultures of cells which bear graft donor antigens. The earliest forms of this assay demonstrated, using morphological criteria, the cytotoxic potential of lymphoid cells from animals with transplantation immunity (Govaerts, 1960; Rosenau and Moon, 1961) or experimental autoimmunity (Koprowski and Fernandes, 1962) with respect to antigenic target tissues in cell cultures. Some cellular, kinetic and metabolic aspects of this reaction have now been described (Wilson, 1965; Cerottini and Brunner, 1972; Brondz, 1972) including the binding of sensitized cells to target cells (Golstein *et al.* 1971). Although lysis of some antigenic cells has been reported as early as one hour after the start of incubation (Brunner *et al.*, 1970), in general longer incubations of 24 or 48 hours are used.

It is commonly accepted that antibody and exogenous complement are not involved in the cytocidal activity of immune lymphocytes (Wilson, 1967). However, the mechanism of cell destruction remains unknown. Suggestions of mechanisms are not scarce, for some authors have demonstrated that cytotoxic material(s) are released from lymphocytes

into tissue culture supernatants upon exposure to (1) specific tissue antigens (Granger and Kolb, 1968), (2) soluble antigens (Ruddle and Waksman, 1968a,b,c), (3) homologous leukocytes in MLC-type cultures (Granger and Williams, 1968) and, (4) more commonly with the mitogens, phytohemagglutinin (PHA) and Pokeweed mitogen (Williams and Granger, 1969). The released material(s) added to cultures of various cell lines was shown to have disruptive metabolic effects; however no direct evidence for a parallel role in vivo is available.

The relation of the cytotoxic properties of immune lymphocytes in vitro to graft destruction in vivo is unclear. Wilson (1965) showed that thoracic duct cells, obtained from grafted animals, which would normally have entered the circulation and would thus be available for invasion into graft tissue, could kill target cells. However, the failure to demonstrate large numbers of sensitized cells at rejection sites with the use of radioactively labeled cells suggests that the lymphocytotoxic reaction observed in vitro may not be of very great importance in vivo.

(c) Inhibition of cell migration

Another assay of seemingly general application to the study of transplantation immunity in vitro is the inhibition of migration assay, which contributed much to the understanding of delayed hypersensitivity. In a brief communication by Al-Askari et al. (1965) it was reported that the technique had been successfully adapted to transplantation immunity in mice. Since this assay provides the basis for the present study, it has been considered in detail on page 20.

vi. The mouse as a model for transplantation studies

Transplantation studies have been refined to a high degree with the use of inbred strains of mice with defined histocompatibility antigen differences at known genetic loci. As in all species studied, the mouse has a major transplantation barrier, termed the H-2 locus. Differences in histocompatibility antigens at this complex locus cause the most vigorous of allograft reactions. Other transplantation loci of varying strength, but weaker than H-2, exist, as determined by longer skin graft survival times. In these cases chronic rejection of skin grafts may continue for months, while differences at the H-2 locus cause rejection in about 10 days (Snell and Stimpfling, 1966). Serological studies, using antisera produced subsequent to repeated immunization with histocompatibility antigens (usually lymphoid cells), have provided a means for the analysis of the H-2 locus. Using tissue absorption techniques and serological typing it was possible to subdivide antigens determined by the H-2 locus into composite serological specificities, each defined by an antiserum (Shreffler and Snell, 1969). Thus far at least 36 such specificities and over 19 strains of mice which differ at the H-2 locus have been defined (Klein and Shreffler, 1971), and, since each of the strains may have up to 15 of these 36 specificities, it follows that some specificities are common to many strains.

It has been shown that a transplant reaction (rejection) can be determined by a single specificity at the H-2 locus (Klein, 1967). In these experiments the mice were otherwise genetically identical,

i.e. congenic. This suggests that some specificities may reflect a complete immunogen and not merely a haptenic determinant. This fact may be of considerable importance if reactions to transplantation antigens are governed by carrier specificity requirements similar to those of antigens involved in reactions of delayed hypersensitivity.

There is some additional evidence in vivo (Berrian and Jacobs, 1959; Eichwald et al., 1966) which suggests that H-2 specificities can act as simple antigens. Thus, mice immunized to a group of specificities determined by an H-2 incompatibility and subsequently exposed to transplantation antigens of a third strain, which carries some of these specificities, showed secondary-type immune reactions. Such an effect is not surprising since cross-reactions are common immunological observations. However, the latter study is subject to criticism since congenic strains were not used, and reactions could have been directed to antigens determined by non-H-2 loci. Analysis of transplantation immunity at a cellular level is necessarily more complex than similar studies of delayed hypersensitivity considered earlier, since the nature of the antigens determined by histocompatibility genes is unknown, while in delayed hypersensitivity the molecular properties of some antigens are well defined.

Considering the relationship of such serologically defined antigens to transplantation reactions it follows, that, since all specificities are serologically determined by hemagglutination or complement mediated lymphocytotoxicity, it is likely that there exist transplantation antigens which evoke little or no antibody, or are not

found on mouse erythrocytes or lymphocytes used for H-2 typing; this has been recently demonstrated (Boyse et al., 1972).

In summary, although many aspects of the serological nature of H-2 antigens are known, the properties of these antigens and the immunological specificities determined by them which elicit cell-mediated reactions remain to be elucidated.

## Inhibition of Lymphoid Cell Migration

### i. Historical

One of the earliest investigations of the action of antigen on cell movement was a report in 1932 by Rich and Lewis. These authors studied the emigration of cells from explanted spleen fragments and buffy coat leukocytes aggregated in plasma clots. The experimental animals were guinea pigs which had been immunized with $R_1$ virulent human tubercule bacilli, and the authors used the tubercule bacillus extract, Old Tuberculin (O.T.), as antigen in their tissue cultures. They reported a failure of cells to migrate out of the fragment if the antigen was incorporated into the tissue culture medium during the culture period which was up to 4 days in duration. Connective tissue cells and lymphocytes were not greatly affected and continued to migrate, but macrophages and polymorphonuclear (PMN) leukocytes did not migrate, and underwent degenerative changes and cytolysis. The authors found that these changes occurred in animals with delayed allergy, but not in animals having anaphylactic sensitivity (antibody-mediated), however, they had little idea of the underlying cellular and humoral mechanisms.

More recently Svejcar and Johanovsky (1961) studied coagulated fragments of guinea pig spleen, peritoneal exudate cells and buffy coat cells. They quantitated the number of viable cells which had migrated from the fragments in tissue culture and found inhibition of this migration in the presence of antigen, but did not observe cytotoxic changes in the inhibited cells. The use of coagulated PEC did not result in strong inhibitions in the presence of antigen. Much of such early work must be regarded with caution for the authors employed questionable tissue culture conditions, non-standardized antigens and substances such as chick embryo extract in the culture media.

ii. The capillary tube migration assay

In their modification of the capillary tube migration assay, George and Vaughn (1962) used oil-induced guinea pig peritoneal exudate cells (PEC) packed by centrifugation into capillary tubes, and allowed the cells to migrate horizontally out of the tubes onto a flat surface adjacent to the tube mouth during the 37°C incubation period. Animals with delayed hypersensitivity to PPD or ovalbumin were used as PEC donors. When the immunizing antigen was present in the tissue culture medium the PEC migrated very poorly. PEC from animals with Arthus reactivity to the antigen (i.e. circulating antibody), but exhibiting no delayed skin reactions, were not inhibited by antigen in the medium. In addition this *in vitro* phenomenon of "migration inhibition" correlated directly with the ability of the same PEC to effect local passive transfer reactions, (see p. 11) reinforcing the relevance of this *in vitro* assay to delayed type reactions *in vivo*. This work

was the first definitive study demonstrating the dependence of migration inhibition on the presence of delayed hypersensitivity in the animals used as the source of the PEC.

iii. Migration inhibition as a correlate of delayed hypersensitivity

In 1964 David and his co-workers confirmed the findings of George and Vaughn and began a thorough investigation of the migration inhibition assay which has disclosed many aspects of cell-mediated immune reactions *in vitro* and suggesting new mechanisms for the *in vivo* manifestations of cellular immunity. In their early experiments (David *et al.*, 1964a), guinea pigs were immunized in various ways to produce animals having delayed hypersensitivity only, delayed hypersensitivity and concomitant antibody production, or antibody production only, to the antigens tuberculoprotein, ovalbumin or diptheria toxoid. Antigens injected in saline produced humoral antibody, and those injected as antigen-antibody complexes or in complete Freund's adjuvant gave rise to delayed hypersensitivity. Inhibition of migration of PEC from capillary tubes occurred when the PEC donor had delayed skin reactivity to the antigen. This inhibition was specific for the immunizing antigen and occurred whether or not circulating antibody was present. It was concluded that precipitating antibody and Arthus reactions had no relation to these inhibitions *in vitro*. Attempts to passively sensitize PEC from normal animals by incubating these cells in serum from immune animals failed, showing that cytophilic antibody was not involved. Since the inhibition occurred in the presence of heat-inactivated serum, the participation of exogenous complement factors was ruled out. The authors concluded that the inhibition of

PEC migration resulted from an interaction between sensitive cells and antigen, and was independent of serum factors.

The specificity of the assay was further studied using hapten-protein conjugates (David et al., 1964c). Antibody-mediated hypersensitivity reactions can be evoked on challenge of the sensitized animal with the immunizing hapten conjugated to many unrelated proteins and is not restricted to the conjugate with the carrier protein used for immunization (as considered previously, p. 7 ). In delayed hypersensitivity however, there exists a major contribution by the carrier protein to the specificity of delayed reactions as described by Benacerraf and Gell (1959) and discussed previously. Using the hapten-protein conjugates, DNP-ovalbumin or DNP-bovine gamma globulin, David et al. (1964c) demonstrated the obligatory participation of the carrier protein in determining the specificity of the inhibition of migration of PEC from animals immunized with the appropriate hapten-protein conjugate. Serum antibodies of the above animals reacted with the hapten irrespective of the carrier protein, as shown by reactions of passive cutaneous anaphylaxis. The results of their migration experiments supported the hypothesis that the specificity of this in vitro assay corresponded to the specificity requirements of the in vivo reaction of delayed hypersensitivity, but not to reactions mediated by circulating antibody.

Also tested were well-defined antigens such as α, DNP-oligo L-lysines for their ability to inhibit the migration of PEC from guinea pigs with delayed hypersensitivity to α, DNP-poly-L-lysine (David and Schlossman, 1968). It is known (Stulbarg and Schlossman, 1968) that

the α, DNP-oligopeptide must contain seven or more lysyl residues in order to act as an immunogen or to be capable of eliciting delayed reactions. However, α, DNP-oligopeptides containing three to six lysine residues can readily react with humoral antibody, but are neither immunogenic nor able to elicit delayed skin reactions.

For migration experiments PEC from guinea pigs with delayed hypersensitivity to α, DNP-oligolysines, with an average chain length of 18 lysines, were assayed in the presence of α, DNP-oligopeptides of varying sizes. None of the lower homologues, α, $DNP(Lys)_3$, α, $DNP(Lys)_4$, or α, $DNP(Lys)_6$ was able to cause inhibition of migration of PEC from guinea pigs immunized with α, $DNP(Lys)_{18}$, but α, $DNP(Lys)_9$ was able to do so. Moreover, experiments with optical isomers of these antigens (i.e. D-lysine-containing stereoisomers of α, DNP-digo-L-lysine) showed that the antigenic requirements for an in vitro migration inhibition were identical to those for the elicitation of delayed skin reactions.

iv. Mechanism of migration inhibition

The same group of workers (David et al., 1964b) showed that when migrating PEC from a normal guinea pig were adjusted so as to contain 10% of PEC from an immune animal, the migration of the cell mixture was inhibited when exposed to the antigen, as though all the cells had originated from the sensitized donor. It was therefore concluded that sensitized cells were able to influence the behavior of normal cells, although it was not possible to distinguish between the alternatives that this reaction was due to "information transfer" or

to the production of pharmacologically active mediators by sensitized cells. The experiments showed that killed sensitized cells were not able to activate normal PEC to the antigen suggesting that this effect depended on a biosynthetic process. To test the above possibility several anti-metabolic drugs were added to the tissue culture medium used in the migration assay. Indeed, puromycin and actinomycin D were shown to reduce the response i.e. to decrease the degree of inhibition of migration of sensitized PEC to specific antigens (David, 1965). This indicates that inhibition of migration did not result simply from the presence of cytophilic antibody* attached to PEC, which could act via antigen to aggregate the cells, consequently blocking migration.

Since a possible basis for the specificity of cell-mediated reactions could be the presence of cell-bound antibody-like receptors on immune cells, a series of experiments was carried out to test this hypothesis. PEC were treated with proteolytic enzymes to see if their in vitro reactivity in the migration assay would be affected (David et al., 1964d). Trypsin or chymotrypsin treatment of sensitized PEC caused the loss of in vitro reactivity, i.e. migrations were not inhibited by specific antigen. However, this reactivity was recovered when the cells were incubated for 24 hours in a suspension culture without enzymes. These results are consistent with the concept of regenerable, enzyme-sensitive receptor sites, and exclude the possibility

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* Boyden and Sorkin (1960) had described the presence of "cytophilic" antibody in immune guinea pig serum by showing its ability to adsorb to macrophage cell surfaces and bind antigen. Binding was demonstrated with radiolabeled antigen, and the formation of rosettes of antigenic cells around the antibody-coated macrophages.

that migration inhibition was due to the interaction of antigen with antibody that had been passively fixed onto cell surfaces *in vivo*.

Despite these results some authors suspected that cytophilic antibody could be responsible for the inhibition of migration. Amos *et al.* (1967) investigated this possibility using sera that contained high titres of cytophilic antibody to PPD or β-lactoalbumin. Under optimal conditions, normal PEC were treated with the antisera to allow binding of the cytophilic antibody and were then exposed to the specific antigen in a migration assay. The results from their careful experimentation sustain the conclusion that cytophilic antibody is not the agent responsible for the inhibition of migration.

Bloom and Bennett (1966) have produced evidence for a likely mechanism underlying the inhibition of PEC migration. Using the property of macrophages to adhere to glass, they removed and purified macrophages from PEC of guinea pigs. Since the PEC population is composed in part of 50% to 70% macrophages and 15% lymphocytes, it was thought that analysis of the contribution of each of these two cell types to the inhibition of migration could provide clues to the mechanism of this reaction. The experimental animals were either normal or sensitized guinea pigs which gave strong delayed skin reactions to PPD. The addition of purified sensitized lymphocytes to either PEC or purified macrophages from normal animals, produced inhibition of migration of the cell mixture, when PPD was added to the tissue culture medium. As few as 0.6% sensitized PEC lymphocytes could cause this inhibitory effect. Purified lymphocytes did not migrate and purified

macrophages from sensitized animals were not inhibited by specific antigen. It was apparent that the migration of the peritoneal macrophage was not affected directly by antigen, but was an indication of the immunological activity of the sensitized peritoneal lymphocyte. The possibility that the lymphocyte elaborated a soluble mediator which then acted on macrophages was tested. Purified peritoneal lymphocytes from immune animals were placed in a tissue culture medium containing the antigen, and 24 hours later the cell-free supernatant was assayed for its ability to inhibit the migration of normal PEC. In these experiments strong inhibitions occurred and the factor responsible for this activity was shown to be non-dialyzable. Similar conclusions were derived by David (1966) with lymph node cells (LNC), in analogous experiments. Firstly a mixture of LNC (comprised of 95% lymphocytes) from sensitized guinea pigs with PEC from normal guinea pigs was shown to cause inhibition of migration of the entire cell population in the presence of antigen; addition of 59% to 35% immune LNC to normal PEC could cause this effect. Secondly, as in the experiments of Bloom and Bennett, a migration inhibitory factor was present in the supernatant of immune LNC cultured with the specific antigen. The factor responsible for the activity in the supernatant was cryostable and termed migration inhibition factor (MIF).

This work was essentially confirmed by Bartfeld and Kelly (1968) using PPD-sensitive guinea pigs. On culturing peripheral blood lymphocytes from these animals in the presence of antigen they recovered a supernatant which was able to inhibit the migration of normal PEC.

This activity found in the supernatant was heat stable (56°C, 30 minutes) but cryolabile, in contrast to the findings of David. For cytological studies they cultured sensitized lymphocytes mixed with normal PEC on a flat surface and found clumping of the lymphocytes and macrophages in the presence of antigen. However, the cells were not morphologically altered, and no change in acid phosphatase staining was observed. In a more recent publication (Bartfeld et al., 1969) the authors described inhibition of migration when peripheral blood lymphocytes from sensitized animals were added to migrating (lung) alveolar macrophages in the presence of antigen and suggested a possible adaptation of this procedure to man. In addition, Thor et al. (1968) reported MIF production by human peripheral blood lymphocytes cultured with specific antigen. These supernatants could inhibit the migration of normal guinea pig PEC. These results are of particular importance since they demonstrate that the effect of MIF is not species specific as regards the source of MIF and PEC and suggest possible applications of this procedure to clinical problems.

Continuing their studies on MIF, Bennett and Bloom (1967) reported that sensitized lymphocytes in tissue culture could produce the inhibitory factor for 4 days, i.e. preceding and during blastogenesis of the lymphocytes, and that once the cells were triggered by antigen to make MIF they continued to do so in the absence of antigen.

It is generally agreed that MIF is eluted on gel filtration in the volume range corresponding to that of albumin and that no MIF activity is recovered in fractions containing higher molecular weight

proteins such as immunoglobulins. Careful physico-chemical analysis by Remold et al. (1970) shows that more MIF elutes just after albumin and, that MIF is more acidic and of lower molecular weight than albumin.

Reports have appeared in the literature suggesting that MIF may have antigen specificity. Bennett and Bloom (1967) reported that the addition of antigen to MIF-containing supernatants that had been antigen depleted enhanced the inhibitory activity of such supernatants. Svejcar et al. (1968), studied the properties of MIF from rabbit lymphocytes and found that the activity of supernatants prepared with minimal amounts of PPD could be increased by the addition of antigen. Recently Amos and Lachman (1970) showed that MIF could be prepared in tissue culture by exposing sensitized lymphocytes to particulate polystyrene-antigen conjugates thereby obtaining cell-free, antigen-free, MIF-rich supernatants. The inhibitory activity of such supernatants was latent, since it was apparent only after the addition of specific antigen. These experiments show that the activity of MIF may be greatly increased in the presence of specific antigen, suggesting that MIF may have antigen specificity. However, these results contradict the findings of Remold et al. (1970) who found that antigen-free MIF isolated in good purity was active in the inhibition of migration assay. In addition, Yoshida (1972) has failed to reproduce the results of Amos and Lachman.

Other observations indicate that MIF may not be the only active agent causing inhibition of PEC migration. Bloom and Bennett (1966) have shown that if an antigen and its antiserum are added to the

tissue culture medium used for migrating normal PEC, inhibition of migration results. Other workers (Heise et al., 1968) have shown that cytophilic antibody could passively sensitize (lung) alveolar macrophages to be inhibited in their migration on exposure to antigen. Using another approach, Spitler et al. (1969) treated human erythrocytes with human-anti-erythrocyte-antibody, and sheep erythrocytes treated with 7S or 19S fractions of rabbit anti-sheep erythrocyte serum. On mixing the treated erythrocytes with normal guinea pig PEC they showed that migration was inhibited by erythrocyte-IgG (7S) complexes, but not by erythrocyte-IgM (19S) complexes. They suggest that while cytophilic antibodies bind poorly to macrophages, antigen-antibody complexes bind very firmly to them and may "agglutinate" the migrating cells.

In conclusion, while there is strong evidence that MIF may be responsible for the in vitro inhibition of migration of guinea pig PEC, the precise nature and immunological specificity of this material to antigen remains to be elucidated. In certain circumstances additional substances have been shown capable of inhibiting the migration of PEC, such as humoral antibody considered above, thus interpretations of the mechanism of inhibition in migration assays of altered protocol must be advanced cautiously.

The role of MIF, if produced in vivo, is uncertain. Tissue culture supernatants containing this activity have been shown to have leukochemotaxic activity for mononuclear cells in vitro (Ward et al., 1969) and probably in vivo (Ramsier, 1969), to induce vascular permeability

upon injection in vivo (Maillard et al., 1972) (however this activity is present among molecules having a molecular weight of 39,000) and to evoke a rapid delayed-type dermal reaction in guinea pigs following intradermal injection (Bennett and Bloom, 1968); this reaction exhibits erythema and induration and, histologically, an accumulation of mononuclear leukocytes. Thus, although the precise role(s) of the bioactive substance(s) described is unclear, it is plausable to consider that they participate in cellular reactions in vivo, perhaps serving as mediators for the attraction of cells from the circulation into foci where traversing lymphocytes have been triggered by antigen and perhaps conferring on these recruited cells new roles such as aggressor cells in cytotoxic reactions, as has been recently described for macrophages in vitro by Grant et al. (1972).

Since the migration inhibition assay was demonstrated to be correlated with cellular hypersensitivity in vivo, other cell-mediated immune responses were explored with this technique, viz. tumor immunity, autoimmunity, delayed hypersensitivity in man, and transplantation immunity.

To demonstrate tumor immunity in vitro, guinea pig hepatoma cells were mixed with PEC from guinea pigs immunized with a hepatoma tumor. Inhibition of migration resulted, whereas cells from another tumor did not produce inhibition (Kronman et al., 1969). These experiments demonstrated that the reaction was specific for the immunizing tumor and not transplantation antigens, since inbred animals were used. In analogous experiments in mice, using methylcholanthrene-induced

tumors similar results have been reported (Halliday and Webb, 1969).

David and Paterson (1965) induced auto-immune allergic encephalomyelitis in guinea pigs and showed that extracts of nervous tissue inhibited the migration of PEC from these animals. Similar work by other authors continues in an attempt to elucidate the role of lymphoid cells, the nature of the immunogen and the target antigen involved in the pathogenesis of this disease (Brockman et al., 1968; Hughes and Newman, 1968).

The inhibition of migration assay has been applied to transplantation immunity in inbred mice (Al-Askari et al., 1965). PEC from CBA mice, immunized with a skin graft from the allogeneic strain A/J, were inhibited in their migration when mixed with PEC from the strain of the skin graft donor. The reaction was shown to be specific since inhibitions did not occur when PEC from the immunized CBA mice were mixed with PEC from a third strain, C57BL/6. However, except for this small study further investigations employing the assay of inhibition of migration in attempts to explore transplantation reactions of mice in vitro have not yet been reported.

Perspective

As indicated in this introduction there appears to be a strong relationship between the factors mediating delayed hypersensitivity and homograft immunity both in vivo and in vitro. In view of this evidence and the importance of understanding the mechanisms of transplant rejection at a cellular level, it is suggested that there exists justification for the adaptation of a highly refined in vitro

correlate of delayed hypersensitivity for the study of transplantation immunity in mice. The successful application of this technique to transplantation studies may provide for a sensitive assay of transplantation immunity and a method for quantitating the intensity of this response. Moreover, the technique developed in this thesis can provide a means for examining the specificity of transplantation reactions at a level which relates the known serology of histocompatibility antigens to antigenic requirements for lymphoid cell activation, i.e., migration inhibition. In addition, the parameters and sensitivity of the assay to be described may allow the analysis of some of the mechanisms underlying the genesis of immune rejection in vivo.

## MATERIALS AND METHODS

### i. Animals

All mice used in these studies were of the highly inbred strains A/J, C57BL/6J, CBA/J and C3H/HeJ and were purchased from the Jackson Laboratory, Bar Harbor, Maine. Animals were shipped at eight weeks of age and were kept at 70-76° F with food and water *ad libitum*. In all experiments young adult mice from nine to sixteen weeks of age were used. In any given experiment animals of one sex only were used.

### ii. Immunizations

#### (a) Tumor grafts

A spindle-cell sarcoma designated SaI, which had been originally obtained from the Jackson Laboratory, was maintained in ascites form in A/J mice. This rapidly growing single-cell suspension was harvested weekly in the following manner. The tumor-bearing animal was sacrificed by cervical dislocation and 3.0 ml of Hanks balanced salt solution (HBSS, Difco) was injected intraperitoneally. After swabbing with 70% ethanol the abdominal skin was reflected in two flaps, bilaterally, from the linea alba, exposing the abdominal wall. The abdominal muscles were seized with a hemostat and a sterile Pasteur pipette was passed through the abdominal wall to aspirate the contents. About 2.0 ml of cell suspension was aspirated and deposited in a sterile centrifuge tube containing 5.0 ml of HBSS. The tube was immediately centrifuged at 150 g for seven minutes. The cell pellet was resuspended

in fresh HBSS and the cell concentration was determined with a hemocytometer using as the diluent, Turk's solution (Campbell et al. 1970). The tumor was maintained by serial passage into new A/J recipients, by injecting 1 x $10^6$ of the harvested cells intraperitoneally. Some of the harvested cells were used to immunize strain C57BL/6J mice. This was accomplished by injecting 0.1 ml of a cell suspension, containing 1 x $10^7$ cells per ml subcutaneously, in two sites overlying the two scapulae. In general the tumors became palpable after the fourth day, grew progressively for a further four to five days, and then began to regress.

(b) Skin grafts

Orthotopic skin grafts were applied according to the manner of Billingham and Aldawar (1950) with some modifications. The abdominal skin surface of the donor was shaved, swabbed several times with 70% ethanol, and then excised and reflected. Subcutaneous fascial and fatty tissue was scraped off with a scalpel, and the skin sheet was cut into rectangles of approximately 1.0 $cm^2$ area. Recipient mice were anaesthetized with sodium pentobarbital (0.06 mg/g body weight, given intraperitoneally). A graft bed was prepared on the lateral thorax of the recipient. In some cases beds were prepared for two grafts on one recipient. To prepare a graft bed the thorax was shaved and swabbed with 70% alcohol. A pair of curved dissecting scissors were used to remove a rectangular patch of skin corresponding to the size of the skin graft, by means of many small excisions exposing the vascular network above the panniculus carnosus. Care was exercised

not to damage the blood vessels. The grafts, which had been kept in sterile HBSS, were applied to the graft bed, blotted with a gauze pad, sprayed with paraplast cellulose dressing (Parke, Davis and Co., Detroit, Michigan) and bound with cellulose tape and a band-aid which encircled the thorax. Dressings were usually not removed; when removal was necessary, the band-aid was cut with scissors, and the cellulose tape released by sponging it with water.

iii. Cytological preparations

Clean glass slides were coated with calf serum and allowed to dry. Cell suspensions from which smears would be prepared were adjusted to contain 50% calf serum and spread evenly on the slide surface. After drying (12 hours) the slides were coated with thirty drops of Wright stain (Difco) for three minutes, then an equal volume of distilled water was added. Four minutes later the slide was rinsed in tap water and dried; Canada Balsam and coverslips were applied to the slides which were then viewed at high power (1250 X) with an oil emersion objective. In general 300 cells in at least 10 random fields per slide were counted to determine the differential composition of cell preparations.

iv. Production of peritoneal exudate cells

Peritoneal cells in large numbers were induced by the injection of several materials into the peritoneal cavity. Light paraffin oil, sterilized by autoclaving, was injected in a single 1.5 ml dose intraperitoneally. Thioglycolate fluid medium (Difco) was injected as

a 1.5 ml dose in the same manner, in other mice receiving this irritant. A third material, dextran, having a molecular weight of 5 to 40 x $10^6$ (Pharmachem. Bethlehem, Penn., U.S.A.) was used routinely. It was dissolved in a phosphate-buffered saline, at a concentration of 35.5 mg/ml, autoclaved and then administered as a single 1.5 ml dose intraperitoneally. Stock solutions of dextran were stored at -20°C.

To collect the induced PEC, animals were sacrificed by decapitation and exsanguination, and 3-4 ml of HBSS were injected into the peritoneal cavity. The abdomen was massaged to mix the contents and swabbed with alcohol. The abdominal skin was reflected as a single flap from a lateral incision. Cells were then aspirated as was described for the recovery of SaI cells, in aseptic conditions using a Pasteur pipette with a fire-polished tip. The PEC were maintained in an ice bath at all times, except for the brief centrifugation steps at room temperature. Immediately after harvest, the PEC were centrifuged at 150 g for seven minutes and the supernatant discarded and replaced with fresh cold HBSS. The cells were resuspended and the centrifugation-resuspension procedure repeated to give a total of three cell washes. Following this, the cell suspension was passed through a sterile 400 mesh stainless steel sieve to eliminate small clumps, and the cell concentration determined with a hemocytometer using Turk's solution as the diluent.

v. Use of lymph node and thymus cells

Axillary and brachial lymph nodes were used as a source of sensitized lymphoid cells or normal (control) lymphoid cells, since

they drain the lymphatic areas where the immunizing grafts were placed. The lymph nodes were surgically removed in a sterile manner, dissected free of connective and fat tissues and fragmented in HBSS at room temperature with hypodermic needles in a sterile disposable petri dish (Falcon Plastics). The thymus was exposed by dividing the rib cage. It was grasped with a curved forceps and pulled free of other structures, and then fragmented in the same manner as the lymph nodes. The resulting cell suspensions were aspirated into sterile plastic disposable centrifuge tubes and centrifuged at 150 g for seven minutes. The pellet was resuspended in HBSS and the suspension passed through a stainless steel sieve as previously described. The cell suspension was washed twice more in HBSS and the cell concentration determined using Turk's solution. The suspension was kept at room temperature until used.

vi. PEC migration

The tissue culture medium in all migration experiments consisted of medium TC 199 (Difco), reconsistuted from the dry powder with double distilled water. It was passed through a 0.45 $\mu$ millipore filter (Millipore Corp.) for sterilization and stored at $4^{o}C$ for not more than four weeks. Fetal calf serum (Gibco) was added to the TC 199 to give a final concentration of 20% v/v of serum. The serum was previously heat-inactivated at $56^{o}C$ for 30 minutes and filtered through a millipore membrane as above. In addition, penicillin and streptomycin antibiotics (Gibco) were added to give final concentrations of 100 units per ml and 100 $\mu$gm per ml, respectively. All components were combined just before use; the final medium prepared for the migration culture is termed

complete tissue culture medium (CTCM).

The capillary tubes used were made of Kimax glass, 0.9 to 1.1 mm in diameter and 100 mm in length. They were washed in 1 N sodium hydroxide, rinsed repeatedly in distilled water and autoclaved. Cells to be placed in the capillary tubes were first dispensed into a series of sterile disposable polystyrene tubes. The volume of cell suspension(s) placed in each tube was determined by the concentration of LNC, thymus cells or PEC suspension calculated in advance. A total of $2 \times 10^7$ cells were innoculated into each tube. While some tubes received $2 \times 10^7$ cells from the PEC suspension only, other tubes received, in part, PEC suspension and in part lymph node or thymus cell suspensions, to give a final total amount of $2 \times 10^7$ cells. In early experiments where PEC from different strains of mice were combined (see Results), a similar procedure was used. The polystyrene tubes were centrifuged (simultaneously) at 150 g for 7 minutes, the supernatants discarded, and each cell pellet resuspended in 0.45 ml of CTCM with the aid of a vortex mixer. The resultant cell suspension was kept in an ice bath while the capillary tubes were loaded.

Capillary tubes were held with a sterile hemostat and cell suspensions were drawn into them from the polystyrene tubes by capillary action until the fluid extended to 1 cm from the distal end. This end contained the heat-insulating air space required to protect the cell suspension while the tubes were sealed with a natural gas-oxygen flame. Seven capillary tubes could be prepared from each tube of 0.45 ml cell suspension by this procedure. The sealed capillary tubes were placed

horizontally (to prevent sedimentation) in sterile glass tubes and maintained at 4°C while the remainder were filled. When all tubes had been filled they were centrifuged at 150 g for 7 minutes at 4°C and then stored vertically in an ice bath during assembly of the migration chambers.

Migration chambers were of the Mackaness design (1952), made of lucite having a 2.0 ml capacity each. They were washed in 1.0 N sodium hydroxide, rinsed in distilled water and autoclaved before use. Glass microscope slide cover slips (24 x 24 mm) were washed in 1.0 N sodium hydroxide, then distilled water, and stored under ethanol until use. During chamber assembly the cover slips were held over a Bunsen flame to burn the alcohol and then placed on a sterile surface. For assembly of the chambers two capillary tubes were removed from the same centrifuge tube. The cell pellet in these capillary tubes was approximately 0.7 cm in length and the tube was cut with a diamond-tipped glass cutter 1.0 mm below the cell-fluid interface, insuring that the opening would expose the packed cells only and not the supernatant above them. The cut tubes were quickly fixed to a glass cover slip with sterile silicone grease (Dow Corning) and the open ends of the tubes were immediately covered with a few drops of CTCM to prevent drying. The cover slip was then inverted and applied to the open side of a lucite chamber. The edges of the glass were sealed to the lucite with molten paraffin wax and the chamber filled with 2.0 ml of CTCM via an access channel which was then sealed with wax. The chambers were placed horizontally with the cover slip serving as the bottom surface

in a 37°C incubator for 24 hours. In each experiment three chambers of two capillary tubes each, for a given cell mixture, were assembled. In some experiments 10 x 35 mm sterile disposable plastic petri dishes (Falcon Plastics) were used as culture chambers. In these cases six capillary tubes were placed in one chamber and six ml of complete tissue culture medium were added.

Following the 24 hour incubation period, the cell migration areas were assessed. The culture chambers were placed on a travelling microscope (Beck, London) equipped with a projection viewer (Hudson Photographic Industries) having dimensions of about 4 x 5 inches. The image appearing on the screen was magnified by a factor of twenty-two. The mushroom-like profile of the migrated cells was traced from the screen, onto transparent plastic sheets and the traced area was integrated directly with a planimeter. The six replicate areas which comprised each of the control and test migrations were averaged to give mean areas of migration. For the control, usually, the extent of migrations of PEC alone was determined, for the test(s) usually PEC with added LNC or thymus cells were the migrating populations. The migration area of PEC alone was considered 100% migration, and the mean area of the test cultures, where other cell types had been added into the capillary tubes was related to this with the following expression:

$$\frac{\text{mean area of control migrations} - \text{mean area of test migrations}}{\text{mean area of control migrations}} \times 100$$

= percent inhibition of migration

vii. Additional procedures

As an adjunct to cytological studies it was of interest to investigate some biological properties of the cells used in these experiments. The ability of PEC to phagocytose colloidal material was examined. Gunter-Wagner shellac-free ink (Pelican, C11/1431a) was mixed with an equal volume of 0.3 M sodium chloride and autoclaved. For in vivo injection this suspension was diluted ten-fold in HBSS and 0.5 ml was injected intraperitoneally into ten-week-old C57BL/6J female mice which had received an injection of 1.5 ml of the dextran solution 48 hours earlier. In this way, an attempt was made to observe localization of carbon particles as a consequence of phagocytosis in the different cell types constituting the 72-hour PEC.

The ability of the different cell types in the PEC to adhere to plastic was observed. Sterile disposable tissue culture flasks (Falcon Plastics) were innoculated with $2 \times 10^7$ PEC in 5.0 ml of complete tissue culture medium. The flasks were incubated in an air-$CO_2$ incubator at $37^oC$ for 24 hours with gas flow rates adjusted to give a stable pH of 7.3. The flasks were not agitated. After the incubation the bottom surface of the culture flask was examined with an inverted microscope fitted with phase-contrast optics.

viii. Anti-lymphocytic serum (ALS)

Sheep anti-mouse ALS was supplied by Dr. E. Sabbadini. It was prepared in a single ewe by an intramuscular injection of $5 \times 10^8$ $B6AF_1$ lymphoid cells (spleen, thymus, lymph node), as an emulsion in an equal volume of complete Freunds adjuvant (Difco). After a period

of 3 weeks the sheep received four injections (spaced two days apart) i.m. of $5 \times 10^8$ $B6AF_1$ lymphoid cells in saline and were bled 7 days after the last injection. The blood was allowed to clot at room temperature and after centrifugation the serum was sterilized by filtration through a millipore membrane and stored at $-20^{\circ}C$. As a control, serum from the same animal prior to immunization was used. The ALS or control serum was added to migrating PEC from normal unimmunized C57BL/6J female mice.

The effect of ALS on PEC migration was related to another property of ALS, i.e., to leukoagglutination. This procedure was carried out in the manner described by Currey and Ziff (1968). Lymph nodes from C57BL/6J female mice were collected and fragmented with needles in EDTA buffer composed of 1.96 g $Na_2HPO_4$, 0.65 g $NaH_2PO_4$, 3.0 g $Na_2EDTA$ and 3.5 g NaCl in one liter of distilled water. Large fragments were decanted and the suspended cells aspirated into centrifuge tubes. The cells were sedimented at 150 g for 7 minutes. The supernatant was discarded and the cells were washed twice more in the EDTA buffer. Finally the cells were resuspended at a concentration of $5 \times 10^7$ per ml in the EDTA buffer supplemented with 1% heat-inactivated (at $56^{\circ}C$ for 30 min) fetal calf serum. ALS was diluted two-fold serially in 'U' shaped wells of a haemagglutination plate (Cooke Engineering) in EDTA buffer with microdilutors. The volume of ALS plus diluent was 0.05 ml per well. To each well 0.05 ml of the lymph node cell suspension was added dropwise. Mixing was accomplished by pressing the plate against a vortex mixer. The trays were then stored for 4 hours at room temperature. After this period the cells in each

well were gently resuspended using separate Pasteur pipettes for each well, and a drop from each well was placed on a glass slide and observed at 50 to 100X magnification using a light microscope. The endpoint of agglutination of the leukocytes was considered to be reached when the appearance of the cell suspensions in the test wells did not differ from those in the control wells, i.e., containing normal serum or no serum at all.

# RESULTS

## i. Production and characteristics of PEC

In order to adapt the migration assay for use in mice, a suitable PEC population was sought. Desirable properties were: (i) the ability of the cells to migrate out of capillary tubes, (ii) high yields of cells per mouse, for reasons of economy, and (iii) high proportions of the exudate to comprise the lymphoid cells (lymphocytes and macrophages) known to be involved in the migration inhibition assay in the guinea pig (Bloom and Bennett, 1966). Several peritoneal cavity irritants were selected and studied in young adult (9 - 12 week old) C57BL/6J and A/J female mice. Dextran, light paraffin oil and thioglycollate fluid medium were used. In some cases comparisons of exudates at 24, 48, 72 and 96 hours after injection of the irritant were made, using at least 5 mice per group.

Cell identification using Wright stain was efficient and rapid. Typical polymorphonuclear (PMN) leukocytes were identified. They had pale pink cytoplasm and multilobed nuclei. The majority of cells appeared to be neutrophils and a minority of PMN leukocytes appeared to be damaged, which made identification difficult. Lymphocytes were distinctive, having blue cytoplasm and intensely basophilic nuclei. In general, about 75% of the lymphocytes in all preparations to be described were of the small variety, having diamters of less than 8 $\mu$ and a thin rim of blue cytoplasm. The remainder were medium and large lymphocytes which stained in a manner similar to that

of small lymphocytes but had larger nuclei and cytoplasm and were up to 15 $\mu$ in diameter. A third population of cells having diameters from 15 to 25 $\mu$ are referred to as macrophages. This cell type had a basophilic nucleus and a 'foamy' highly vacuolated cytoplasm which stained pale blue. These cells were somewhat fragile since bare nuclei, resembling macrophage nuclei, were frequently observed in the smear preparations. This cell type constituted a majority among the three cell types recovered, and because of its large volume constituted an even greater proportion with regard to cell mass. While lymphocytes, PMN leukocytes and macrophages appeared in exudates induced with all irritants, the 'foamy' appearance of the macrophages was observed only after the injection of dextran.

Peritoneal contents of mice injected with the dextran solution were analyzed at various times following the injection. Visual inspection of the peritoneal cavity of mice sacrificed at 24 hours following the injection revealed that small amounts of the viscous dextran solution, about 0.5 ml (of the 1.5 ml injected) remained. By 48 hours all liquid had disappeared, and the peritoneal cavity continued to remain free of liquid until the last observation which was made at 96 hours. Removal of the immigrant cells thus depended upon rinsing the peritoneal cavity with HBSS. Heparin was not used since in preliminary experiments it was found that the anticoagulant inhibited cell migration, and was difficult to wash off the PEC.

Although care was exercised to minimize trauma to visceral blood vessels and to the liver during aspiration of the PEC, peritoneal bleeding, which contaminated the PEC with blood, was a frequent problem. This was largely resolved by exsanguination of the mice. As a result, most C57BL/6J PEC preparations were entirely free of red blood cell contamination. When red cells were present, they were apparent after centrifugation as a red layer which sedimented atop the packed white PEC. The aspirated cell suspension was deposited in sterile plastic tubes and placed in an ice bath to prevent the attachment of cells to the plastic surfaces, which occurred at ambient temperatures (20-39$^{o}$C).

Experiments were performed in order to select the time following the injection of dextran which would provide PEC of suitable composition. Accordingly, the dextran solution was injected into otherwise untreated C57BL/6J and A/J female mice, and groups of mice were sacrificed at various times thereafter, and the total cell yield and composition determined. It is evident from the data in Table 1 that the proportion of PMN leukocytes in both strains of mice decreased steadily from 24 to 96 hours. Conversely, the proportion of macrophages increased steadily to a maximum at 96 hours in C57BL/6J mice. Both these findings favour a long induction period in order to obtain PEC rich in macrophages. As can be seen, the proportion of lymphocytes appears to have remained relatively constant. While the lymphocyte proportion appears not to vary with time, it was noted that approximately 75% of the lymphocytes were of the small variety at 24, 48 and 72 hours, but at 96 hours more than 50% were of the large type, for both A/J and C57BL/6J mice.

TABLE 1

The Relationship between Length of the Induction Period on Yield and Composition of Dextran-Induced PEC

| | Average yield per mouse[1] | | Percentage of total leukocytes[2] | | |
|---|---|---|---|---|---|
| strain | cells x $10^{-6}$ | hour | lymphocyte | macrophage | PMN |
| A/J | 4.0 | 24 | 33 | 28 | 36 |
| | 8.3 | 48 | 19 | 57 | 24 |
| | 5.1 | 72 | 23 | 73 | 4 |
| | 2.5 | 96 | 44 | 50 | 0 |
| C57BL/6J | 18 | 24 | 14 | 34 | 52 |
| | 24 | 48 | 17 | 63 | 20 |
| | 17 | 72 | 14 | 79 | 7 |
| | 12 | 96 | 16 | 84 | 0 |

[1] In all experiments mice were 9 to 12 weeks of age.

[2] Differential counts were performed on a pool of cells from 5 to 6 female mice. At least 300 cells were counted in ten or more random fields.

From a practical viewpoint large cell yields were desirable as well. The total cell yield per mouse at different times following the injection of dextran is also shown in Table 1. It is clear that while maximum cell yields occur at 48 hours, the total numbers of lymphocytes and macrophages are comparable at both 48 and 72 hours. In view of the relative absence of PMN leukocytes in the 72-hour PEC, this induction period was routinely employed.

In the experiments to be described, 72-hour PEC were produced in normal (otherwise untreated) mice and in skin-grafted or sensitized mice. The induction of PEC (3 days) in sensitized mice was identical to the method used for normal mice, and a single dextran injection at day 5 to day 19 after skin-grafting allowed for the recovery of 'sensitized PEC' 8 to 22 days after grafting. The effects of immunization on the yield and composition of the PEC is shown in Table 2. While the proportions of constituent cell types were not affected by grafting, the total cell yield increased by 70% in C57BL/6J mice and by 60% in A/J mice. This effect was of practical value, since, when experiments required PEC from immunized mice, fewer mice were required for grafting and for harvesting of PEC.

From a survey of the literature, it appears that for the production of PEC in guinea pigs, mineral or light paraffin oil is a common choice as a peritoneal irritant, e.g., David et al. (1964). The use of oil to induce PEC in mice was attempted, and the 72-hour exudate populations induced with light paraffin oil or dextran in C57BL/6J mice were compared. The first observation concerning oil-induced PEC was

TABLE 2

The irritants used were 1.5 cc of dextran or 1.5 cc of light paraffin oil.

PEC were harvested from A/J mice on day 8 to 12 following orthotopic grafting with skin from C57Bl/6J mice, and from C57BL/6J mice on day 9 to 12 following grafting with A/J skin.

Female mice aged 9 to 12 weeks of age were used. Cell yields are based on averaged yields for 5 to 11 mice in all but one case. Differential counts were performed as for Table 1.

* Over 50% were of the large variety of lymphocyte.

TABLE 2

The Composition of Induced, 72-Hour PEC in Normal and Skin-Grafted Mice

| Strain | Number of mice | Treatment | Percentage of Total Cells | | | Yield per Mouse $X\ 10^{-6} \pm 1$ S.D. |
|---|---|---|---|---|---|---|
| | | | Lymphocyte | Macrophage | PMN | |
| A/J | 55 | normal | 21 | 74 | 5 | $6.7 \pm 1.3$ |
| A/J | 33 | grafted | 16 | 83 | 1 | $10.7 \pm 5.0$ |
| C57BL/6J | 123 | normal | 8 | 83 | 9 | $13.3 \pm 3.0$ |
| C57BL/6J | 39 | grafted | 14 | 86 | 0 | $22.6 \pm 2.7$ |
| C57BL/6J | 5 | oil PEC | 38* | 42 | 20 | 17.0 |

that, despite the precaution of exsanguination, a considerable number of erythrocytes was present in the leukocyte population. In addition, the HBSS aspirated from the peritoneal cavities contained large amounts of oil which coated all the tubes and pipettes necessitating frequent and time consuming transfers of the cell suspension to fresh sterile tubes. Oil-induced PEC migrated in a manner similar to the PEC induced with dextran; however, occasionally, when red cell contamination was severe, abnormally large migrations, likely due to cells spilling from the capillary tubes, were observed. While the total cell yield obtained with oil was similar to that obtained with dextran, it may be seen (Table 2) that the PEC contained a high proportion of lymphocytes and PMN leukocytes. In addition, there was a greater number of large lymphocytes than could be found in dextran-induced PEC. Macrophages appeared as typical blood monocytes having kidney-shaped and eccentrically-placed nuclei.

The decision to use dextran as the agent to induce PEC was based principally upon the practical considerations of obtaining the maximal harvest in terms of numbers of cells, the desirability of large numbers of macrophages and few granulocytes, and the observation that migrations of oil-induced PEC were not very reproducible.

Thioglycollate fluid medium has been used previously to induce PEC in mice (Feldman and Gallily, 1967), and was employed in several of the present experiments. The 72-hour total cell yield was as high as $2\text{-}3 \times 10^8$ cells per mouse, and the preparation was free of red blood cell contamination. However, these cells were completely unable to migrate out of capillary tubes therefore studies with this agent were terminated.

Several experiments were performed to identify more precisely the 'foamy' cell type considered to be a macrophage, by testing for properties commonly attributed to macrophages such as phagocytosis and adherence to glass or plastic. Cell smears were prepared from 72-hour dextran-induced PEC from mice which had received an injection of colloidal carbon intraperitoneally at 48 hours. All of the large foamy cells had dense black cytoplasm while none of the PMN leukocytes contained observable intracellular carbon. Thirty-eight percent of the lymphocytes had minute carbon inclusions in their pale blue cytoplasm . At least 50 cells from each morphological class were examined in random fields.

The ability of the macrophages in the 72-hour dextran-induced PEC to adhere to plastic was confirmed upon microscopic examination of the culture flask surface after 10-12 hours of incubation at $37^{o}C$, as described in the section 'Materials and Methods'. It was found that all the large macrophage-like cells had spread on the plastic surface and were virtually absent from the fluid phase even after gentle agitation. Only a small proportion of the lymphocytes attached to the plastic and most were easily released into the fluid medium by gentle agitation. No PMN leukocytes were observed, indicating that these cells degenerated rapidly during the period of tissue culture.

An observation was made regarding the sedimentation properties of dextran-induced PEC. It was apparent that some cells sedimented very rapidly when a tube containing a suspension of PEC was allowed to stand for several minutes. Microscopically, the cells were seen

to consist, to a large degree, of the large macrophage-like cell. An attempt was made to exploit this physical property for the isolation of these large cells. It was found that if PEC were suspended in 3 to 4 ml of Hanks solution, centrifuged for 30 seconds (peak acceleration about 150 g) and the supernatant discarded, the cell pellet was greatly enriched in large cells. After this process was repeated two additional times, the macrophage-like cells constituted more than 99.5% of the sedimented cells.

In summary, the dextran-induced PEC was an economical and convenient preparation to use, and it contained the cell types (lymphocytes and macrophages), known to be responsible for the phenomenon of migration (and its inhibition) as described for the guinea pig system.

ii. Nature of the migration

After assembly of the migration chambers and the start of the incubation, the chambers were observed at various times in order to study the nature and progress of the migration. Migration began at 4 to 6 hours after incubation in the form of a small monolayer of cells on the coverslip at the mouth of the capillary tube. Cells continued to move out of the capillary tube and onto this cell layer which consequently became several cells deep, while the periphery of the cellular field spread farther from the mouth of the capillary tube in a radial manner. By 24 hours the migration usually covered an area of 12 to 16 $mm^2$. Microscopic examination of the 24-hour migration suggested that each cell moved independently, since at the

periphery of the translucent migration area the cells were monodisperse.

When inhibition of migration occurred, the periphery of the cell field always appeared as a dense cohesive mass, continuous with the non-translucent migration area, (since darkfield illumination was used). When the fluid content of the culture chamber was agitated, the cells forming the migration area of non-inhibited cultures were dispersed into a fine suspension. When inhibitions occurred and the chambers were similarly agitated, the migration field was more slowly dispersed and only large aggregates or clumps of cells would separate. It appears, therefore, that the force underlying migration inhibition may be an increased propensity of cell-to-cell adhesion.

Since the mechanism of migration of PEC from capillary tubes is unknown, the possibility that the cells spilled out of the capillary tubes passively was tested. Migration chambers were assembled and replicates incubated at $4^{o}C$ and $37^{o}C$. A complete failure of migration was observed in chambers incubated at $4^{o}C$.

iii. Inhibition of migration with antiserum

Results of David et al. (1969) showed that antiserum, when present in the tissue culture medium, was able to cause inhibition of migration of PEC from mice against which the antiserum was formed. In addition, transplantation antisera, when mixed with leukocytes carrying donor antigens, are known to result in the agglutination of the leukocytes (Amos, 1953). Thus, it was of interest to compare a given antiserum for its ability to cause inhibition of PEC migration and the ability of the same serum to give a leukoagglutination reaction at varying dilutions. The leukoagglutination titer was determined as

described in 'Materials and Methods' and found to be 128 for a preparation of sheep-anti mouse lymphocyte serum (ALS). In contrast, the preimmunization control serum gave a negative leukoagglutination reaction at dilutions as low as 1:4.

ALS was added to the cultures of migrating C57BL/6J PEC and the preimmunization serum served as the control. The control serum at the high concentration of 1/50 was not inhibitory, but stimulated the migration by 15%. This may be the effect of foreign protein on non-sensitized PEC, observed previously* (David et al., 1964a). The ALS had a profound inhibitory effect on the PEC migration. At low dilutions, i.e., at 1:100, inhibitions in excess of 70% were observed. Even at dilutions of 1:5000 ALS was able to inhibit PEC migration by 20-28% and the data plotted in Figure 1 demonstrate that the inhibition was proportional to the logarithm of the ALS dilution, at dilutions higher than 1/500.

iv. Inhibition of migration as an in vitro correlate of transplantation immunity

(a) PEC as cellular antigens

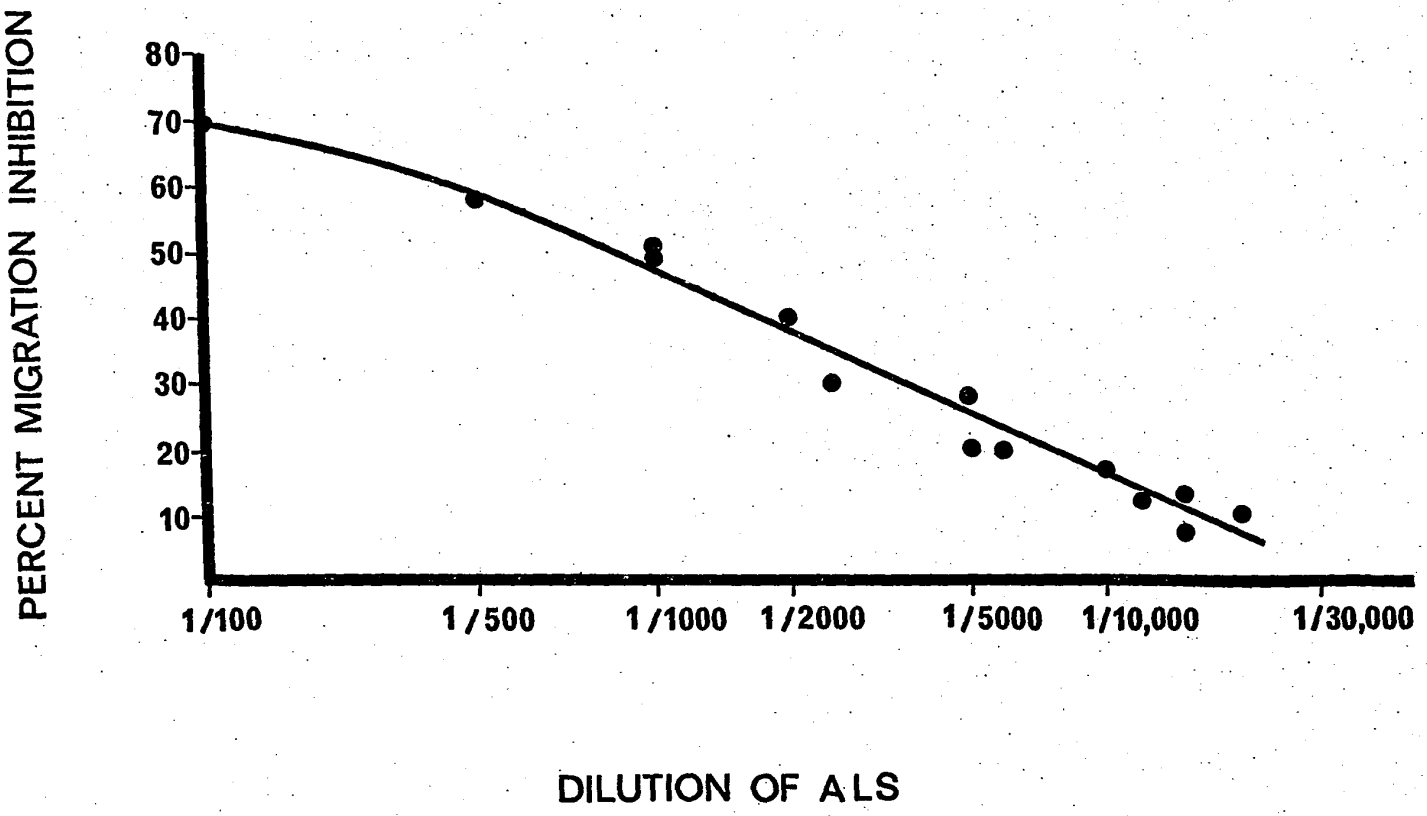
Having shown that dextran-induced PEC were capable of migration, it was necessary to investigate whether this cell preparation could show immune reactivity, i.e., the ability to be inhibited by antigen, when derived from immunized animals. 'Normal' dextran-induced PEC to

* In these experiments, ovalbumin or diptheria toxoid at concentrations of 30 μg/ml in the culture medium enhanced the migration of PEC from non-immunized guinea pigs.

FIGURE 1

The Effect of Sheep-Anti-Mouse ALS on the Migration of Dextran-Induced PEC from C57BL/6J Mice.

The dilution of ALS was recorded as the partial volume of ALS in the culture medium. Female mice, 9 to 12 weeks of age were used. Each point was calculated from the average value of six capillary migration areas.


PERCENT MIGRATION INHIBITION
80
70
60
50
40
30
20
10
1/100
1/500
1/1000
1/2000
1/5000
1/10,000
1/30,000
DILUTION OF ALS

be used in control experiments were obtained from C57BL/6J female mice, and 'immune' dextran-induced PEC were obtained from C57BL/6J mice which had been immunized to strain A/J antigens by a subcutaneous innoculation of the SaI tumor, which shows only a transient growth in C57BL/6J mice.

The C57BL/6J PEC were exposed to A/J antigens by mixing the C57BL/6J PEC with PEC from otherwise untreated A/J mice.

When the PEC from unimmunized C57BL/6J were combined with an equal number of A/J PEC, inhibitions with a mean of 10.5% were obtained (Figure 2). Inhibitions were calculated by comparing the area of the migration of the cell mixture with the average of the two areas of the two PEC preparations migrating independently. Under similar conditions, however using C57BL/6J PEC from SaI-immunized mice, inhibitions of 40% were observed (Figure 2). Owing to the poor yields of A/J PEC, other experiments utilized a migrating population comprised of 20% A/J PEC and 80% C57BL/6J PEC. Under these conditions, using normal C57BL/6J PEC, inhibitions with an average of 13.6% were obtained, while after immunization with SaI inhibitions of 31.6% were recorded (Figure 2). Apparent as well from the data in Figure 2 (see detail) is that the degree of inhibition remained constant from day 8 to 22 after grafting.

In four experiments of this series the effects of varying the cell ratios was studied. It may be seen in Figure 3, that at day 8 following grafting, increasing the proportion of antigenic cells resulted in increased inhibition only at high antigenic cell content,

FIGURE 2

The Effects of Mixing PEC from A/J and C57BL/6J Mice on the Migration of the Resultant Cell Population.

Each point was calculated from the average value of six capillary migration areas obtained in a single experiment. N.B. This applies to all subsequent figures. The mean value and range of the percents migration inhibition for each group is indicated.

The calculation of percent migration inhibition for mixtures of PEC of unequal proportions (e.g. 20% A/J PEC and 80% C57BL/6J PEC) was based on the expression:

$$\frac{\text{(Area of PEC mixture, i.e. 20\% A/J + 80\% C57BL/6J)}}{\text{0.2 (Area of A/J alone) + 0.8 (Area of C57BL/6J alone)}} \times 100$$

Detail in Figure 2: In the experiments where 20% A/J PEC were used, PEC from C57BL/6J mice were obtained on days 8, 9, 11, 13, 19 and 22 after skin grafting, and the resultant inhibitions were 33%, 33%, 21%, 38%, 32% and 33%, respectively (considered in the text).

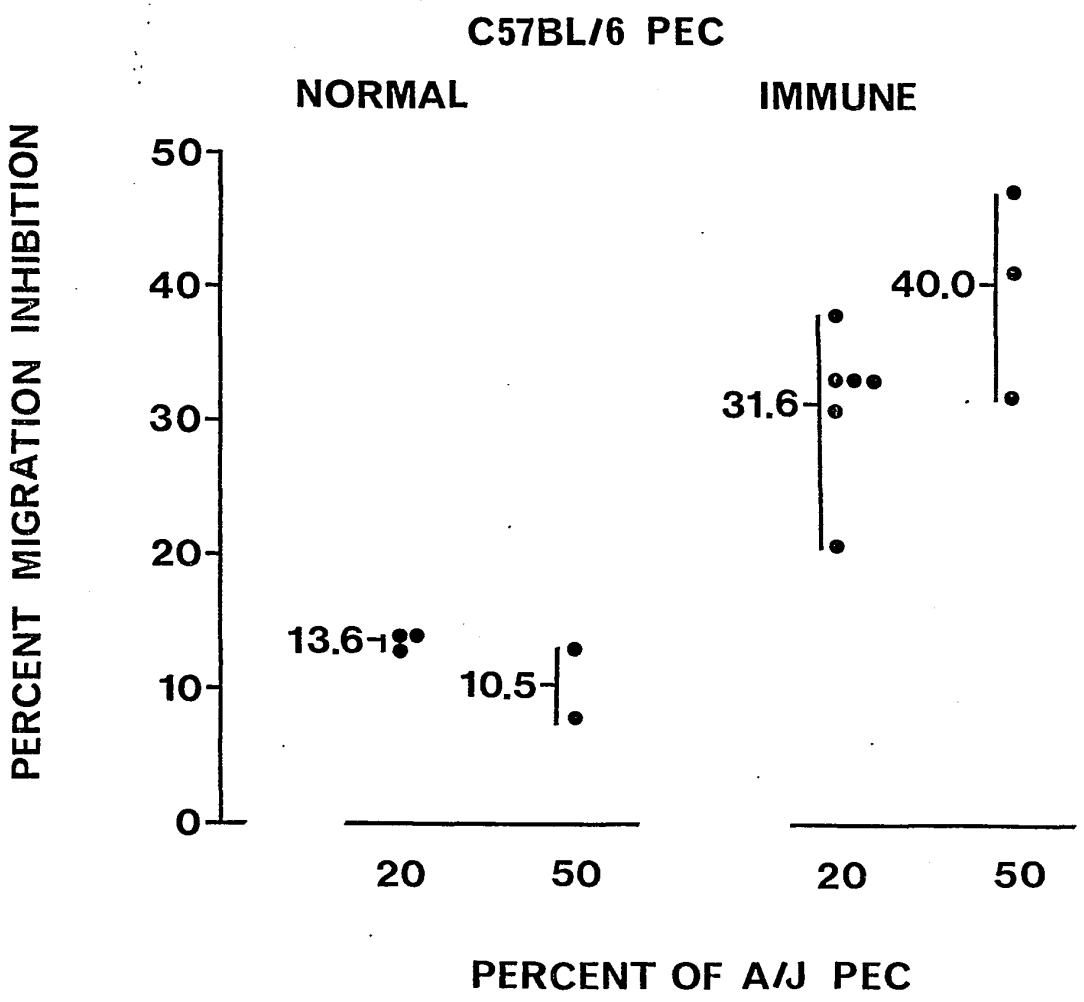
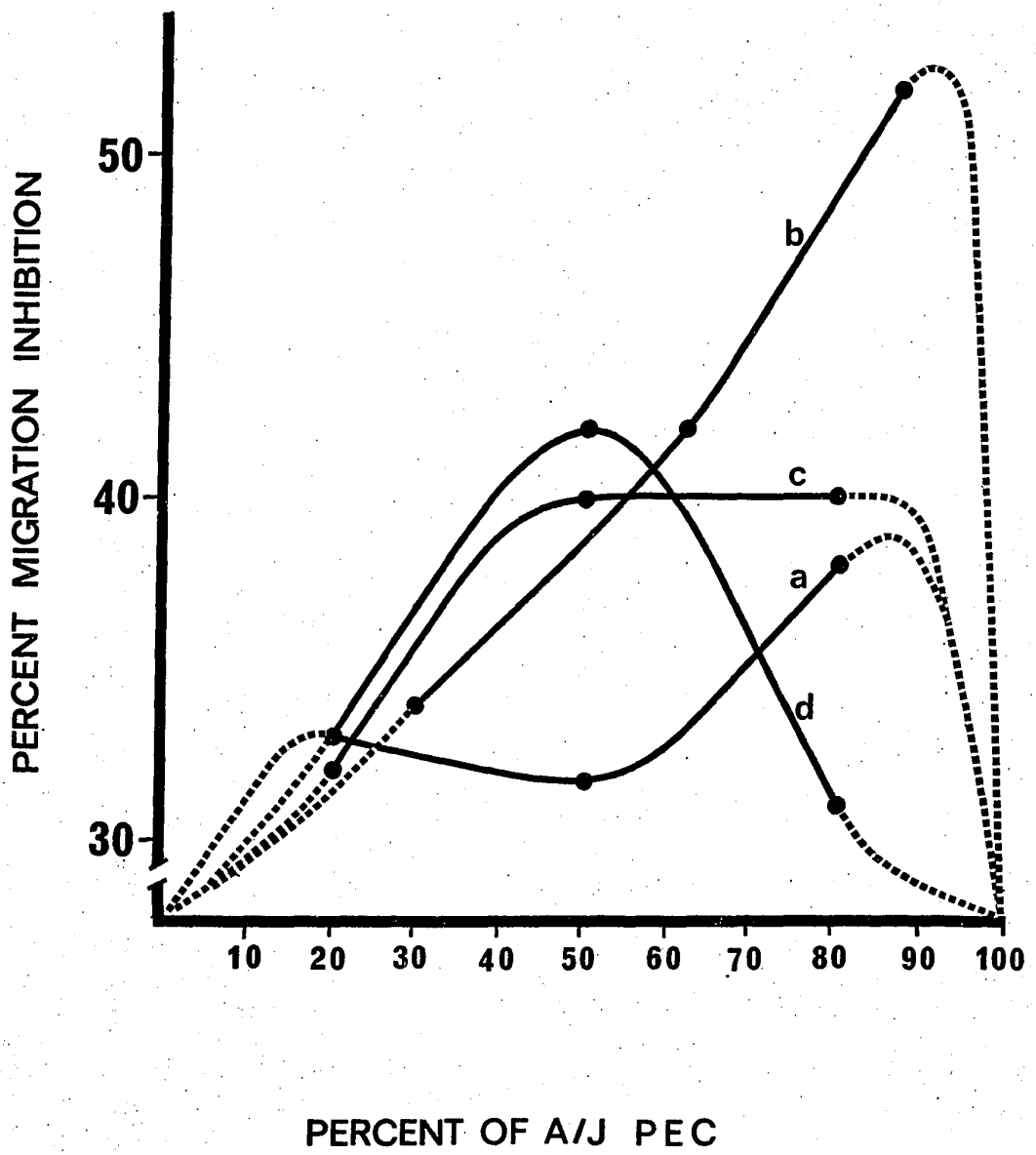

C57BL/6 PEC
NORMAL
IMMUNE
PERCENT MIGRATION INHIBITION
50
40
30
20
10
0
13.6
10.5
31.6
40.0
20
50
20
50
PERCENT OF A/J PEC

FIGURE 3

The Effect of Varying the Proportions of C57BL/6J and A/J PEC on the Inhibition of Migration of the Resultant Cell Mixture.

PEC from C57BL/6J mice immunized with Sarcoma SaI.
C57BL/6J PEC from mice grafted 8 days earlier .... a
10 days earlier .... b
19 days earlier .... c
22 days earlier .... d

Each point was calculated from the average value of six capillary migration areas. All mice were female and 9 to 12 weeks of age.


PERCENT MIGRATION INHIBITION
50
40
30
b
c
a
d
10
20
30
40
50
60
70
80
90
100
PERCENT OF A/J PEC

while at day 10 this effect was evident at both intermediate and high antigenic cell concentrations. At day 19 this increased inhibition was apparent at an intermediate antigenic cell concentration only, but not at high concentrations, and at day 22 increased inhibition was again present at intermediate antigenic cell concentrations but now less inhibition was observed at the higher concentration. These results demonstrate that PEC from sensitized mice can be inhibited in their migration on mixing with antigenic cells and can show altered reactivity which can be related to the time elapsed after immunization, thus confirming and extending the experiments of Al-Askari et al. (1965).

(b) Thymocytes as cellular antigens

Thymus cells from non-immunized animals are known to be poorly immunocompetent when compared with lymph node cells (Billingham and Silvers, 1964), while PEC (likely the lymphocytic elements) are highly immunocompetent (Kornfeld and Weyzen, 1968). In the assay just described use was made of the antigenic properties of donor A/J PEC, but contributions to the observed inhibitions, due to some immunological event resulting from reactions of A/J PEC against C57BL/6J PEC antigens, could not be ruled out. Therefore in this series of experiments donor thymocytes were used in place of donor PEC, since they provide an adequate source of H-2 antigens (Winn, 1962; Billingham and Silvers, 1964) and could enable a more simple interpretation of the results.

Preliminary experiments in which syngeneic or allogeneic thymocytes were added to migrating PEC from normal C57BL/6J mice were carried out to test for possible non-specific inhibitory effects

attributable to the thymus cells. In Figure 4 it can be seen that when the thymocyte content of the PEC-thymus cell mixture was 20%, 43%, or 60% there was no detectable difference in the migration of this mixture from the migration of normal C57BL/6J PEC. Syngeneic and allogeneic (A/J) thymus cells behaved alike in this respect.

For routine experiments the cellular content of the capillary tubes was adjusted to contain 20% thymus cells and 80% PEC. In the first series of experiments PEC from either normal A/J mice, or A/J mice grafted 9 to 12 days previously with single C57BL/6J skin grafts, were employed. The PEC were mixed with thymus cells from the graft donor C57BL/6J (test), or with thymus cells from syngeneic A/J mice (control), or thymus cells from C3H/HeJ mice used as an additional control. The results in Figure 5 show that the addition of thymus cells of syngeneic or allogeneic origin to PEC from normal A/J mice was without effect. However, the migration of PEC from A/J mice grafted with skin from C57BL/6J mice was markedly inhibited (43%) by thymus cells of C57BL/6J origin; however thymus cells from syngeneic A/J mice and from C3H/HeJ mice did not significantly inhibit the migration of the immune PEC, demonstrating the immunological specificity of this reaction.

In another group of experiments C57BL/6J mice were immunized with a single skin graft from A/J mice. The experimental design remained unchanged from the previous experiments, i.e., thymus cells were added to the migrating C57BL/6J PEC in an adjusted ratio, whereby the migrating population contained 20% thymus cells and 80% PEC. As

FIGURE 4

The Effect of the Addition of Various Proportions of Syngeneic or Allogeneic Thymocytes to PEC from Unimmunized C57BL/6J Mice on the Migration of the Resultant Cell Mixture.

The mean value and range of the percents migration inhibition is indicated for each group. Each point was calculated from the average value of six capillary migration areas. Female mice 9 to 12 weeks old were used.

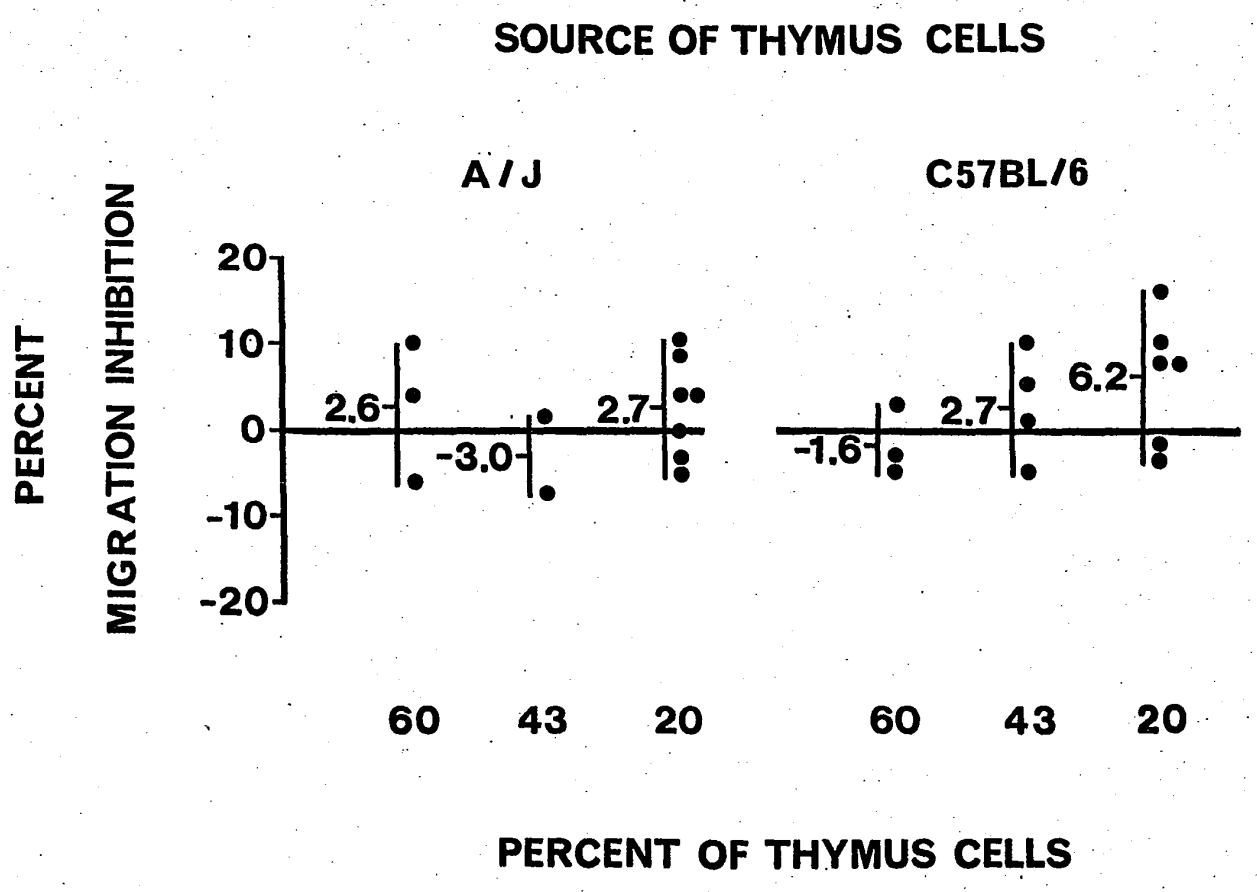
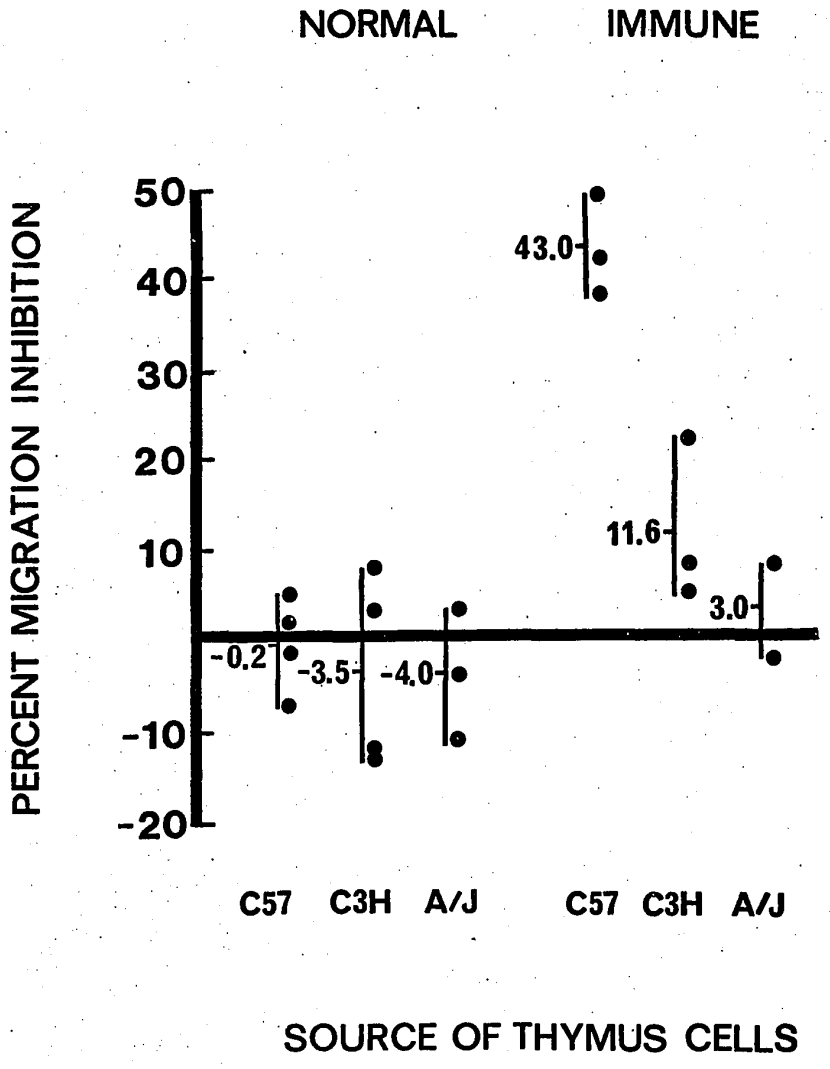

SOURCE OF THYMUS CELLS
A / J
C57BL/6
PERCENT MIGRATION INHIBITION
20
10
0
-10
-20
2.6
-3.0
2.7
-1.6
2.7
6.2
60
43
20
60
43
20
PERCENT OF THYMUS CELLS

FIGURE 5

The Effect of the Addition of Thymus Cells from Various Mouse Strains to PEC from A/J Mice on the Migration of the PEC.

"Normal" PEC were obtained from non-grafted A/J mice while "immune" PEC were obtained from A/J mice grafted with C57BL/6J skin 9 to 12 days earlier. Each point was calculated from the average value of six capillary migration areas; the mean value and range of the percents migration inhibition is indicated for each group. In each experiment mice of one sex only were used.


NORMAL
IMMUNE
PERCENT MIGRATION INHIBITION
50
40
30
20
10
-10
-20
43.0
11.6
3.0
-0.2
-3.5
-4.0
C57
C3H
A/J
C57
C3H
A/J
SOURCE OF THYMUS CELLS

illustrated in Figure 6, the addition of syngeneic or allogeneic thymus cells to migrating PEC from C57BL/6J mice was without effect. However, when the PEC were obtained from mice immunized 9 to 14 days prior with a single A/J skin graft, the migration of the PEC was inhibited (29.7%) when thymus cells from A/J were added, and 35.5% when thymus cells from C3H/HeJ mice were used, thus showing inhibition due to graft-donor thymus cells and to third-party thymus cells. Syngeneic C57BL/6J thymus cells produced no inhibition, demonstrating the specificity of the reaction.

In a study of the time course of this in vitro immune reactivity it was found that PEC from C57BL/6J mice grafted 7 days previously with A/J skin were not inhibited by A/J thymus cells (-9%), and that PEC from mice immunized 8 days previously were only moderately inhibited (16%).

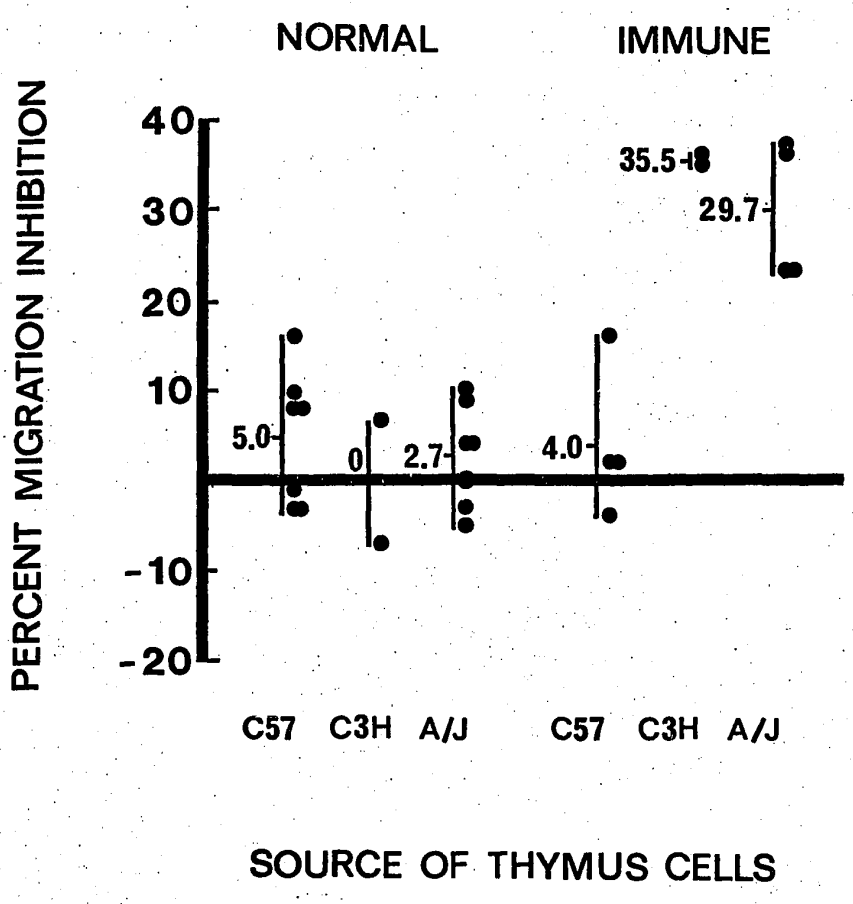
The choice of 20% thymus cells in the migrating population was based on data which showed that 'non-immune PEC' remained unaffected in their migration by this or even a greater number of thymus cells (see Figure 4). It was therefore of importance to know whether the presence of 20% thymus cells was a suitable antigen concentration to affect 'immune PEC', since the possibility remained that this antigen dose could be limiting, and that a higher percent content of thymus cells in the migrating population might result in greater inhibitions.

In an experiment designed to test this possibility the percent of A/J thymus cells was reduced in a population of 'immune C57BL/6J PEC' (obtained 10 days following an A/J skin graft), and the

FIGURE 6

The Effect of the Addition of Thymus Cells from Various Mouse Strains to PEC from C57BL/6J Mice on the Migration of the PEC.

"Normal" PEC were obtained from non-grafted C57BL/6J mice. "Immune" PEC were obtained from C57BL/6J mice grafted with A/J skin 9 to 14 days earlier. Each point was calculated from the average value of six capillary migration areas. The average value and range of the percents migration inhibition is indicated for each group. In each experiment mice of one sex only were used.


NORMAL
IMMUNE
PERCENT MIGRATION INHIBITION
40
30
20
10
-10
-20
35.5
29.7
5.0
0
2.7
4.0
C57
C3H
A/J
C57
C3H
A/J
SOURCE OF THYMUS CELLS

inhibitions of migration recorded. When the percent of A/J thymus cells was 20, 11.1 or 5.9 the percents migration inhibition were identical (40%), while at 3.0% thymus cells the inhibition of migration decreased (23%). These results suggest that immune migrating cells containing 20% thymocytes are exposed to a non-limiting quantity of antigen, and demonstrate the sensitivity of the assay.

In summary, it was shown that strain A/J and C3H/HeJ thymus cells were able to inhibit the migration of PEC from C57BL/6J mice immunized to skin from A/J mice. Although C3H/HeJ was not the graft donor these findings suggest that C3H/HeJ carries some antigens in common with A/J that evoke the inhibition of migration. Furthermore, these experiments show that thymocytes can serve as an adequate source of cellular antigens in the migration assay, and can evoke inhibition of migration in an immunologically specific manner. A migrating population of PEC from immune mice containing twenty percent of antigenic thymus cells was shown to carry antigen in amounts more than four-fold excess; this suggests that the maximum degree of inhibition attainable with the migration assay may approximate 40%.

(c) A migration assay for sensitized lymph node cells

The model system for the inhibition of migration assay in the guinea pig was considered in the 'Introduction'. The mechanism for the inhibition of PEC migration proposes an interaction between a lymphocyte-like immune cell and the antigen, resulting in the release of factor(s) which inhibit the migration of other motile cells (macrophages).

It was considered that cells derived from lymph nodes draining the graft site 9 to 11 days following skin grafting might serve as the first component of the model (as similar cells were used by David (1966) to first demonstrate MIF). If the migrating cells were obtained from the strain serving as the graft donor, and the two cell types mixed together, the migration system would be complete. In such a situation the sensitized lymph node cells (LNC) might recognize the histocompatibility antigens carried by the migrating cells and secondarily inhibit their migration.

In these experiments immunizations consisted of placing bilateral grafts of C57BL/6J skin on A/J recipients. This allowed for economy in the strain serving as the source of LNC. The LNC from normal A/J mice or from A/J mice grafted 9 to 12 days prior with C57BL/6J skin were added to PEC induced in A/J, C3H/HeJ or C57BL/6J mice. The cell proportions comprising the migrating population were adjusted to contain 20% LNC and 80% PEC.

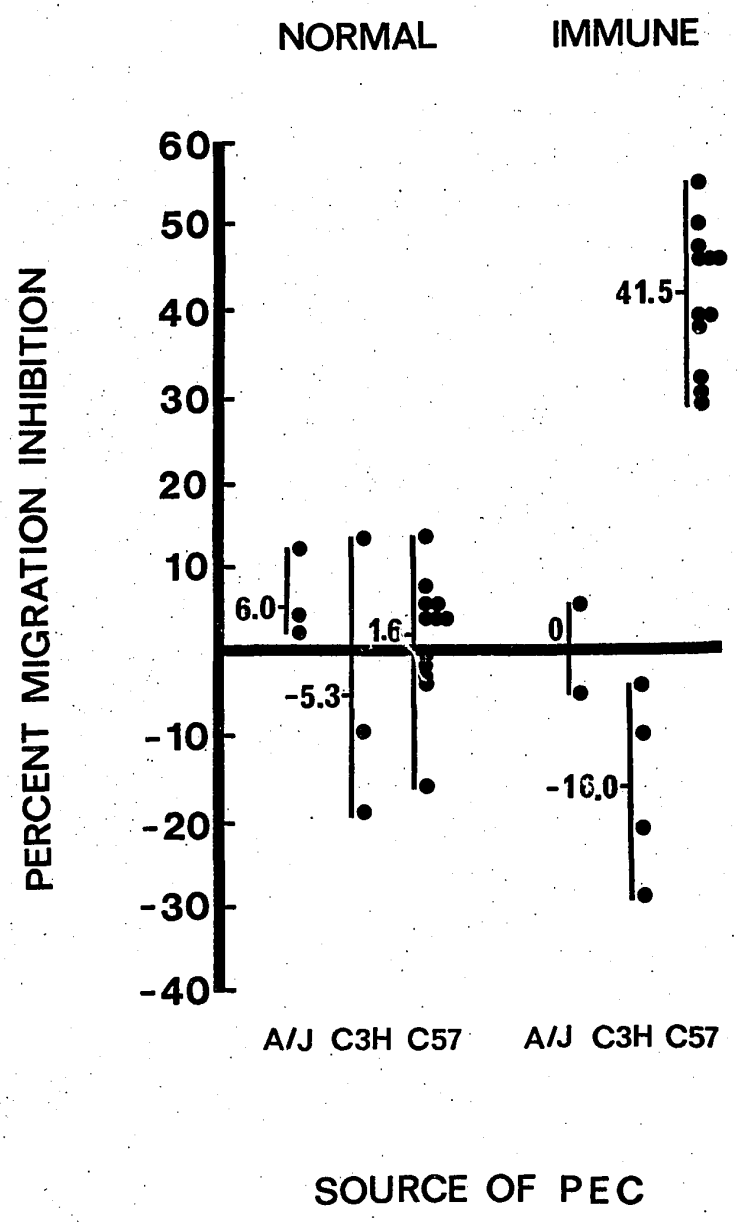
As can be seen from Figure 7, normal A/J LNC when added to syngeneic A/J or allogeneic (C3H/HeJ or C57BL/6J) PEC produced no inhibitions, but the variability within the groups may be slightly greater than when thymus cells were added to normal PEC. The extreme group averages were from 6% inhibition of migration of syngeneic A/J PEC to 5.3% stimulation of migration of allogeneic C3H/HeJ PEC.

In contrast, LNC from A/J mice grafted with C57BL/6J skin strongly inhibited the migration of C57BL/6J PEC (average of 41.5%) but were without significant effect on the migration of syngeneic

FIGURE 7

The Effect of the Addition of Lymph Node Cells from Untreated A/J Mice and from A/J Mice Grafted with C57BL/6J Skin to PEC from Various Mouse Strains on the Migration of the PEC.

"Normal LNC"were obtained from non-grafted A/J mice. "Immune LNC"were obtained from A/J mice grafted with C57BL/6J skin. Each point was calculated from the average of six capillary migration areas. The average value and range in percent migration inhibition is indicated for each group. In each experiment mice of one sex only were used.


NORMAL
IMMUNE
PERCENT MIGRATION INHIBITION
60
50
40
30
20
10
-10
-20
-30
-40
41.5
6.0
1.6
0
-5.3
-16.0
A/J C3H C57
A/J C3H C57
SOURCE OF PEC

A/J PEC. PEC from C3H/HeJ were not inhibited, confirming the specificity of the assay. In reciprocal experiments C3H/HeJ served as the graft donor and it was shown that C3H/HeJ PEC could be inhibited; values of 18% and 21% inhibition of migration were obtained.

These results show that lymph node cells from skin-grafted mice, when mixed with PEC from the strain which served as the graft donor, can inhibit the migration of the cell mixture in an immunologically specific manner. Further consideration of the data follows in the 'Discussion'.

(d) The relationship between the degree of inhibition of migration and the concentration of lymph node cells

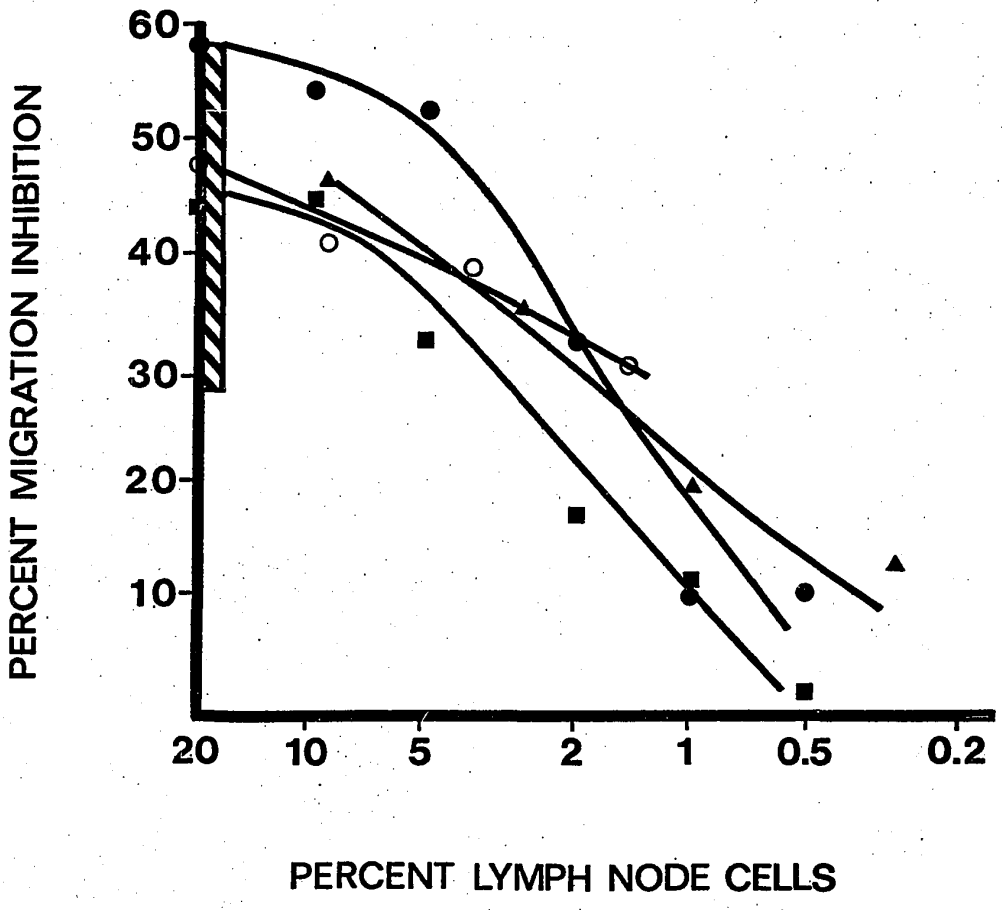
A series of experiments were carried out to assess the sensitivity of the above assay which employed sensitized LNC and migrating PEC and, possibly, to gain insight into the mechanism underlying the inhibition of migration that occurs in this modification of the assay.

LNC from A/J mice, grafted with C57BL/6J skin 9 to 12 days previously, were added to PEC from normal C57BL/6J mice. The percent of LNC present in the migrating population was varied from 20% to 0.15% and the corresponding inhibitions of migration were recorded. Figure 8 shows, on a semi-logarithmetic plot , the percent of LNC contained in the migrating populations and the resultant inhibitions in four experiments. The hatched area represents the range of inhibitions obtained in twelve experiments using 20% LNC, (the average was 41.5% inhibition). It is evident that at concentrations of 20% LNC the inhibitions approached a maximum of 40 to 60 percent. Apparent as

FIGURE 8

The Effect of the Addition of Various Proportions of Lymph Node Cells from Immunized A/J Mice to C57BL/6J PEC on the Migration of the Cell Mixture.

Each point was calculated from the average value of six capillary migration areas. Each curve is derived from a separate experiment, in which LNC were obtained from A/J mice grafted 9 to 12 days earlier. The hatched area represents the range of inhibitions obtained in twelve experiments using 20% LNC, (the average was 41.5% inhibition).


PERCENT MIGRATION INHIBITION
60
50
40
30
20
10
20
10
5
2
1
0.5
0.2
PERCENT LYMPH NODE CELLS

well is a linear relationship between the logarithm of the LNC concentration and the percent of inhibition of migration.

This latter finding suggests that the assay can be employed for a comparison of the immunity of different cell populations, on a quantitative basis, in terms of inhibition of migration. For example, in other experiments LNC obtained from A/J mice 6 days following grafting were able to inhibit the migration of C57BL/6J PEC by 33% at a concentration of 20% LNC and by 22% at a concentration of 11% LNC. LNC from mice immunized 9 to 12 days earlier do not show (proportionately) such a rapid decrease of activity when similarly diluted (see Figure 8). This rapid decrease does occur (Figure 8) with LNC concentrations beginning at 4 or 5%. Thus, it may be inferred that LNC from mice immunized 9 to 12 days prior to cell harvesting contain sensitized cells in frequencies of 4 to 5 times greater than are present in lymph nodes at 6 days after grafting. Similarly, when the LNC were taken from mice grafted 22 days earlier with C57BL/6J skin, an inhibition of 26% was obtained when LNC were present at a concentration of 20%, but with a concentration of 10% LNC the inhibition fell to 7%, well within the range of inhibitions caused by normal LNC (control). In this case the proportion of sensitized cells in the draining lymph nodes at day 22 may have differed by a factor of 20 when compared with the proportion of sensitized cells in the lymph nodes of mice grafted 9 to 12 days prior to cell harvesting.

In summary, the mixture of lymph node cells from skin-grafted mice with PEC from the strain which served as the graft donor was shown to provide the basis for a highly sensitive assay, i.e., as few as 2% or 1% LNC were able to inhibit the migration of antigenic PEC. In addition, this experimental design provides a method for quantitative comparison of the immunological activity of different populations of sensitized cells.

# DISCUSSION

## i. Properties of murine peritoneal exudate cells

Peritoneal exudate cells (PEC) induced with dextran in mice showed properties very similar to those reported for guinea pig PEC. The relative proportions of macrophages and lymphocytes (approximately 70% and 30%, respectively) agree well with the findings for the guinea pig (Bloom and Bennett, 1966). The lymphocyte population was cytologically typical, but cells of the macrophage population were unusually large in size and contained many cytoplasmic vacuoles. These latter cells were motile, and were the predominant cell type in the migration field. In addition, they were able to adhere to plastic surfaces and to phagocytose colloidal carbon. These findings support the suggestion that these large cells are similar in kind to the mononuclear phagocytic cells which appear in relatively high numbers in the peritoneal cavity of normal animals.

This large 'foamy' cell, considered to be a highly vacuolated macrophage, closely resembles cells of macrophage myeloid colonies that grow in vitro from bone marrow cells cultured in agar (Pluznik and Sachs, 1966). Ingestion of non-degradable polysaccharides of agar or of dextran is the likely cause of the sustained vacuolization of these cells.

According to van Firth and Cohn (1968), these peritoneal phagocytes arise from newly-formed (half-time 22 hours) circulating monocytes and, in their experiments, a rapid entry of these cells into the peritoneum could be evoked by inducing a sterile peritoneal inflammation.

Inbred strains of mice differ from each other in many respects such as hematocrit, body weight(Green, 1966) and the ability to respond to certain antigens (McDevitt and Tyan, 1968). It was, therefore, not surprising to find that the yield of PEC formed after 72 hours in C57BL/6J mice was greater (about 3 times) than that of A/J mice. However, the similarity of the two exudates with regard to differential cellular composition suggests that the exudates are indeed comparable.

Of interest was the finding that PEC induced with thioglycollate fluid medium were unable to migrate. This suggests that such PEC have altered properties and that interpretations of experiments in which they are used should be advanced cautiously.

When exudates were induced 6 to 8 days after grafting, the cell yield at 72 hours was markedly increased. This effect is likely due to the stress and trauma of grafting and to possible local infections attributable to the surgical procedure. These factors likely result in increased numbers of circulating leukocytes which could be recruited into the peritoneal cavity.

The PEC population induced with dextran was able to migrate, forming a mushroom-like cell field on the glass surface. The areas of migration formed by these cells (12-16 $mm^2$) were comparable to the areas described by George and Vaughn (1962) for the migration of oil-induced PEC of the guinea pig.

No cell migration was detected in culture chambers incubated at $4^{\circ}C$ for 24 hours, which is interpreted as indicating that the migration is an active, metabolism-dependent process.

The observations of David et al. (1969), that antibody, capable of reacting with antigens of the migrating cells, was able to inhibit the migration of PEC, was confirmed. Thus, a preparation of sheep-anti-mouse ALS having a leukoagglutination titre of 128 could inhibit the migration of mouse PEC at dilutions up to 1:5000. It therefore appears that the migration assay is markedly more sensitive than the leukoagglutination test for detecting antibody activity.

The sensitivity of this assay may be ascribed to at least three processes mediated by antibody. The first may be due to a simple agglutination of the PEC by antibody, preventing the cellular deaggregation needed for migration. Support for this suggestion is found in the report of Thompson et al. (1968), who showed that human peripheral blood leukocytes, treated with anti-HL-A antibody, remained aggregated in pellet form in capillary tubes which were inverted at $45^{o}$ for 2 hours at room temperature; by contrast non-treated cells flowed freely down the capillary tube. A second mechanism could proceed via antibody molecules bound to migrating cells acting as opsonins (Tizard, 1969), attracting and binding other macrophages to the exposed Fc end of the molecule. In fact, the work of Spitler et al. (1969) supports this hypothesis, since they demonstrated that antigenic cells coated with IgG antibody when mixed with PEC from normal guinea pigs inhibited the migration of such PEC. A third mechanism may involve the activation of lymphocytes by antigen-antibody complexes (Möller, 1969; Oppenheim, 1969) and the release of substances which inhibit PEC migration. The production of such bio-active substances

by transformed lymphocytes is well documented (Bloom, 1969).

Of the suggested processes by which ALS may inhibit migration, it is attractive to consider the mechanism of opsoninization, since it has been shown that the immunosuppressive potency of ALS can be correlated with its ability to act as an opsonin in vivo (Martin and Miller, 1969). The authors correlated the immunosuppressive potency of different ALS preparations with the ability of these sera to cause the removal of radiolabeled lymphocytes from the circulation by the reticuloendothelial system.

Thus the migration assay may provide a new test for assaying the activity of ALS in vitro and the linearity of the plot relating the logarithum of ALS dilution to inhibition of migration (Figure 1) provides an accurate measure for quantitative comparisons of different antisera.

ii. Inhibition of migration as an in vitro correlate of transplantation immunity

Initial experiments employed PEC from SaI-tumor-immunized C57BL/6J mice. These mice had received bilateral subcutaneous inoculations of tumor cells on day 0 and an intraperitoneal (booster) injection of $1 \times 10^7$ tumor cells on day 7. Dextran-induced PEC from such mice harvested on day 10 migrated very poorly, as if already inhibited, when compared with PEC from mice which did not receive the i.p. booster injection; therefore, in subsequent experiments the secondary immunization on day 7 was omitted. The poor migration observed was not unexpected, since Nelson and North (1965) had shown

that peritoneal cells of guinea pigs having delayed hypersensitivity to an antigen aggregate *in vivo* when exposed to the antigen.

Experiments designed to test the efficacy of dextran-induced PEC to be inhibited, and to confirm the findings of Al-Askari *et al.* (1965), were successful. PEC obtained from C57BL/6J mice injected with the SaI tumor, when mixed with A/J PEC, produced migration areas markedly smaller than the combined average areas of C57BL/6J PEC and A/J PEC, recorded in the same experiment when these cells were migrating separately. As indicated in 'Results', PEC which were inhibited could be dispersed as large aggregates into the culture fluid, when the culture chambers were agitated. By contrast, non-inhibited PEC could be dispersed into a fine suspension, suggesting that an aggregation of inhibited PEC had occurred. The macrophage disappearance reaction *in vivo* has been attributed to such a phenomenon (Nelson and North, 1965) and more recently, the aggregation of PEC from guinea pigs (having delayed hypersensitivity) when cultured *in vitro* in the presence of antigen has been described (Somsak *et al.*, 1970). Thus the underlying mechanism of inhibition of migration may be due to metabolic processes causing cell-to-cell adhesion.

Microscopic observations showed that the periphery of the migration fields of inhibited cultures had very clear boundaries with no single cells, confirming that the cell population had aggregated during migration. Uninhibited migration fields always displayed a 'halo' of single cells about the periphery of the main migration zone.

These experiments indicated that PEC from normal C57BL/6J mice mixed with allogeneic PEC from A/J mice migrated quite well, but showed slight inhibitions having an overall average of 12.4% (range 8-14%). However, when the PEC were induced in C57BL/6J mice grafted with SaI from strain A/J mice, inhibitions were observed with an overall average of 34.4% (range 21-46%). No notable differences were observed regarding the degree of inhibition and the day of experimentation after immunization (from day 8 to day 22) using the above experimental procedure.

Experiments were performed with PEC from C57BL/6J mice immunized to SaI tumor to compare the inhibitions resulting from mixing different ratios of A/J PEC and C57BL/6J PEC, and to observe the nature of the resulting inhibitions with time. When PEC were obtained 10 days after tumor grafting and the percent of C57BL/6J PEC was decreased from 70% to 12%, there resulted a continuous rise in the degree of inhibition (from 34% to 54%), as illustrated in Figure 3. These results suggest that the PEC are inhibited by a process other than aggregation of PEC from the immune C57BL/6J mice. This conclusion is based on the hypothesis that, if the mechanism of inhibition of C57BL/6J PEC by A/J PEC was simply an aggregation of C57BL/6J 'immune PEC' in the presence of A/J cellular antigens, e.g., by cytophilic antibodies carried by C57BL/6J macrophages (thereby impeding the movement of the PEC mixture), it could be argued that decreasing the capillary tube content of C57BL/6J PEC would decrease the inhibition. However, the opposite effect was seen. An hypothesis that would satisfy this

observation is that A/J antigenic cells act on C57BL/6J PEC not via an aggregation mechanism but evoke the production of a migration inhibitory factor (MIF) from the immune cells as has been characterized in the guinea pig system (Bloom and Bennett, 1966).

While the absolute values of inhibitions obtained on different days may not be justifiably compared, since minor experimental differences likely exist, the shapes of the curves, each drawn from a single experiment, can be compared. The interpretation of the data is based on the following considerations. It is obvious that as the proportions of A/J PEC increase, the number of C57BL/6J cells capable of effecting the inhibitory influence decreases. However, the remaining immune cells are now subject to higher cellular antigen concentration, increasing the probability of a recognition event between a sensitized cell and an antigenic cell. Thus graphic representation of such a study may be expected to show no inhibitions at 0% antigenic cell content nor at 100% antigenic cell content, but a curve with a maximum at some optimal cell ratio. The final consideration is the assumption that a given proportion of reacting immune cells can inhibit PEC of the immune strain C57BL/6J and PEC of the donor strain A/J to the same degree. This may be safely assumed since it has been demonstrated that PEC from unrelated strains such as B10.BR and C3H/HeJ or A/J and B10.A, but having common antigens, are inhibited equally by immune cells reacting to their antigens (Lake, unpublished results ). The results may be interpreted (subject to the above contingencies) to indicate that in the population of C57BL/6J cells shortly after

immunization there is a high proportion of sensitized cells, since high values of migration inhibition are observed when these cells are highly diluted, and that this proportion may be higher on day 10 than on day 8, since the slope of curve b. (see Figure 3) on day 10 is much steeper and the degree of inhibition higher than that of a. (day 8). On day 19 and day 22 after grafting, the curves show opposite trends. Beyond 50% content of antigenic cells inhibitions do not increase, but are seen to decrease for PEC of day 22. This finding indicates that the proportion of sensitized cells in the C57BL/6J exudate has decreased at this time.

Since peritoneal leukocytes are considered to be derived from blood-borne cells (van Firth and Cohn, 1968), it is suggested that the proportion of sensitized cells in the peritoneal cavity and in the blood reaches a peak at about day 10 after grafting. These results agree well with the findings of Brunner *et al.* (1970) who showed that at 10 to 11 days following allografting, blood leukocytes of recipient mice have maximal *in vitro* cytotoxic activity.

While dextran-induced PEC from immune mice were capable of being inhibited in experiments analagous to those of Al-Askari *et al.* (1965), the experimental design includes at least two serious shortcomings. The first is the possibility of cell destruction occurring within the capillary tubes. Granger and Weiser (1964) have shown that PEC from mice sensitized to transplantation antigens of another strain, produced lytic plaques (death of both types of PEC), when such cells were placed onto monolayers of donor PEC. Thus PEC from the immune

mice could simply destroy the migrating elements thereby producing inhibitions. A second drawback is that while the inhibitory effect observed may be attributed to the immune population, an augmentation of this inhibition due to activities of the immunocompetent antigenic population is possible. In this case an MLC type of reaction (in both directions) may potentiate the activity of the sensitized cells; as may be seen in Figure 2 mixtures of allogeneic PEC did result in small inhibitions.

To reduce the likelihood of these two possibilities, thymocytes were chosen as the source of antigenic cells in subsequent experiments.

iii. Thymocytes as cellular antigens

Thymocytes were considered to be the most suitable source of cellular antigens since this cell population, being largely lymphoid, is known to carry a relatively high density of H-2 antigens (Winn, 1962), and is quite homogeneous, consisting of lymphocytic elements in excess of 99%. In addition, thymocytes are known to be relatively non-immunocompetent as shown by their poor ability to induce graft-versus-host reactions (Billingham and Silvers, 1964) and antibody formation (Claman et al., 1966). Thus, if inhibitions occurred using these cells as the antigen source, interpretation of the results would be much simplified.

Control experiments showed that a 20% content of syngeneic or allogeneic thymus cells in a population of PEC from normal mice had no detectable effect on the migration of these PEC. In the experiments

employing thymus cells as antigen, normal A/J mice grafted with C57BL/6J skin were used as the source of PEC. Strong inhibitions occurred when thymus cells from C57BL/6J mice were added to the migrating cells, while in the two experiments in which C57BL/6J thymus cells gave the greatest inhibitions thymus cells from C3H/HeJ produced no inhibitions. In the third experiment a weak inhibition by C3H/HeJ thymus cells was observed. Arguing against the significance of this reaction is an experiment which demonstrates that thymocytes are present in values as high as four-fold antigen excess, vide infra. Thus the antigenic effect, if any, of the C3H/HeJ cells would be at most one-seventh that of C57BL/6J. No inhibitions were produced by syngeneic A/J thymus cells.

This experiment demonstrates the immunological specificity of the inhibition observed. According to available H-2 serotyping data (Klein and Shreffler, 1971), C3H/HeJ carries none of the H-2 antigens to which A/J reacts, when immunized with C57BL/6J grafts. However, cross-reaction at the weaker non-H-2 loci may account for the questionable inhibition produced by C3H/HeJ in the one experiment.

In experiments in which C57BL/6J mice were the recipients of skin grafts from A/J mice, the addition of A/J thymus cells to PEC from immune C57BL/6J mice produced inhibitions of migration of the PEC. In these experiments thymus cells from C3H/HeJ mice were able to produce

strong inhibitions as well. This result was expected since cells of C3H/HeJ mice carry 7 of the 15 H-2 specificities that C57BL/6J mice recognize when immunized with A/J grafts. In addition, there may be recognition of non-H-2 transplantation antigens common to both A/J and C3H/HeJ mice.

The finding that despite the absence of 8 H-2 specificities strong reactions were observed, points to the sensitivity of the assay.

Another experiment which illustrates the sensitivity of this test shows the relationship between the degree of inhibition and the capillary tube content of antigenic cells. PEC from C57BL/6J mice grafted ten days earlier with A/J skin were mixed with A/J thymus cells to give thymus cell proportions of 20%, 11.1%, 5.9% and 3.0%. The degrees of inhibitions observed were 40%, 40%, 40% and 23% respectively. This finding indicates that only very few antigenic cells are required to produce maximal inhibitions and suggests that all experiments in which thymus cells were used as antigen were performed with antigen in three to four-fold excess.

Using this experimental design (immune PEC mixed with antigenic thymocytes), inhibition of migration was first detectable at day 8 following grafting. This finding agrees well with the observations of Brunner *et al.* (1966), where the ability of blood leukocytes to inhibit tumor growth *in vitro* was first detectable at 7 to 8 days following an allograft.

In summary, thymocytes were shown to function as effective cellular antigens, inhibiting in a specific manner the migration of PEC from immunized mice. Cross-reaction (inhibition by thymocytes from a third-party strain) was observed when H-2 specificities were shared by the graft donor and a third strain. In this case, C3H/HeJ carries all the specificities determined by the K end of the H-2 locus of A/J (Klein and Shreffler, 1971). The latter observation is in good agreement with the observations of Brondz and Snegirova (1971), that cell-mediated cytotoxity in vitro can be elicited both by target cells from the graft donor strain and by target cells from another strain carrying all the specificities of either the D or K subloci.

iv. Activity of sensitized lymph node cells

Bloom and Bennett (1966) first demonstrated that lymphocytes purified from PEC of guinea pigs with delayed hypersensitivity, reacted in tissue culture containing the specific antigen and elaborated a soluble mediator(s) capable of inhibiting the migration of normal peritoneal exudates. Also, the addition of as few as 0.6% to 2.0% purified lymphocytes (from "immune PEC") could, in the presence of specific antigen, significantly inhibit the migration of PEC from normal guinea pigs with which they were mixed.

David (1966) demonstrated that a similar substance could be released by lymph node cells, from guinea pigs with delayed hypersensitivity, when they were cultured with specific antigen, and termed the material, migration inhibitory factor (MIF). In support of the hypothesis that the modified migration assay described in this thesis

for use in the mouse, was an analogue of the guinea pig PEC assay was the demonstration that lymph node cells (LNC) from skin-grafted mice could inhibit the migration of PEC carrying donor antigens. Thus, the specific immunologic events could be attributed to cells of lymphocytic character. Cytologic observations showed that more than 95% of these lymph node cells were lymphocytes (in agreement with the observations of David).

In previous experiments the possibility existed that macrophage cytophilic antibody could contribute to the inhibition of PEC migration induced by thymus cells, since these antibodies could remain adsorbed to macrophages of "immune PEC", and act by aggregating antigenic cells. This possibility was discussed previously (see page 25). Since lymph node cells from immunized mice were able to inhibit the migration of PEC from normal mice, the above possibility was greatly reduced since lymphocytes were shown not to bind cytophilic antibody (Jonas et al., 1965; Howard and Benacerraf, 1966). In the present studies the migrating population contained 20% lymph node cells (LNC) and 80% of cells from PEC of different strains. The presence of 20% LNC from normal mice did not alter the migration of syngeneic or allogeneic PEC. When "sensitized LNC" (from A/J mice immunized with C57BL/6J skin grafts) were mixed with PEC from normal A/J, C3H/HeJ or C57BL/6J mice, only the migration of C57BL/6J PEC was inhibited, demonstrating the specificity of the reaction. The enhancement of the migration of C3H/HeJ PEC is attributable to the poor ability of these cells to migrate; the migration areas being rather small. The addition of

LNC to these cells may act as a disaggregant. Similarly, although PEC from C3H/HeJ mice can be inhibited by A/J LNC from mice sensitized by C3H/HeJ skin grafts, the poor migration of these cells may not allow for high values of inhibition. It is clear from the data obtained in above experiments that LNC from immune mice can strongly inhibit the migration of PEC of mice of the graft donor strain in a specific manner. The degrees of inhibition obtained using this assay (Figure 7) and the assay using PEC and thymus cells (Figure 5) are almost identical suggesting that 40% inhibition of migration is the maximum extent to which PEC may be inhibited.

In summary, this experimental design demonstrates that macrophages (the predominant cell type) of PEC, from one strain of mice, can be inhibited by "sensitized LNC" from another strain, and that these PEC provide an ample source of antigen for the inhibition of migration. In addition these findings agree well with the observed activity of lymphocytes in the guinea pig migration systems as considered earlier.

Experiments in which different numbers of "immune LNC" were mixed in different concentrations with antigenic PEC demonstrate the high sensitivity of the assay, since only 1-2% of "sensitized LNC" were able to detectably inhibit the migration of antigenic PEC, moreover, only a fraction of the 1-2% of LNC can be assumed to be 'specifically sensitized'. To further appreciate this reaction one can consider the potency of only a few lymphocytes to act on macrophages which are present not only in numbers one hundred fold greater than themselves,

but each macrophage having a twenty-fold greater volume than each lymphocyte. The ability of lymph node cells, even when present in very low numbers (1-2%) to inhibit the migration of antigenic PEC, suggests that the inhibition is not attributable to the lysis of the migrating cells by the sensitized LNC, since it is rare that lysis occurs even when sensitized cells are present in numbers of 50 to 100 times greater than this (Wagner et al., 1972). In addition, it is unlikely that the inhibition is due to antibody released from the LNC (which could act to agglutinate the PEC) since inhibitions due to immune LNC (or sensitized PEC*) reach maximums of about 40% despite the presence of LNC or antigenic thymocytes in measurable excess, while antibody-mediated (ALS) inhibition of migration was shown to attain a degree of inhibition as high as 70%.

One possible explanation for the sensitivity of this assay is that a MIF-like inhibitory product of sensitized lymph node lymphocytes is released subsequent to contact of these cells with antigenic macrophages. As suggested previously, such a substance(s) released within the capillary tubes, in very high local concentrations, could then act on the migrating PEC and inhibit their migration (Lake et al., 1971).

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* As a further argument against the production of antibody in the migration assay (which employs the mixture of thymus cells and 'sensitized PEC'), it has been shown both in vitro (George and Vaughn, 1962) and in vivo (Kornfeld and Weyzen, 1968) that PEC from immune animals are not actively engaged in antibody production as are lymphoid cells from other anatomical sources.

The sensitivity of this assay could be exploited to examine the characteristics of transplantation antigens that trigger cell-mediated immunity. One approach to this problem would be to compare the ability of "sensitized lymphocytes" to react to transplantation antigens (i.e., inhibit the migration of antigenic PEC) of strains of mice which share serologically defined (cross-reacting?) H-2 specificities with the graft donor. Valuable knowledge could be gained from such a comparison; for example, while some antigens may show serological cross-reactions it is possible other properties of the antigen (carrier effects, sub-immunogenic size, etc.) may cause it to evoke no cell-mediated reaction. The clinical implications of such phenomena would be great for both transplantation and cancer problems.

v. Relation of inhibition of migration to transplantation reactions in vivo

Effector mechanisms for delayed hypersensitivity in the guinea pig have been suggested subsequent to phenomena observed with the in vitro migration experiments as described in the Introduction. The suggestion that circulating lymphocytes encounter antigen and subsequently release trophic factors which recruit, in a non-specific manner, inflammatory cells into lesions of delayed hypersensitivity, has been borne out in experiments using radiolabeled cells (considered earlier, p. 3 ).

With regard to effector mechanisms in rejections of certain transplants, where cell-mediated precesses are clearly implicated, such as skin grafts or solid tumor grafts, an accumulation of cellular elements having similar radiolabeling properties to those found in lesions of delayed hypersensitivity has been described, i.e., there is a failure of radiolabeled immune cells (obtained from sensitized mice and transferred to normal skin-grafted recipients) to accumulate in high numbers in the skin grafts (Gowans and McGregor, 1965).

The striking ability of only a few lymphocytes to influence the motile activity of high numbers of cells considered to be derived from circulating monocytes, in the experiments described in this thesis, points to the possibility that an analogous mechanism of inflammation to that proposed for reactions of delayed-type hypersensitivity may occur in the process of graft rejection in the mouse.

It is possible that leukochemotactic and MIF-like factors exist in vivo to attract and activate cells from vascular passage through tissues in which sensitized cells have reacted to the histocompatibility antigens. These cells might then serve in cytopathogenic processes directly, or, secondarily, perform phagocytic and endocytic roles after tissue damage.

Solid-tissue graft rejection by cell-mediated processes is considered to be a consequence of infiltrating cells, comprising lymphocytes of various sizes, macrophages, and a minority of plasma cells as established by both light and electron microscopy (Weiner et al., 1964). Waksman (1963) concluded that the major destructive processes in skin

grafts in the rat were due to the local accumulation of mononuclear cells within and outside of the blood vessels, leading to vascular arrest, and due as well to a direct cytopathogenic action of these cells on the foreign cells, further, (using ear skin as the graft tissue) he observed that macrophages constituted a high proportion of the infiltrating cells. In a more direct approach, Gillette and Lance (1971) labeled macrophages *in vitro* with radioactive chromium. Following the intravenous injection of these cells into mice undergoing the rejection of skin allografts, an accumulation of radioactivity was demonstrated in the allografts when compared with healing autografts. In addition, Poulter *et al.* (1971) have described a histochemically active population of macrophages in the cellular infiltrate of mouse skin allografts. Their results led to the conclusion that the accumulation of these cells (having, with time, increasing lysosomal proteolytic activity) could be at least partly responsible for skin allograft rejection. Thus macrophages appear to be strongly implicated in processes of immune rejection *in vivo*, and the data in this thesis may point to some of the mechanisms underlying their involvement.

## CONCLUSIONS

The assay of inhibition of PEC migration as described by Al-Askari et al. (1965) for the in vitro demonstration of transplantation immunity in mice was confirmed and refined in this thesis.

PEC were induced with dextran which resulted in an efficient and economical procedure for cell recovery*. Large cell yields were obtained having similar properties and compositions to those induced with oil irritants. Thus, large numbers of macrophages, which were able to adhere to plastic surfaces and to phagocytose colloidal carbon, were recovered together with a typical lymphocytic population. The PEC were able to migrate out of capillary tubes and to form characteristic radial patterns similar to those reported for oil-induced guinea pig PEC. The PEC were shown to be highly sensitive to ALS* since inhibition of migration was detectable at dilutions of 1:5000 of the antiserum, while the leukoagglutination titre was only 128. Since ALS-mediated inhibition of migration may be due to opsonization, this procedure may be valuable in ascertaining the immunosuppressive potential of an ALS preparation.

Dextran induced PEC from mice immunized to transplantation antigens by skin grafting were able, when mixed with equal numbers of (antigenic) PEC from the donor strain, to inhibit the migration of the cell mixture, confirming the study of Al-Askari et al. It was concluded from the results of experiments in which the two types of PEC

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Asterisk denotes claim to original work by the author.

were mixed in different ratios that the proportion of specifically sensitized cells present in PEC from immune mice varied with the time elapsed after immunization*.

In another series of experiments, thymus cells were shown to be able, when mixed with PEC from graft-immunized mice, to cause inhibition of migration of the cell mixture in an immunologically specific fashion*. Furthermore it was concluded that these experiments were conducted in conditions of antigen saturation since a four-fold reduction in the number of thymus cells did not affect the degree of inhibition. It was also shown that inhibition of migration could be elicited by thymus cells from a strain sharing some of the transplantation antigens of the graft donor, suggesting that this approach may be useful in the interpretation of the properties of transplantation antigens presently defined in terms of complex serology.

Sensitized lymphocytes (LNC) were shown to have the ability to inhibit the migration of antigenic PEC when mixed with these cells*. Experiments in which the proportions of LNC were varied provide a quantitative method for assessing the activity of sensitized lymphoid cells and demonstrate the high sensitivity of this test. Since a very small number of lymphocytes (1-2% of the migrating cell mixture) was shown to inhibit PEC migration it was concluded that all experiments were performed in conditions of "immune cell" saturation.

Since the average inhibitions of the thymus-PEC mixture and the LNC-PEC mixture were 43.0% and 41.5%, respectively, and both protocols are considered to be of non-limiting designs, it is apparent

that PEC cannot become inhibited in excess of 40%. In contrast, antibody-mediated inhibition (ALS) reached values as high as 70% and did not show saturation effects. Consequently, the mechanism underlying the inhibition of migration in the protocols using thymus cell-PEC and LNC-PEC mixtures is not attributed to antibody. LNC are not considered to cause inhibition of migration by direct cytotoxic effects on the migrating cells, since it is rare that cytotoxic effects are reported even when the ratio of sensitized to antigenic cells is 50 to 100 times greater than used in this work.

The release of a pharmacologically active mediator(s) (such as MIF) from sensitized lymphoid cells, which could then act within the capillary tubes at high local concentrations, is the likely mechanism underlying the inhibition of PEC and is probably the basis for the high sensitivity of the assay.

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