The Specificity of Cell-Mediated Transplantation Immunity

by

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A thesis submitted to the Faculty of Graduate Studies, University of Manitoba, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Ph. D. Thesis

ABSTRACT

The Specificity of Cell-Mediated Transplantation Immunity

The specificity of cell-mediated transplantation reactions to antigens determined by the major histocompatibility region (H-2) in mice has been studied. The approach used was to compare the effector cell responses to antigenic cells of donor origin with responses to antigenic cells of other third-party strains sharing some antigens (identified serologically) with the donor. The in vitro techniques of inhibition of lymphoid cell migration and target cell destruction were adapted and refined for these studies.

In one form of the migration assay thymus cells served as antigen and were mixed with sensitized peritoneal exudate cells (PEC). Inhibitions resulted only when antigenic cells shared a donor H-2 region (K or D). In addition a second form of the migration assay was used in which sensitized lymph node cells (LNC) were mixed with antigenic PEC. This assay was shown to be highly sensitive since 1 to 2 per cent of LNC in the migrating population caused detectable inhibitions and assays could be performed using non-limiting numbers of immune LNC. Results with this assay revealed that reactions (inhibitions) could be detected against antigenic cells from strains which did not share an H-2 region with the donor but that such reactions were weaker than those directed at the donor.

The relationship of this assay to cell-mediated responses was supported by the finding that anti-θ serum could abrogate LNC-mediated inhibitions, indicating that a cell of thymus-derived lineage is integral in this reaction.

The data from the migration studies was inconsistent with the published specificity of the assay of cell-mediated cytotoxicity which prompted a new investigation of the latter system. A new type of target cell was employed consisting of ^{51}Chromium-labeled macrophages which were shown to be highly suited for quantitative comparisons of cytotoxicity. Aggressor spleen cells from skin-grafted mice lysed only target cells which shared a donor H-2 region in agreement with the migration data. A second source of aggressor cells was the peritoneal cavity of mice which had rejected an ascites tumor allograft. Such cells were 16 to 32-fold more potent than spleen cells, in terms of lytic capacity, and enabled quantitative comparisons of cytotoxicity ranging over three orders of magnitude of aggressor cell numbers. Lysis of third-party target cells, having disparity with the donor at both H-2 regions, was now detected but was extremely weak, from 1/32 to 1/64 of that of donor cells.

The effector cells in this system were sensitive to anti-θ serum.

The above findings of very weak reactions to non-donor related antigens were confirmed in an in vivo assay of immunity to an allogeneic tumor graft. In this assay mice were immunized with skin grafts from congenic strains and subsequently challenged with with a tumor allograft from a third-party strain not sharing a donor H-2 region. Suppression of tumor growth was observed but was weak relative to the effects of donor skin grafts.

The data indicate that public H-2 antigens (H-2 antigens of a strain not sharing a donor H-2 region) may evoke only very weak cell-mediated immunity. Thus the importance ascribed to such antigens in tissue-typing serology differs markedly from their importance in cell-mediated (rejection) reactions. Cell-mediated immunity would appear therefore to be directed at private or other undefined H-2 antigens.

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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 the 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, Kantor and Benacerraf, 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 peri-

vascular 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 seem 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 throiditis 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, Benacerraf and McCluskey (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 (1961;1962 and 1963) 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 and Converse, 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, Brown and Medawar, 1958, 1962).

In summary, the morphological picture of the cells infiltrating antigenic sites and the histopathological events in transplantation and delayed-type reactions are 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. Furthermore both forms of immunity are thymus-dependent (Osoba and Miller, 1967), which underlines the possible involvement of cells of common lineage.

(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, considered 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.

Two in vitro assays, i.e., the blast-cell transformation reaction and the technique of inhibition of peritoneal exudate cell migration, 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, Bach, Kolodny, Forscheim and Hashem, 1963). While Oppenheim's adaptation of this technique in humans and guinea pigs (Oppenheim, Wolstencroft and Gell, 1967; Oppenheim, 1968) 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. Helper T cells may have been detected. 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 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 monolayers 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.

A third assay, first described by Ruddle and Waksman (1967) is a cytotoxicity test whose mechanism was suggested as a common basis for the diverse in vitro manifestations of cellular hypersensitivity and for the tissue damage accompaning severe tuberculin reactions, lesions of autoimmunity and homograft rejection. In this system lymphocytes from animals with delayed hypersensitivity released a cytotoxic substance into the tissue culture medium, upon reaction with specific antigen. 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, Sabbadini and Sehon (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. However the mechanism of this reaction is now held suspect since antigen-antibody complexes may be involved (Golstein, Schirrmacher, Rubin and Wigzell, 1973).

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 stated previously for cell-mediated phenomena in vivo, there appear to be common manifestations 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 using mice of several

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 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, Spiro and Russel, 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, Mikulska and O'Gorman (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, Algire and Prehn (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, Gesner and McGregor (1961) and Billingham, Silvers and Wilson (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), and 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 classical evidence that transplantation immunity is due to cell-mediated effector processes is found in experiments of passive transfer of immunity. The work of Mitchison (1953, 1954) and of Billingham, Brent and Medawar (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).

More recently it has been shown that thymocytes, sensitized by in vivo passage in X-irradiated allogeneic hosts and incapable of antibody formation were able to confer allograft immunity adoptively

(Freedman, Cerottini and Brunner, 1972)

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 suppress 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 of the same species 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, Vos and Lowenstein 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, Fariday and McLean, 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 Ginzburg (1968), Berke, Ax, Ginzberg and Feldman (1969), Wagner and Feldman (1972) and of Alter, Schendel, Bach, Bach, Klein and Stimpfling (1973) who showed that co-cultured xenogeneic or allogeneic lymphoid cells become transformed and then cytotoxic to appropriate target cells. Such experiments may reflect the entire cycle of immunocompetent cells triggered by antigen, becoming transformed and finally maturing through sequential mitoses into aggressor cells cytotoxic to antigenic cells.

(b) Target cell destruction

Lymphoid cells sensitized *in vivo* or *in vitro* to transplantation antigens or other cell surface 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. Many cellular, kinetic and metabolic aspects of this reaction have now been described and are considered further on page 35.

(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 24.

vi. The mouse H-2 complex as a model for transplantation studies

The immunological rejection of tissue and organ grafts is attributed to cellular antigens of the donor recognized as foreign by the recipient. Such antigens are designated histocompatibility antigens and are controlled by histocompatibility genes located at 30 or more separate genetic loci in the mouse (Snell and Stimpfling, 1964; Bailey, 1970). As in all species studied, the mouse has a histocompatibility locus, termed the H-2 locus, which results in rejections of the greatest vigor. More recently this genetic region has been termed the H-2 gene complex as it is apparent that several genes clustered in this segment of the ninth linkage group control a complex system of alloantigenic products (Klein and Shreffler, 1971). This locus is the apparent evolutionary homologue of the HL-A region of man (Amos, 1969) and is a highly complex and accessible mammalian genetic region which may therefore be studied as a valuable resource in applied and basic immunogenetic research.

Identification and analysis of the H-2 complex has been greatly aided by the development of congenic resistant lines of mice, i.e., strains which are genetically identical except for disparity at a single genetic locus, conceived by Dr. G.D. Snell (Stimpfling and Reichert, 1970).

The H-2 complex has been the subject of intensive serologic and immunogenetic study and appears to comprise at least four genetic regions separable by genetic recombination; the H-2K, Ir, Ss-Slp and H-2D regions. The closely linked K and D regions (0.5% recombination)

were considered to contain one or several genes each which governed all the antigens detected in serological studies (Stimpfling, 1971). However recent studies indicate that some of the antigens detected serologically may be determined by genes located in the Ir region (David Shreffler and Frehinger, 1973; Hauptfeld, Klein and Klein, 1973) and may have been previously attributed to the closely linked H-2K genes. The Ss locus controls the amount of a β-globulin in mouse serum and the Slp locus controls a sex-limited (to males) serum protein (Passmore and Shreffler, 1970) and is apparently unrelated structurally or otherwise to other H-2 gene products involved in transplantation. The significance of the location of the Ss-Slp locus (which has not been subdivided by recombination) i.e., between H-2K and H-2D, is entirely unknown, except that it strengthens the possibility that the K and D regions are derived by gene duplication from a single ancestral gene (Shreffler, David, Passmore and Klein, 1971) and thus it provides a fortuitous marker within the H-2 complex.

The Ir region apparently comprises a large number of genes which regulate immunological responsiveness to many natural and synthetic antigens (Benacerraf and McDevitt, 1972). One recombinant has revealed the independence of Ir-IgA locus (which controls the response to an IgA myeloma allotype, (Lieberman, Paul, Humphrey and Stimpfling, 1972) from the Ir-IgG locus (similarly defined) while Ir-1, which controls the immune response to synthetic polypeptides (McDevitt and Benacerraf, 1969), has not yet been separated from Ir-IgA.

The allo-immune response to H-2 antigens includes the production of antibody-plaque forming cells and humoral antibody (Brunner,

Mauel, Cerottini, Rudolf and Chapuis, 1967; Brondz and Golberg, 1970) as well as the appearance of sensitized lymphoid cells active in transplantation reactions *in vivo* and *in vitro* (Brunner, Mauel, Cerottini and Chapuis, 1967). Hyperimmune antisera obtained subsequent to prolonged immunization with H-2 incompatible lymphoid tissues have been used in the analysis of the fine structure of H-2 antigens. Using techniques of absorption *in vivo* or *in vitro* and by selection of donor and recipient strains it has proven possible to analyze the hemagglutinating and lymphocytotoxic activity of the antisera. Most such antisera are complex and react with the antigen-donor strain and varyingly with other strains of independent origin. As shown by Gorer and Mikulska (1959) the total number of specific H-2 antigen-antibody systems (antigens) which may be defined, assuming one antigen evokes one antibody, is related to the number of H-2 types (n) in the expression 2^n-2. At present approximately 12 H-2 types have been carefully studied and more than 30 specificities have been defined, compiled, and are represented in the H-2 chart (Klein and Shreffler, 1971) portions of which are represented in Tables 7 and 26.

The above information concerning H-2 serology has been analyzed in accordance with one of two cognitive models (Hirschfeld, 1965). In the simple-complex (antibody-antigen) model a complex antigen, having several different antigenic determinants, evokes the production of a number of antibody molecules each specific for its corresponding antigenic determinant and non-reactive with other distinct H-2 determinants. In this interpretation, positive reactions with cells of third-party strains are attributed to the presence in a homologous antigen of the

"cross-reacting" (third-party) strain of some antigenic determinants identical with those of the donor. In the antithetical model the H-2 antigen is considered to be simple, having only one determinant but can elicit the production of antibodies having a mixture of recognition sites which are capable to react with other H-2 antigens.

Both the above models are idealized (and thus falsified) and it may be expected that biological reality is some accommodation of both interpretations. H-2 serology is generally interpreted in terms of the simple-antibody, complex-antigen concept, i.e., each H-2 chromosome of independent origin determines the expression of several discrete specificities as evidenced in the H-2 Tables 7 and 26. However, using the alternative reasoning, it is equally possible that each H-2 chromosome determines a single antigen only, and that the reactions of such cells with another antiserum (rendered monospecific by suitable absorption) is due to the presence of antibody molecules formed against the other H-2 type which are cross-reactive with the cells in question. Thus H-2 serology would be greatly simplified and much of the complexity would be discarded as artifactual; the HL-A system has been traditionally interpreted in this manner (Thorsby, 1971). Evidence in possible support of this concept was provided by Snell, Cherry and Demant (1971). They identified in many H-2 types the presence of certain antigens which have exceptionally high titers in the cytotoxicity assay and which can be arranged into two mutually exclusive (or allelic) series located at the K and D regions of the H-2 complex in homology with the HL-A system. Further, these antigens have a restricted distribu-

tion among the inbred strains, i.e., they are found in only one H-2 chromosome and in chromosomes derived from it by recombination between the two H-2 loci and therefore absent in wild mice. These antigens have been termed "private" specificities (Klein, 1971). In contrast it is possible to distinguish a number of "public" H-2 specificities which are widely shared by different H-2 chromosomes of inbred and wild mice (Klein, 1972). The occurrence of most of these shared specificities in H-2 chromosomes is not random, but highly organized in inclusion systems which would be consistent with the concept of undirectional cross-reactive antibodies (Thorsby, 1971). These antigen systems would be considered artifactual in a complex-simple interpretation as discussed above.

Resolution of this dilemma will ultimately reside in the immunochemical analysis of the H-2 antigenic product. However, in another approach to the analysis of H-2 antigens, the specificity of cell-mediated immunity to H-2 antigens has been studied. In several reports (Brondz, 1968; Ginsburg, 1968; Brondz and Golberg, 1970; Mauel, Rudolf, Chapuis and Brunner, 1970; Ax, Koren and Fischer, 1971; and Berke and Levey, 1972) it was shown that while target cells from the graft donor strain were destroyed <u>in vitro</u> by recipient spleen cells, target cells from third-party strains having some of the public H-2 specificities of the graft donor were not affected. However, antisera from similar immunizations were cytotoxic. The basis for this discrepancy is unknown but would appear to be significant for the understanding of the specificity of cellular recognition of H-2 antigens. It may be relevant to the cognitive interpretation of H-2 serology and may thus signal a need for the re-

evaluation of the simple-complex position of the murine histocompatibility system.

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. These 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 suggested new mechanisms for the *in vivo* manifestations of cellular immunity. In their early experiments (David, Al-Askari, Lawrence and Thomas, 1964), 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 occured 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, Lawrence and Thomas, 1964a). 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 Gel (1959) and discussed previously. Using the hapten-protein conjugates, DNP-ovalbumin or DNP-bovine gamma globulin, David et al. (1964a) 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-oligo-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., 1964c). 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 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

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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.

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 mechanism underlying the inhibition of PEC migration. Using the ability 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 (comprising 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 ($56^{\circ}C$, 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, Atoynatan and Kelly, 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, Jureziz, Veach, Miller and Dray (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, Katz, Haber and David (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, Pekarek and Johanovsky (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, Janeway and Paul (1972) have 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, Han and Weiser, 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, Huber and Fudenberg (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 a more recent study (Pick, Krejei and Turk, 1972) it was shown that a material released from sensitized lymphocytes in the presence of antigen could induce the release of MIF and SRF from non-immune lymphocytes. MIF release from non-sensitized 'B' lymphoid cells has been documented (Yoshida, Sonozaki and Cohen, 1973); it is therefore possible that interaction of two lymphoid cells may be in part responsible for the mechanism of MIF release.

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 chemotactic activity for mononuclear cells in vitro (Ward, Remold

and David, 1969) and probably *in vivo* (Ramsier, 1969), to induce vascular permeability upon injection *in vivo* (Maillard, Pick and Turk, 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, Currie and Alexander (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, i.e. 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 to 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, Stiffey and Tesar, 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 this work has been extended only slightly. Friedman (1971) showed that spleen cells from grafted animals could be inhibited in their migration if incubated with antigenic extracts of donor mice and that immune lymph node cells produced MIF which was active to inhibit the migration of guinea pig PEC. The release of MIF in the murine transplantation system was confirmed by Al-Askari and Lawrence (1972) and points to the possibility that these phenomena are common to cell-mediated reactions of different species.

Target cell destruction in vitro

i. General characteristics of the phenomenon

Lymphoid cells, cytotoxic in vitro to target cells of graft-donor origin have been recovered from all of the peripheral lymphoid tissues. However the activity of these cells and the duration of this activity varies with the source of the sensitized population. Following an intraperitoneal tumor allograft cytotoxic cells were first detectable in the spleen on day 4 and in the peripheral blood leukocytes on day 6; the peak specific activity was found in the spleen, lymph nodes and blood on day 10 - 11. While the activity in the spleen and nodes declined rapidly to undetectable levels, that of the circulating leukocytes was observed to plateau between days 20 - 60 (Brunner, Maucl, Rudolf and Chapuis, 1970) which may indicate a physiological compartmentalization of cytotoxic cells into the re-circulating pool of lymphocytes. In support of this concept, the above authors demonstrated that cells obtained in the first 17 h of thoracic duct drainage had a much superior cytotoxic potential to cells obtained in the subsequent 24 h, indicating that the recirculating cells are more active than the mobilizable pool of cells.

In early studies, target cell death was considered to be an indolent progressive phenomenon involving attachment of sensitized cell to the target cell followed by visible signs of cellular damage at approximately 20 h in the form of retraction and rounding-up of the cytoplasm (e.g., Wilson, 1965). However it was shown in other assays involving non-morphologic criteria that irreversible cell

damage can occur more rapidly. Thus, in an assay in which plaque forming cells were lysed upon mixing with cytotoxic cells (Friedman, 1964) and in a colony inhibition assay (Brunner, Mauel and Schindler, 1966) irreversible damage was detected in 2 h for the former and in 3 h for the latter assay. More recently it has been shown that cell damage is detectable within 10 min of mixing of sensitized lymphoid cells and target cells (Berke, Sullivan and Amos, 1972b). In this study cytotoxic cells comprised a very high proportion of the aggressor cell population, which suggests that the latency to detect significant cytotoxicity is in part related to the proportion of cytotoxic cells and the sensitivity of the test.

In a qualitative study of lymphocyte-mediated cytotoxicity, Wilson (1965) described the complex direct relationship between the numbers of aggressor cells added to cultures of target cells and the resultant degree of cytotoxicity. It was found that the logarithm of percent survival was linearly related to the dose of lymphocytes added. This inverse logarithmic relationship was interpreted statistically as to indicate that one lymphoid cell was adequate to destroy one target cell and to be inconsistent with the premise that two or more lymphoid cells were required per cytotoxic episode.

In the above study cell destruction was determined by enumeration of nuclear isolates of surviving cells which differed in volume from the aggressor lymphocytes. Other assays include 1. changes in metabolic functions such as the incorporation of radiolabelled amino acids into protein (Granger and Kolb, 1968), 2. destruction of plaque-forming cells (Friedman, 1964), 3. cloning inhibition subsequent to

the encounter of tumor cells with the cytotoxic cells (Brunner et al., 1966) and 4. the radiochromium assay of target cell destruction. The radiochromium assay has proven to be sensitive, objective, simple and more versatile than most other systems and is considered in further detail below.

ii. ^{51}Cr release as a correlate of cytotoxicity

Sanderson (1964) and Wigzell (1965) extended the original work of Goodman (1961) verifying the suitability of the radioactive chromium salt, sodium chromate, to serve as an intracellular label which is released upon cellular disruption caused by antibody and complement. Sanderson showed that spontaneous release of isotope was not unreasonably rapid (5 percent per hour at 37°C) and that a maximum of 70 - 80 percent the label could be released into the tissue culture supernatant when cytotoxicity reached 100 percent (as determined by the exclusion of the vital stain Trypan blue). Wigzell demonstrated the spontaneous release to be linear with time and, that while staining with Trypan blue was slightly more sensitive, likely due to kinetic parameters of dye uptake versus the slower release of intracellular macromolecules labeled with ^{51}Cr, the chromium method was more precise.

The chromate ion has been demonstrated to bind in a non-specific manner to intracellular proteins of molecular mass between 80,000 and 250,000 daltons (Bunting, Kiely and Owen, 1963; Henney, 1973). In a study of various intracellular markers as indicators of the break-down of membrane impermeability, it was found that the time required

for indicator release was a function of molecular size. Thus ATP and ^{86}Rb, which are present intracellularly in the free state, efflux within 20 min of the addition of sensitized lymphocytes, while ^{51}chromate and ^{3}H-thymidine, both associated with high molecular weight constituents, efflux within 1 h and 1.5 h respectively. For all these indicators, release was a linear function of time (Henney, 1973).

The correlation of ^{51}Cr release with target cell death, induced in a variety of ways, suggests that this isotope is a reliable indicator of cell destruction and indirectly, of residual viability. Thus, target cells lysed by freezing and thawing, by PHA-induced, lymphocyte-mediated cytotoxicity (Holm and Perlman, 1967) by antibody and complement (Wigzell, 1965) or by specifically sensitized lymphoid cells (Brunner, Mauel, Cerottini and Chapuis, 1967) result in similar maximum percents ^{51}Cr liberated into the tissue culture medium and values of cytotoxicity compatible with results obtained in other systems. In a comparison of cloning inhibition and release of ^{51}Cr, parallel findings support the premise of uniform distribution of cellular label among the target cells and similar extents of release by target cells lysed at different times during the experiment (Brunnet et al., 1967). And in a recent study (Sullivan, Berke and Amos, 1972), with the use of an aggressor population rich in cytotoxic cells, an excellent correlation between ^{51}Cr release and dye uptake was obtained with suspensions of target and aggressor cells. This confirmed similar findings obtained using target cell monolayers (Berke, Ax, Ginsberg and Feldman, 1969).

A further virtue of the chromium label is the absence of detectable reutilization of labeled material which becomes released during tissue culture spontaneously or due to cytotoxicity both in short term (Bunting et al., 1963) and longer term cultures (Holm and Perlmann, 1967). Reutilization would cause an underestimation of the extent of lysis.

In summary the chromium label has been shown to be highly suited for measurements of cytotoxicity as defined by the loss of selective membrane permeability (revealed by the efflux of labeled, high molecular weight intracellular protein).

iii. Characteristics of cytotoxic cells and cellular receptors

It is known that contact of the immune cell with the target cell is essential for cytotoxicity in almost all systems of cell-mediated cytotoxicity studied to date (Perlmann and Holm, 1969). Intimate contact and bonding of the aggressor and target cell was an early observation in assays of cell-mediated cytotoxicity and was termed contactual agglutination by Koprowski and Fernandez (1962). This phenomenon was confirmed by Wilson (1965) who detected strong binding of a small percentage of lymphocytes in sensitized populations after 6 - 8 hours of incubation which could cause as much damage as the entire population. Further, it was concluded that the property of contactual agglutination was relatively temperature independent (Wilson, 1967) in that similar degrees of clustering occurred in cultures maintained at 27°C and 37°C, however subsequent lysis occurred only in cultures maintained at 37°C. This suggests that

binding of the two cells may be due to a preformed receptor substance on the surface of the lymphoid cell while the destructive process may involve metabolism-dependent events.

Extending these findings, Brondz (1968) demonstrated that populations of sensitized cells could be depleted of cytotoxic cells by prior absorption (for 6 h at 27°C or 1 to 3 h at 37°C) on monolayers of antigenic macrophages (donor-type) in a specific manner, i.e., macrophages from third-party strains unrelated to the donor did not remove cytotoxic cells. It was further shown that cytotoxic cells from mice sensitized to antigens of both the K and D regions of the H-2 complex could be separately absorbed on cell monolayers having antigens of each region, with little reduction in remaining activity to antigens of the other region (Brondz and Snegirova, 1971). Similar results have been obtained with mice immunized with grafts from two unrelated strains (Golstein, Svedmir and Wigzell, 1972) and argue against the possibility that cytotoxic cells act via passively absorbed cytophilic antibody. The latter authors showed also, that following absorption, the aggressor cells could be recovered by trypsinization and that the cells retained their cytotoxic properties. Similar results have been obtained by Berke and Levey (1972). Further arguing against a role for humoral antibody serving this receptor function are the repeated demonstrations that passive antibody inhibits the cytotoxicity mediated by sensitized cells (Moller, 1965; Wilson, 1965; Brunner, Mauel and Schindler, 1967). This blocking effect suggests that the cytotoxic cells recognize antigenic sites identical to, or very close to those against which humoral antibody is formed.

Cytotoxic lymphocytes, which in some experiments were generated by the passage of thymus cells in lethally irradiated allogeneic hosts, were shown to be not inhibited or removed by heteroantisera directed at μ-heavy chains or Fab fragments (Canty and Wunderlich, 1970; Chapuis and Brunner, 1971, Golstein, Schirrmacher, Rubin and Wigzell, 1973). Such sera have been demonstrated to be capable of inhibiting cell-mediated cytotoxicity of the type induced by conventional antibody (Chapuis and Brunner, 1971; Golstein et al., 1973; Van Boxel, Stobo, Paul and Green, 1972). Thus conventional immunoglobulin has not been demonstrable as the receptor substance.

The ability of cytotoxic cells to bind to target cells in an immunologically specific manner however indicates the presence of stereospecitic receptors. Attempts to analyze such receptors have met with only scant success. Canty and Wunderlich (1970) showed that the receptors were not removed by repeated washing and were thus unlikely to be cytophilic antibody. Mauel, Rudolf, Chapuis and Brunner (1970) demonstrated that the receptor activity was sensitive to trypsin and was regenerated during subsequent tissue culture.

Attempts to inhibit cell-mediated cytotoxicity with soluble transplantation antigens, based on the premise of blocking receptor sites with antigen have failed. Such experiments have entailed the addition of soluble antigen, which could react with anti-target cell antibody (blocking antibody) to mixtures of aggressor and target cells (Brunner et al., 1967b)

In summary, aggressor T cell populations have been shown to have trypsin-labile, non-cytophilic, antigen-binding receptors which can be synthesized by the immune cells, thus confirming other evidence failing to demonstrate a role for humoral antibody in their activity.

IV. Specificity and mechanism of cell destruction

Since the earliest descriptions of cell-mediated cytotoxicity *in vitro*, the strict specificity of the cytotoxic reaction has been repeatedly confirmed. It has been shown that sensitized cells can be absorbed *in vitro* onto target cells of donor genotype but not onto target cells of another strain of independent origin (Brondz and Snegirova, 1971; Berke and Levey, 1972). Similarly lysis of only donor target cells ensues without deleterious effects to target cells from third-party strains (Wilson, 1963; Mauel *et al.*, 1970). A qualification to this rule is that target cells from strains having recombinant H-2 chromosomes are able to absorb and be destroyed by lymphoid cells sensitized against antigens determined by genes at either part of the crossover site (Brondz and Snegirova, 1971).

In some studies attempts have been made to define the relationship between the antigens recognized by the aggressor cells and those antigens defined serologically i.e., the H-2 chart (Klein and Shreffler , 1971). In a number of studies it was shown that while target cells from the graft donor strain were destroyed *in vitro* by recipient spleen cells, target cells having only some of the relevant H-2 specificities of the donor (i.e., public ones, see also page 138) were not affected (Brondz, 1968; Ginsburg, 1968; Brondz and Golberg,

1970; Mauel et al., 1970; Brondz and Snegirova, 1971; Ax, Koren and Fischer, 1971; Berke and Levey, 1972; MacDonald, Phillips and Miller, 1973). The implications of the discrepant findings between humoral and cellular specificity are considered further elsewhere (see Rationale).

The mechanism of cell destruction has been an intreguing problem since the incipience of the in vitro phenomenon of target cell destruction by lymphoid cells. It is clear that for initiation of cytotoxicity cell to cell contact is required. No cytotoxicity is detected when sensitized cells and target cells are separated by a cell-impermeable membrane (Wilson, 1965). Furthermore, gentle rocking of the culture vessel, which may be expected to increase the frequency of cell to cell encounters, accelerates cytolysis by about four fold (Canty and Wunderlich, 1970). In addition, while immune-target cell binding can occur in the absence of cytotoxicity (27°C) it appears that cytotoxicity requires cell contact. This is supported by evidence which shows that third-party target cells labeled with chromium and mixed with sensitized cells (and their corresponding target cells) exhibit no cytotoxic liberation of label (Canty and Wunderlich, 1970; Cerottini et al., 1971; Berke et al., 1972a; McDonald et al., 1973a). The cell contact leading to cytotoxicity may involve more than the consequence of surface membrane interactions, as aggressor cells labeled intracellularly with fluorescein were observed to transfer some of the dye into the cytoplasm of target cells (Sellin, Wallach and Fischer, 1971).

A role for conventional antibody and complement in cell mediated cytotoxicity appears to be excluded. Antisera to immunoglobulins do not impair cell-mediated cytotoxicity as considered earlier (Canty and Wunderlich, 1970; Chapuis and Brunner, 1971; Golstein et al., 1973), while such sera are active to inhibit antibody-dependent cell-mediated cytotoxicity (Van Boxel et al., 1972). The contribution to cytotoxicity of complement, acting in a conventional manner, is equally doubted. Thus, Wilson (1963) observed that complement was not required for cell-mediated lysis. This was confirmed by Canty and Wunderlich (1970) who added several inhibitors of complement activity including cobra venom factor, EDTA, carageenin and Trypan blue. Further, Henney and Mayer (1971) prepared antisera to three complement components and found that lysis proceeded unimpeded in the presence of the antisera. Thus the participation of antibody or complement appears unlikely in most studies and positive evidence for their participation in reactions of cell-mediated cytotoxicity is confined to systems involving effector cells having an origin distinct from the cells considered in the studies reviewed (see Origin and properties of cytotoxic T cells, page 49).

In a series of reports, Ruddle and Waksman (1967; 1968a; b; c) described the presence of a soluble cytotoxic factor released from sensitized lymphoid cells upon reaction with specific antigen which caused the destruction of syngeneic target fibroblasts. While they suggested that cytotoxicity was a facet of the expression of cellular immunity and the soluble cytotoxin a possible mechanism for cell-mediated cytotoxicity, they failed to exclude the role of antibody

and of antigen-antibody complexes. Further, in critical experiments revealing the existence of soluble cytotoxic activity, controls for immunological specificity were not evident. Other authors have described "cytotoxic" substances released from sensitized lymphocytes upon reaction with specific antigen (Granger and Kolb, 1968) and released from non-sensitized lymphoid cells upon stimulation with mitogens (Granger and Williams, 1968). These "factors" have been the subject of some physical-chemical studies (Kolb and Granger, 1970) but are subject to the criticism that metabolic alterations of the target cells rather than target cell viability was studied. Furthermore the production of the active principles has not proven to be reproduceable (Liske, 1973) which confirms the negative findings of others. Using the ^{51}Cr release assay such factors have never been demonstrable (Perlman and Holm, 1969b). Arguing more firmly against the role of soluble cytotoxic mediators are the many experiments discussed previously in which ^{51}Cr-labeled irrelevant target cells were mixed with interacting cytotoxic cells and their corresponding target cells where lysis of the labeled cells failed to occur. In this system target cells are exposed most intimately to presumptive soluble mediators and even to physical contact with active cytotoxic cells, making a strong case against soluble cytotoxic factors and in favour of the importance of cell to cell contact via specific surface receptors and antigen.

The importance of target cell surface antigen density and its possible relationship to susceptibility of target cells to cell-mediated immunity has been examined. The results indicate that target

cells bearing high concentrations of transplantation antigen are more readily lysed than target cells quantitively deficient in surface antigen and underline a related finding that target cells of different tissue origins but of the same genotype differ widely in their susceptibility to lysis (Brunner et al., 1970).

Several metabolic parameters of the cytotoxic interaction have been described and indicate high complexity of the reaction. As considered earlier trypsin can inhibit the activity of cytotoxic cells which regenerates spontaneously in an enzyme-free culture. This recovery may be attributed to protein reappearance at the cell surface by transfer from intracellular stores or by de novo synthesis. The latter possibility is supported by evidence, that in the presence of cycloheximide recovery fails to occur (Mauel et al., 1970). There is evidence that cytochalasin B, which interferes with actomyosin i.e., inhibits microfilament function, inhibits cell-mediated cytotoxicity in a reversible fashion (Cerrotini, and Brunner, 1972), this finding which implicates cell and membrane movement in target cell destruction. This effect is reversible with shaking (Ferlugo, Asherson and Becker, 1972). In an extension of this work it was found that the cytolytic event proceeds in discrete stages since the events blocked by cytochalasin B are distinct from and precede those stages blocked by EDTA (and require divalent cations) and the latter phase precedes any changes in cell membrane permeability (Henney and Bubbers, 1973). However, in terms of mechanism, it has been shown only that cytochalasin B interferes with the ability of the immune cell to become bound to the target cell (Stutling, Berke and Hiemstra,

1973).

The plant alkaloids colchisine and vinblastine disrupt microtubules thereby affecting the topographical distribution of membrane proteins, the release of intracellular granule stores and cellular secretion. These agents can cause up to 60 percent inhibition of cytotoxicity mediated by sensitized cytotoxic cells or by normal cells in the system of antibody-facilitated non-immune lymphocyte-mediated cell lysis (Strom, Garovoy, Carpenter and Merrill, 1973). Such data would be consistent with a secretion event involving the trans-intracellular movement of toxic material leading to cell lysis as suggested by the transfer of fluorescein discussed previously. A candidate for the toxic agent is an activated esterase present in the aggressor cells prior to the cytotoxic event as indicated by the depression of cytotoxicity by organophosphorus inhibitors (Ferluga et al., 1972). The probably lysosomal location of the active agent is suggested by experiments demonstrating a reduction of cytotoxicity in the presence of the lysosome-active drugs, Trypan blue, chloroquin and predinsolone (Brondz, Snegirova, Yu, Rassulin and Shamborant, 1971). However further experimentation is required to confirm these suggestions.

In summary, the destruction of target cells by aggressor lymphoid cells appears to be independent of humoral antibody and complement. Arole for soluble extracellular mediators is doubtful. Biochemical and cytological studies point to a secretory event, i.e., the possible transfer of a lysosomal esterase into the target cells, which leads to lysis.

V. Fate of cytotoxic cells

In early experiments, using aggressor cells from the thoracic duct, Wilson (1963) obtained no evidence that the cells which mediate cytotoxicity are consumed in the process and cinematographic evidence suggested that a single aggressor cell was capable of multiple sequential cytotoxic encounters (Ax, Malchow, Zeiss and Fischer, 1968). In the non-immune system of PHA-induced cytotoxicity it was observed that the cytotoxic cells were not destroyed (Perlmann, Perlmann and Holm, 1968). Brunner et al. (1970) found in an immune allograft system that a second population of target cells was destroyed at least as rapidly as the first. This was confirmed by Berke et al., (1972c) who found no decrease in the rate of lysis with time in a similar system. The latter authors obtained compelling proof of the ability of an aggressor cell to lyse a target cell and functionally survive in experiments with a highly active cytotoxic population (non-adherent peritoneal exudate lymphocytes). In this study aggressor cells lysed twice as many target cells as their own number. Furthermore, when aggressor cells were labeled with ^{51}Cr, label was not released when these cells mediated cell destruction.

VI. Origin and properties of cytotoxic cells

Several systems concerning cytotoxicity mediated by cells have been described. They may be grouped into three categories, 1. non-immune cytotoxicity mediated by activated cells, 2. immune cytotoxicity mediated by normal cells and dependent upon specific antibody and 3. immune cytotoxicity mediated by lymphoid cells which act in apparent autonomy. Such cells, which are the subject of this review, are thought to be of thymus-derived (T) lineage and evidence supporting this conclusion has been obtained by the analysis of cells of different tissue origins to differentiate into cytotoxic cells and by the study of differentiation antigens of cells obtained following in vivo and in vitro sensitization and/or by the use of various techniques of cell purification.

In the first of such experiments, the intravenous injection of thymus cells into lethally irradiated allogeneic hosts resulted in the production of cytotoxic cells which were recovered from host spleens. This indicated that the cytotoxic cells were likely thymus derived (Cerottini, Nordin and Brunner, 1970a; Blomgren, Takasugi and Friberg, 1970). This concept was confirmed in subsequent experiments in which spleen cells, sensitized in a similar graft-versus-host (GVHR) system, gave rise to cytotoxic cells which became inactivated upon treatment with anti-theta serum and complement- i.e., an antiserum to the cell surface differentiation alloantigen of thymus derived cells (Cerottini, Nordin and Brunner, 1970b). The above studies however did not exclude some contribution of B cells or other cell types. To exclude the possible contribution of B cells and cell types which have

receptors for antigen-antibody complexes, receptors for complement and those cells having surface immunoglobulin, immune spleen cells and GVHR 'educated' thymus cells were passed through a bead column coated with anti-mouse immunoglobulin. Eluted cells retained their characteristic propensity for absorption on antigenic monolayers and specific cytotoxic activity, thus suggesting very strongly that only T cells are involved in target cell killing in this system (Golstein, Wigzell, Blomgren and Svedmyr, 1972).

Studies of the generation of cytotoxic cells in vivo and in vitro support this view also since precursor populations of cytotoxic cells have not been shown to require the presence of cells other than T cells (and apparently a non-lymphoid accessory cell) (Shortman, Brunner and Cerottini, 1972; MacDonald et al., 1973b). Cooperation between two types of T cells in the induction of cytotoxic cells appears operative in that a cell type rich in the lymph nodes and peripheral blood which is θ positive, cooperates synergistically with another θ-positive cell which is rich in the spleen and thymus. The latter cell is the precursor of the cytotoxic cell (Cohen and Howe, 1973; Wagner, 1973).

Other studies corroborate the view of functional independence of the cytotoxic T cell. Thus adherent cells or monocytes, important in some systems of antibody dependent cell-mediated cytotoxicity (Dennert and Lennox, 1973), have been shown to be unnecessary for antibody independent cytotoxicity in allograft immunity in vitro (Brunner and Cerottini, 1971) and in vivo (Freedman, Cerottini and Brunner, 1972; Rouse and Wagner, 1973).

Thus, in studies of allograft immunity *in vitro* using the system of cell-mediated cytotoxicity the most compelling evidence favours the concept of the autonomous action of a thymus-derived aggressor cell.

CHAPTER 1

MATERIALS AND METHODS

i. Animals

All mice used in these studies were of the highly inbred strains CBA/J, DBA/2J, C3H/HeJ, A/J, BALB/cJ, C57BL/6J and congenic C57BL/10, B10.D2 (new), B10.A, B10.BR, and B10.M and were purchased from the Jackson Laboratory, Bar Harbor, Maine. Animals were shipped at eight weeks of age and were kept at 70-76°C 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 were used. For experiments in which cells from a panel of six strains were studied males only were used and females were used in the experiments of tumor growth.

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 on the fifth or sixth post-transplant day and 3.0 ml of Hanks balanced salt solution (HBSS, Difco) was injected intraperitoneally after swabbing the abdomen with 70 per cent 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 fluid 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 solution (Campbell, Garvey, Cremer and Sussdorf, 1970). The tumor was maintained by serial passage into young A/J recipients, by injecting 1×10^6 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 1×10^7 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 Medawar (1950) with some modifications. The abdominal surface of the donor was shaved, swabbed several times with 70 per cent ethanol, and then excised and reflected. Subcutaneous fascial and fatty tissue was scraped off with a scalpel, and the skin sheet selected for non-active hair growth was cut into rectangles of approximately 1.0 cm^2 area using sterile techniques. Recipient mice were anaesthetized with sodium pentobarbital (0.06 mg/g body weight, administered intraperitoneally). A graft bed was prepared on the lateral thorax of the recipient. Mice destined for use as a source of sensitized lymph node cells received

bilateral grafts. To prepare a graft bed the thorax was shaved and swabbed with 70 per cent 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 skin 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, David and Co., Detroit, Michigan) and secured 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. In all experiments, unless otherwise stated, sensitized lymphoid cells were recovered on days 9-11 after grafting.

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 per cent 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 immersion 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

PEC were induced in both normal and skin-grafted mice by an intraperitoneal injection of a solution of dextran, grade 2-P (Pharmachem, Bethlehem, Maryland). The dextran had a molecular weight of 5-40 x 10^6 and was dissolved at a concentration of 35.5 mg/ml in phosphate-buffered saline. After autoclaving, the solution was stored at -20°C. Each animal received 1.5 ml of the solution 3 days prior to the recovery of the PEC. PEC were harvested from exsanguinated mice by injecting 6-7 ml of cold HBSS i.p., massaging the abdomen and aspirating the fluid with a syringe. The PEC suspension was washed 3 times in HBSS (at 150 g for 5 min each), a single cell suspension was decanted from cell aggregates and debris, and the cell concentration was determined as above.

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 underlying the immunizing grafts. 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, grasped with a curved forceps, pulled free of other structures, and fragmented in the same manner as the lymph nodes. The resulting cell suspensions were aspirated into sterile plastic disposable centrifuge tubes, large fragments were allowed to settle for 5-10 min and the cells in the supernatants were centrifuged at 150 g for 7 min . The cell pellets were resuspended in HBSS,

washed twice more in HBSS and the cell concentrations determined using Turk solution. The suspensions were kept at $4^{o}C$ until used.

vi. PEC migration

The methods used were a modification of those described by David, Al-Askari, Lawrence and Thomas (1964). The tissue culture medium in all migration experiments consisted of medium TC 199 (Difco), reconstituted from the dry powder with double or triple distilled water. It was passed under positive nitrogen pressure through a 0.45 μ millipore filter (Millipore Corp.) for sterilization and stored at $4^{o}C$ for not more than four weeks. The TC 199 was adjusted to contain 20 per cent v/v heated ($56^{o}C$ for 30 min) fetal calf serum (Gibco), penicillin 100 U per ml and streptomycin 100 μg per ml (Gibco antibiotics). The serum was filtered through a millipore membrane as above. All components were combined just before use.

The capillary tubes used were #34502 Kimax glass tubes, 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. PEC (20×10^{6}) were dispensed into a series of sterile plastic centrifuge tubes and thymus cells (5×10^{6}) or LNC in variable numbers, from appropriate strains, were added to some of these PEC suspensions while other PEC suspensions, which served as one form of control, received no additional cells. The tubes were centrifuged at 150 g for 5 min and the cell pellet was resuspended in 0.45 ml of the tissue culture medium with the aid of a vortex mixer. The resultant cell suspension was kept in an ice-water 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 served to provide a heat-insulating air space to protect the cell suspension while the tubes were sealed with a small natural gas-oxygen flame. Seven capillary tubes were prepared from each 0.45 ml cell suspension with this procedure. The sealed capillary tubes were placed horizontally (to prevent differential cell sedimentation) in sterile glass tubes and maintained at 4^{o}C while the remainder were filled. When all tubes had been filled they were centrifuged at 150 g for 7 minutes at 4^{o}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 ignite 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 the capillary tubes was approximately 0.7 cm in length and the tube was cut with a diamond-tipped scribe 1.0 mm below the cell-fluid interface, to insure that the tube mouth would expose the packed cells only and not the supernatant. 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 TCM to prevent artifacts due to 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 TCM via an access channel which was then sealed with wax. The chambers were placed horizontally with the coverslip 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 later experiments 10 x 35 mm sterile disposable plastic petri dishes (Falcon Plastics) were used as culture chambers. The culture chambers were assembled from sterile disposable petri dishes with lid #3001 forming the cover and dish #3002 containing a 6.25 cm^2 cover slip, fixed with a drop of silicone grease, forming the base. Capillaries (6 per chamber) were mounted on the cover slip with silicone grease, TCM was applied to the tube mouths, the lid was rapidly welded to the base at several points with a hot Pasteur pipette and the chamber was sealed with molten paraffin wax. TCM (6 ml) was slowly injected into the culture chamber via a temporary access channel.

Following incubation of the chambers for 24 h at 37°C, migration areas were traced onto transparent film from dark-field images using a Nikon Model 6 Profile Projector (20X magnification) and were integrated directly by planimetry. Per cent migration inhibition was calculated from the expression [(mean area of control - mean area of test migrations)/(mean area of control migrations)] x 100.

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 inoculated with 2×10^7 PEC in 5.0 ml of complete tissue culture medium. The flasks were incubated in an air-CO_2 incubator at $37^{\circ}C$ 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 5×10^8 $B6AF_1$ lymphoid cells (spleen, thymus, lymph node), as an emulsion in an equal volume of complete Freund's adjuvant (Difco). After a period of 3 weeks

the sheep received four injections (spaced two days apart) i.m. of 5×10^8 $B6AF_1$ lymphoid cells in saline and was 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 5×10^7 per ml in the EDTA buffer supplemented with 1 per cent 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.

ix. Tumor growth assay

Sarcoma I ascites tumor cells were washed once in Hanks' balanced salt solution (HBSS with centrifugation at 150 g (5 min) and resuspended in HBSS to produce a nucleated cell concentration of 10 x 10^6 cells/ml. Cell viability was in excess of 95 per cent as determined by the exclusion of 0.2 per cent Trypan blue. In each experiment all groups of mice were inoculated subcutaneously on the dorsum with 2 x 10^6 tumor cells from a continuously mixed cell suspension and tumor growth was recorded as the mean of two perpendicular diameters.

x. Studies with anti-theta serum

Anti-θ serum was prepared according to Reif and Allen (1964) in AKR mice, and used neat or diluted in HBSS (pH 7.2) containing 1.5 per cent heated fetal calf serum and 0.01 M N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) buffer. LNC (1.5 - 2.0 x 10^6) were incubated for 30 min at 37°C in antiserum, washed once with the above solution and resuspended in 1.0 ml of agarose-absorbed guinea pig serum following the method of Cohen and Schlesinger (1970) (diluted 1:9) and incubated again for 30 min at 37°C. The cells were washed once and

resuspended in medium RPMI 1640 containing 20 per cent heated fetal calf serum, 0.04M HEPES and antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin) which also served as the medium for migration in these experiments. Some batches of anti-θ sera (diluted 1:2 in HBSS) were absorbed with A/J or AKR brain homogenates (1 vol serum: 5 vol sediment) for 1 h at $4^{o}C$.

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 as having pale pink cytoplasm and multilobed nuclei. The majority of cells appeared to be neutrophilis 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 per cent of the lymphocytes in all preparations to be described were of the small variety, having diameters of less than 8 μ 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 μ in diameter. A third population of cells having diameters from 15 to 25 μ 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.

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 ramined relatively constant. While the lymphocyte proportion appears not to vary with time, it was noted that approximately 75 per cent of the lymphocytes were of the small variety at 24, 48 and 72 hours, but at 96 hours more than 50 per cent were of the large type, for both A/J and C57BL/6J mice.

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 yeild and composition of the PEC is shown in Table 2. While the

Table 1

The relationship between length of the induction period and yield and composition of dextran-induced PED

Strain	Average yield per mouse[1]		Percentage of total leukocytes[2]		
	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	18.0	24	14	34	52
	24.0	48	17	63	20
	17.0	72	14	79	7
	12.0	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.

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 ± 1 S. D.
			Lymphocyte	Macrophage	PMN	
A/J	55	Normal	21	74	5	6.7 ± 1.3
A/J	33	Grafted	16	83	1	10.7 ± 5.0
C57BL/6J	123	Normal	8	83	9	13.3 ± 3.0
C57BL/6J	39	Grafted	14	86	0	22.6 ± 2.7
C57BL/6J	5	Oil PEC	38*	42	20	17.0

The irritants used were 1.5 ml of dextran or 1.5 ml 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 per cent were of the large variety of lymphocyte.

proportion of constituent cell types was not affected by grafting, the total cell yield increased by 70 per cent in C57BL/6J mice and by 60 per cent 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 that, despite the precaution of exsanguination, a considerable number of erythrocytes were present in the leukocyte population. 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, the inconvenience of oil-medium mixtures, and the observation that migrations of oil-induced PEC were not reproducable.

Thioglycollate fluid medium has been used previously to induce PEC in mice (Gallily and Feldman, 1967), and was employed in several of the present experiments. The 72-hour total cell yield was as high as 2-3 x 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 with 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°C, as described in the section Material 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 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 Hank's solution, centrifuged for 30 seconds (peak acceleration about 100 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 per cent 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 $4^{o}C$.

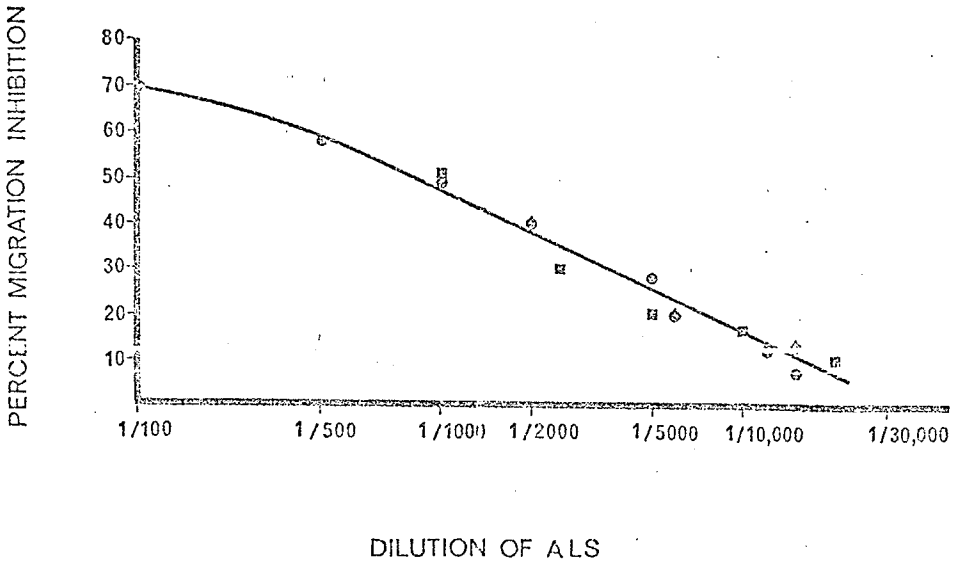

Figure 1

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. The curve represents pooled results from four experiments.

iii. Inhibition of migration with antiserum

Results of David Al-Askari and Lawrence (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 per cent. This may be the migration enhancing 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 per cent were observed. Even at dilutions of 1:5000 ALS was able to inhibit PEC migration by 20-28 per cent 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 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 inoculation of the SaI tumor, which shows only 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 per cent 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 per cent were observed (Figure 2). Owing to the poor yields of A/J PEC, other experiments utilized a migrating population comprised of 20 per cent A/J PEC and 80 per cent C57BL/6J PEC. Under these conditions, using normal C57BL/6J PEC, inhibitions with an average of 13.6 per cent were obtained, while after immunization with SaI inhibitions of 31.6 per cent were recorded (Figure 2). Apparent as well from the data in Figure 2 (see

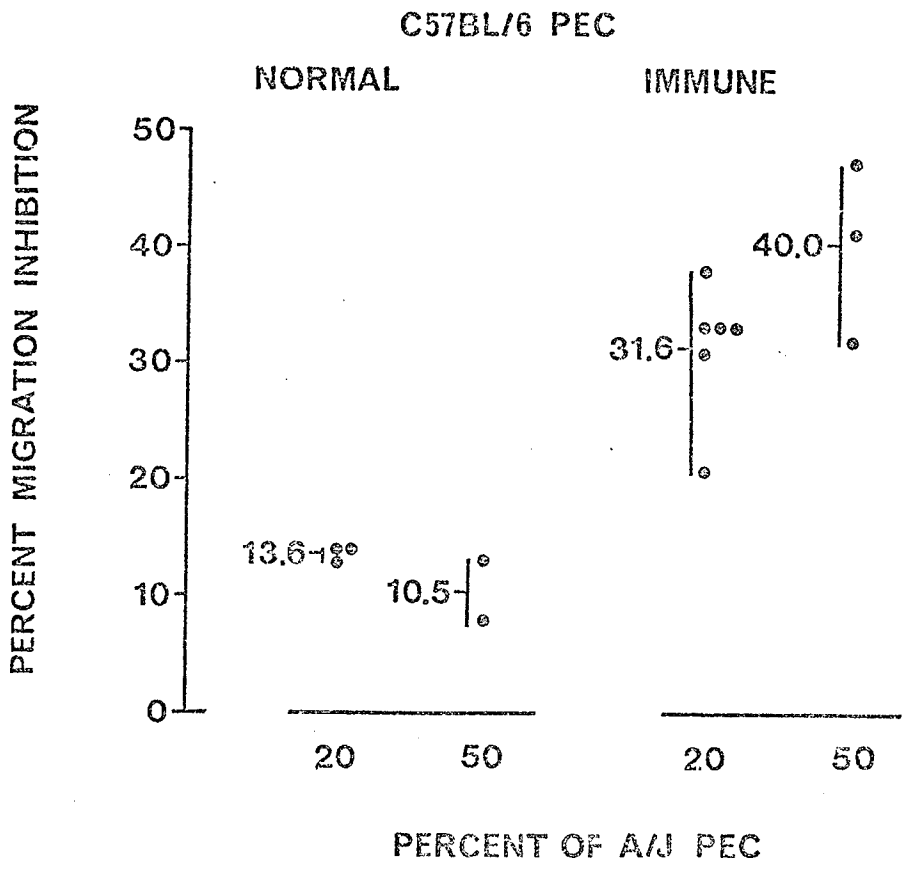

Figure 2

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 per cents migration inhibition for each group is indicated. The calculation of per cent migration inhibition for mixtures of PEC of unequal proportions (e.g. 20 per cent A/J PEC and 80 per cent C57BL/6J PEC) was based on the expression:

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

Detail in Figure 2: In experiments in which 20 per cent 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).

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, 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

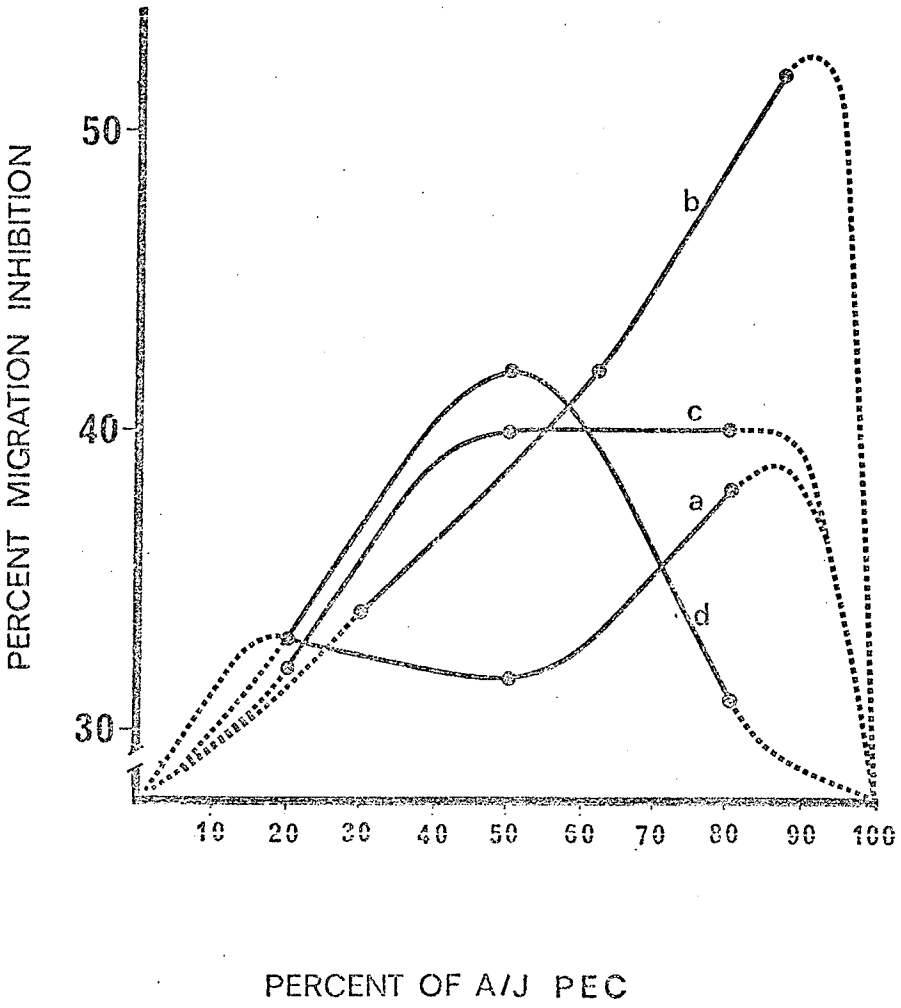

Figure 3

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.

source of H-2 antigens (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 per cent, 43 per cent, or 60 per cent 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 per cent thymus cells and 80 per cent 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 per cent) 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

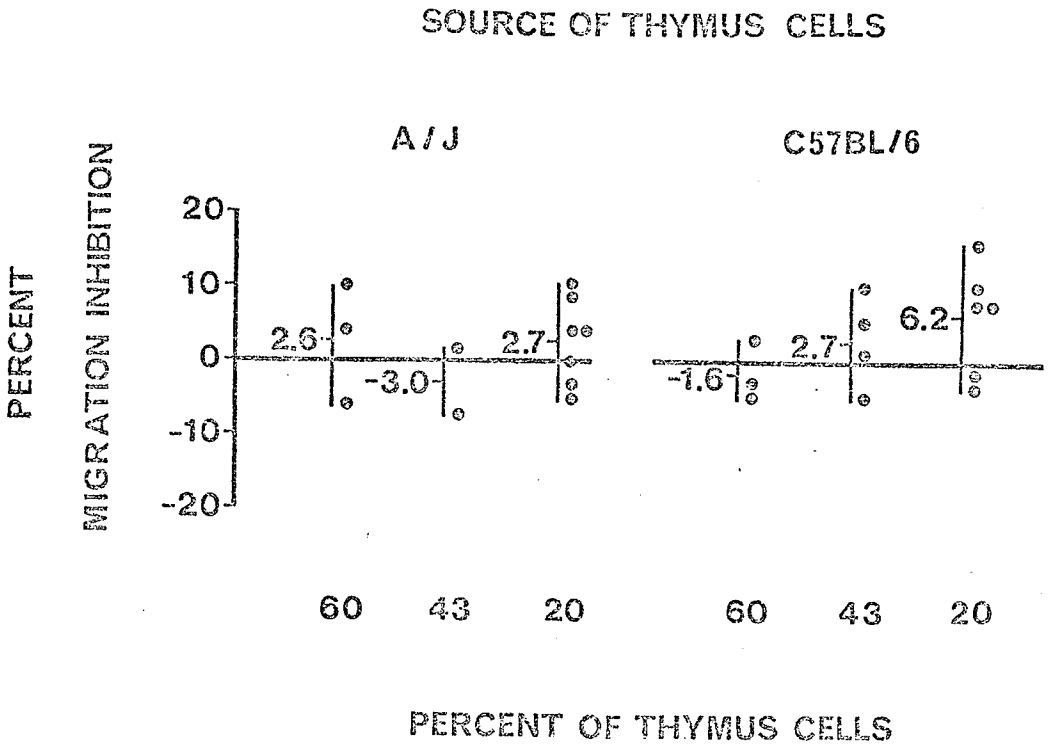

Figure 4

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 per cents 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.

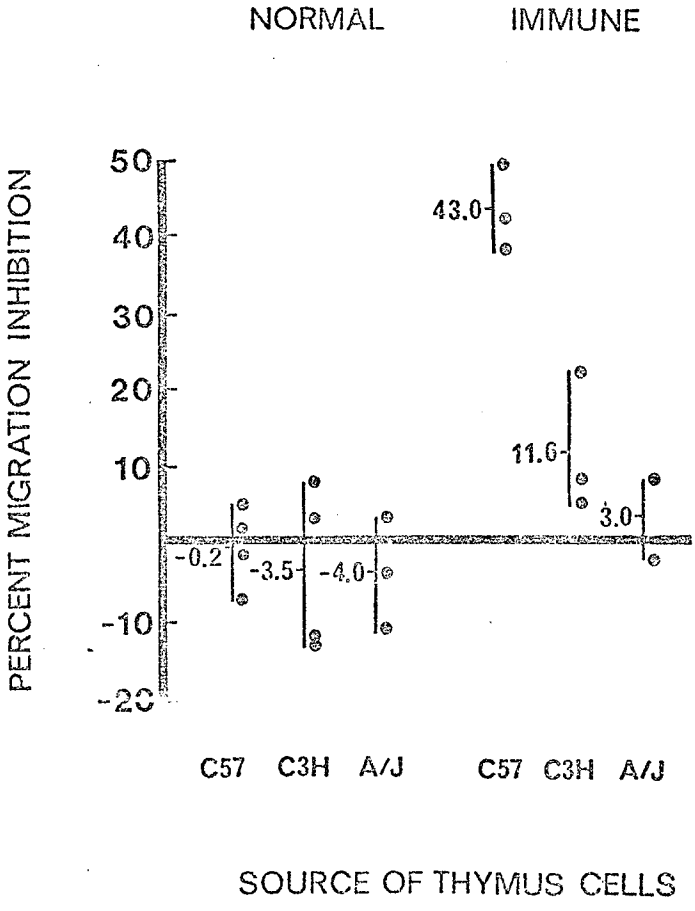

Figure 5

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 obtained in a separate experiment; the mean value and range of the per cents migration inhibition is indicated for each group. In each experiment mice of one sex only were used.

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 per cent thymus cells and 80 per cent PEC. As 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 per cent when thymus cells from A/J were added, and 35.5 per cent 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 per cent), and that PEC from mice immunized 8 days previously were only moderately inhibited (16 per cent).

The choice of 20 per cent 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 per cent 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 per cent content of thymus cells in the migrating population might result in greater inhibitions.

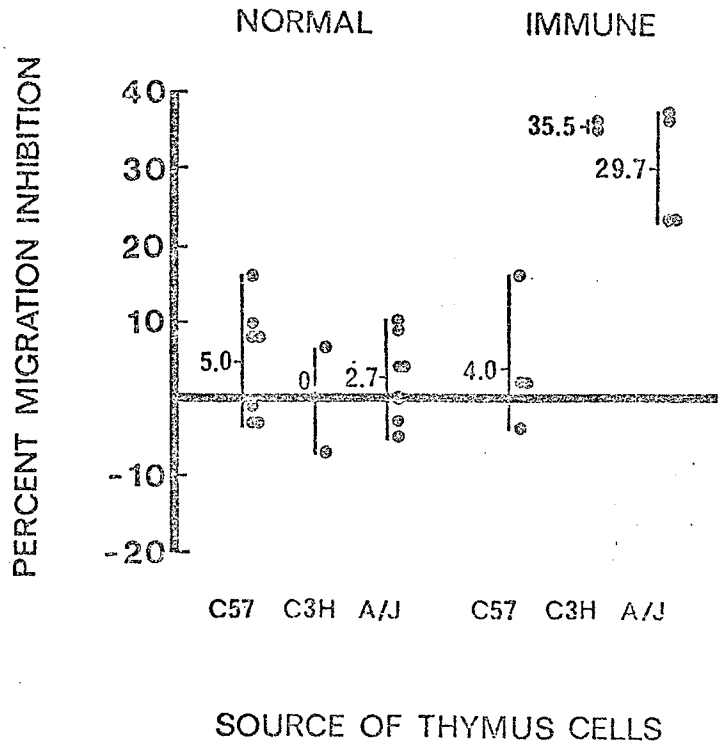

Figure 6

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 obtained in a different experiment and was calculated from the average value of six capillary migration areas. The average value and range of the per cents migration inhibition is indicated for each group. In each experiment mice of one sex only were used.

In an experiment designed to test this possibility the per cent 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 inhibitions of migration recorded. When the per cent of A/J thymus cells were varied from 20 per cent to 5.9 per cent the per cents migration inhibition were identical while at 3.0 per cent thymus cells the inhibition of migration decreased (Table 3). These results suggest that immune migrating cells containing 20 per cent 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 20 per cent 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 per cent.

(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-

Table 3

Effect of the proportion of antigenic thymus cells on the migration of admixed PEC

Per cent of thymus cells	Per cent inhibition of migration ± 1 S.E.M.
20.0	39.2 ± 1.4
11.1	40.3 ± 2.1
5.9	39.7 ± 1.7
3.0	22.9 ± 2.9

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 were 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 per cent LNC and 80 per cent 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 per cent inhibition of migration of syngeneic A/J PEC to 5.3 per cent 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 per cent) but were

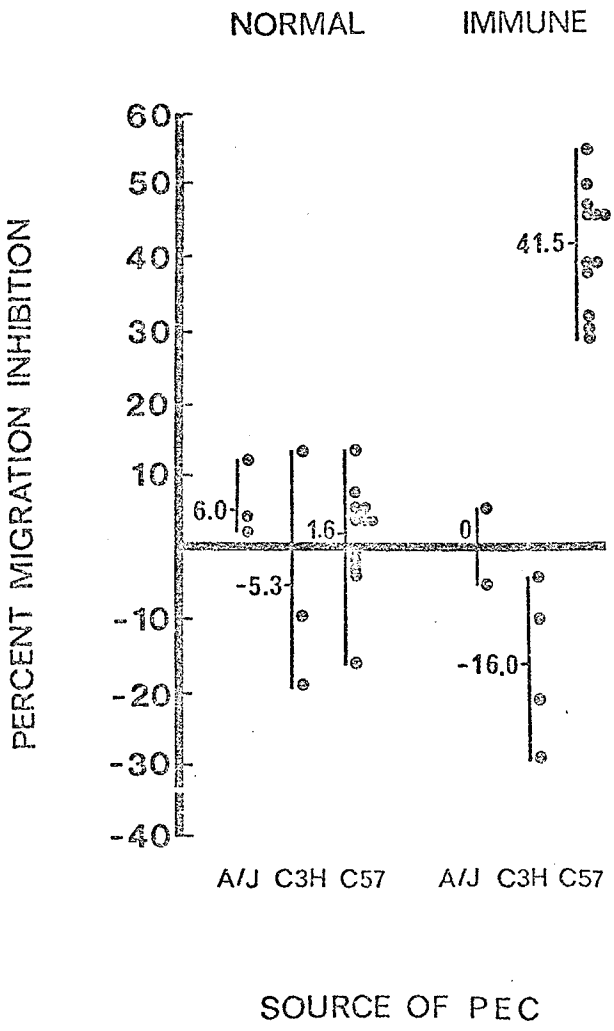

Figure 7

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 obtained in a different experiment and was calculated from the average of six capillary migration areas. The average value and range in per cent migration inhibition is indicated for each group. In each experiment mice of one sex only were used.

without significant effect on the migration of syngeneic A/J PEC and 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 per cent and 21 per cent 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 per cent of LNC present in the migrating population was varied from 20 per cent to 0.15 per cent and the corresponding inhibitions of migration were recorded. Figure 8 shows, on a semi-logarithmic plot, the per cent of LNC contained in the migrating populations and the resultant inhibitions of four experiments. The dashed area represents the range of inhibitions obtained in twelve experiments using 20 per cent LNC, (the average was 41.5 per cent inhibition). It is evident that at concentrations of 20 per cent LNC the inhibitions approached a maximum of 40 to

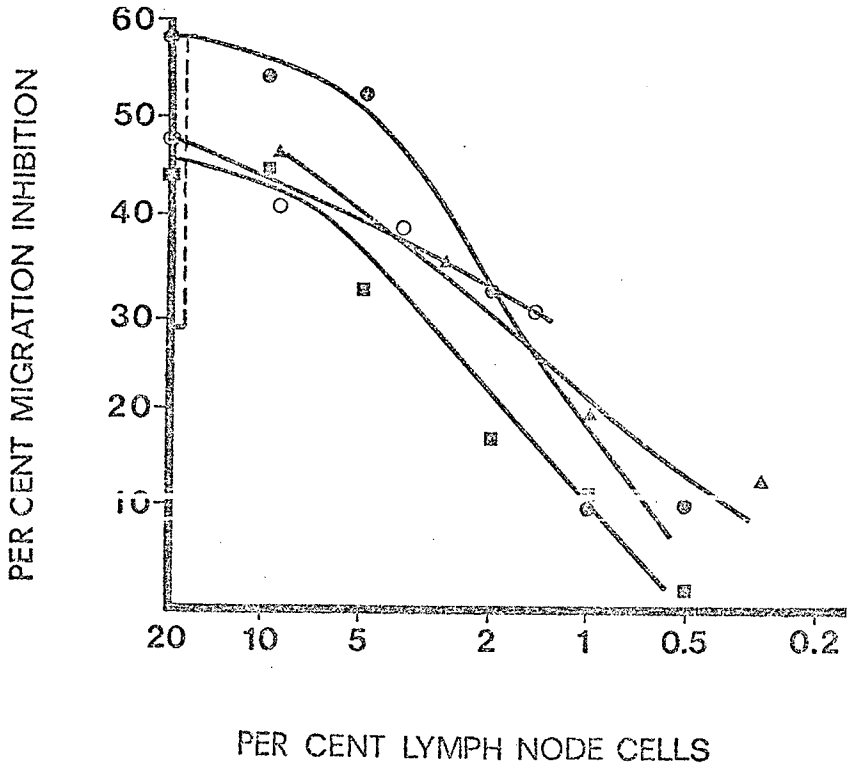

Figure 8

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 represented the range of inhibitions obtained in twelve experiments using 20 per cent LNC, (the average was 41.5 per cent inhibition).

60 per cent. Apparent as well is a linear relationship between the logarithm of the LNC concentration and the per cent 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 per cent at a concentration of 20 per cent LNC and by 22 per cent at a concentration of 11 per cent 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 per cent. 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 are taken from mice grafted 22 days earlier with C57BL/6J skin, an inhibition of 26 per cent was obtained when LNC were present at a concentration of 20 per cent, but with a concentration of 10 per cent LNC the inhibition fell to 7 per cent, 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. These results contrast with the ability of LNC obtained 9-12 days following grafting to sustain near-maximum inhibitions at 10 per cent LNC and illustrate

the suitability of the test to follow response kinetics semi-quantitively.

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 per cent or 1 per cent LNC were able to inhibit the migration of antigenic PEC. In addition, this experimental design provides a method for a semi-quantitative comparison of the immunological activity of different populations of sensitized cells.

(e) Other approaches to immunization

(i) Other alloantigens

In some ancillary studies the ability of different cellular antigens to sensitize host lymphoid cells was tested in inhibition of migration experiments. Antigens used were in the form of skin grafts and involved immunizations to either H-2 incompatibilities only or to non-H-2 incompatibilities only.

In the first series of studies strain B10.129(5M) mice were immunized with bilateral C57BL/10 skin grafts. Since the two strains are known to differ at only one weak histocompatibility locus, H-1 (Graff, Hildemann and Snell, 1966), it was of interest to test if the migration assay could detect weak H-1 transplantation immunity. LNC from graft-recipient mice were mixed (20 per cent) with PEC (80 per cent) from the donor strain or with syngeneic PEC. As shown in Table 4 no inhibition of migration was detected using such LNC from day 10 to day 28 following skin grafting despite the rejection of all the skin grafts by day 24.

Table 4

Test of ability of LNC from B10.129(5M) sensitized to C57BL/10 to inhibit the migration of C57BL/10 PEC

Experiment	Lymph node cells	Day	Grafts rejected / Total grafted	Per cent inhibition of migration (+ 1 S.E.M.) of PEC from C57BL/10	Per cent inhibition of migration (+ 1 S.E.M.) of PEC from B10.129(5M)
1	Normal			+ 4.3 ± 2.2	-
	Immune	10	0/8	-11.7 ± 3.0	-
	Immune	24	8/8	+ 7.7 ± 2.0	-
2	Normal			+ 3.0 ± 3.8	+ 7.8 ± 3.0
	Immune	14	3/4	+ 9.3 ± 2.4	+ 8.3 ± 3.2
	Immune	28	4/4	+10.1 ± 2.9	+11.2 ± 2.4

Male mice only were used in this study.

Although no immunity was detected to the single (H-1) incompatibility in vitro it was of interest to test for immunity to multiple non-H-2 incompatibilities, since it is known that differences at multiple histocompatibility loci are additive in effect (Graff, Silvers, Billingham, Hildemann and Snell, 1966) and would perhaps therefore be detectable in vitro. Accordingly strains C57BL/6 and 129/J mice were used since they share the same H-2^b type but have different non-H-2 constitutions, and immunizations were performed in both directions i.e., 129/J → C57BL/6 or C57BL/6 → 129/J. Both forms of the migration assay were used, thus in one pair of experiments immune C57BL/6 PEC (80 per cent) were mixed with antigenic 129/J thymocytes (20 per cent) and in another pair of experiments immune 129/J LNC were mixed with antigenic C57BL/6 PEC (80 per cent). It is evident from the data (Table 5) that no inhibition of migration was detected even with the use of an immunization involving multiple non-H-2 antigens.

(e) Other approaches to immunization

(ii) Effect of antigen in different forms

Since skin grafts were shown to be potent immunogens it was of interest to test the ability of suspensions of normal allogeneic lymphoid cells to immunize mice for inhibition of migration, as lymphoid cell suspensions are commonly used to presensitize mice in tests for histocompatibility differences. For these experiments A/J mice were inoculated s.c. in the region of the scapulae, bilaterally with a dose of 5×10^7 C57BL/6 spleen cells per site. Recipient, draining lymph nodes were removed 9 to 11 days later and assessed for the ability to inhibit

Table 5

A failure to detect immunity to multiple non-H-2 differences by inhibition of migration

Immunization	Experiment	Day	Per cent (± 1 S.E.) inhibition of migration		
			Antigenic cell		
			A/J	129/J	C57BL/6
129/J → C57BL/6	(I) PEC + Thymus	9	- 4.3 ± 5.3	- 5.3 ± 2.4	+ 0.3 ± 2.5
129/J → C57BL/6	(I) PEC + Thymus	10	+ 4.8 ± 4.5	+ 1.4 ± 8.1	+ 9.4 ± 1.6
C57BL/6 → 129/J	(I) LNC + PEC	9	-	-	+ 0.7 ± 2.3
	(N) LNC		-	-	- 4.1 ± 3.5
C57BL/6 → 129/J	(I) LNC + PEC	9	-	-	+ 4.8 ± 4.2
	(N) LNC		-	-	- 9.7 ± 3.1

(I) = immune; male mice only were used in the above experiments.

donor PEC. Varying ratios of LNC and PEC were used in order to estimate semi-quantitatively the degree of immunity obtained. As is evident in Table 6 the subcutaneous inoculation of 1×10^8 allogeneic spleen cells did confer a substantial degree of immunity upon recipient LNC. However the immunity was of the order of 10 to 20 times less than that induced with skin grafts (compare with Figure 8), since no inhibitions were detected with the use of 4.7 per cent LNC while following skin grafting, in the same strain combination, 5.0 per cent of LNC produced near-maximal inhibitions.

Much of the work which defines the technique of inhibition of migration in guinea pigs involved soluble protein antigens not cell-bound antigens; it was therefore of interest to attempt to induce inhibition of migration in mice immunized with a soluble antigen so as to produce cellular immunity (Crowle and Hu, 1966). Two groups of mice (six animals each) were immunized with 0.25 mg of HSA or methylated HSA (kindly supplied by Dr. Crowle) on day 0 and day 7. The antigen vehicle was a 50 per cent emultion in complete Freund's adjuvant and injected in a volume of 0.1 ml s.c. in an abdominal site. A third group received adjuvant only. On day 13 the animals were skin-tested with 20 µg of methylated HSA in 0.02 ml, injected intradermally and reactions were read at 24 h. Animals injected with adjuvant only had indurated skin reactions of less than 5 mm^2, those injected with HSA and adjuvant had reactions of 12 mm^2 and those injected with methylated HSA had reactions of 10 - 12 mm^2. PEC were obtained from each group of mice (dextran had been administered on day 11) and used in the migration assay. Test cultures contained 75 µg/ml of methylated HSA

Table 6

Inhibition of migration of C57BL/6 PEC mediated by LNC from A/J mice immunized with C57BL/6 cell suspensions

Experiment	Per cent A/J LNC	Per cent inhibition of migration (± 1 S.E.M.)
1	20.0	20.9 ± 2.5
	4.7	0.0 ± 1.8
	20 (normal)	9.3 ± 4.4
2	20.0	15.5 ± 2.4
	4.7	0.8 ± 3.5

Female mice were used in these experiments. Immunization consisted of a bilateral inoculation of 5×10^7 C57BL/6 spleen cells into two s.c. sites. LNC were obtained from recipient mice sacrificed on days 9 and 11.

in the tissue culture medium. PEC from the control group (adjuvant only) exhibited 0.7 ± 3.6 (S.E.) per cent inhibition, the group inoculated with HSA 6.8 ± 1.2 per cent and the group inoculated with methylated HSA, 0.0 ± 3.0 per cent inhibition of migration. Thus delayed hypersensitivity to a soluble protein antigen was not demonstrated *in vitro* with the use of mice. Female C57BL/6 mice of 10 weeks of age were used in these experiments.

(f) Analysis of the antigen specificity for inhibition of migration by antigenic thymocytes

As considered previously no inhibition of migration was observed upon mixing allogeneic thymus cells (20 per cent) with normal PEC (80 per cent). This was confirmed using C57BL/10 PEC and a wide variety of allogeneic thymus cells (Figure 9). To investigate more precisely the antigenic requirements for inhibition of immune PEC migration C57BL/10 mice were grafted with skin from C3H/HeJ or A/J mice and tested for inhibition of migration with thymus cells from a panel of strains having all, part, or none of the H-2 antigens of the graft donor strain as defined serologically (Table 7). In the first series of experiments PEC from C57BL/10 mice grafted with C3H/HeJ skin were inhibited equally by C3H/HeJ and by B10.BR thymus cells (Table 8). Since B10.BR shares the same H-2 chromosome (according to the definition of Klein and Shreffler, 1971) with C3H/HeJ mice but is otherwise genetically identical to the recipient C57BL/10 this finding indicates that antigens determined by the H-2 complex are of paramount importance in this assay. Thymus cells from A/J caused strong inhibition of migration as well.

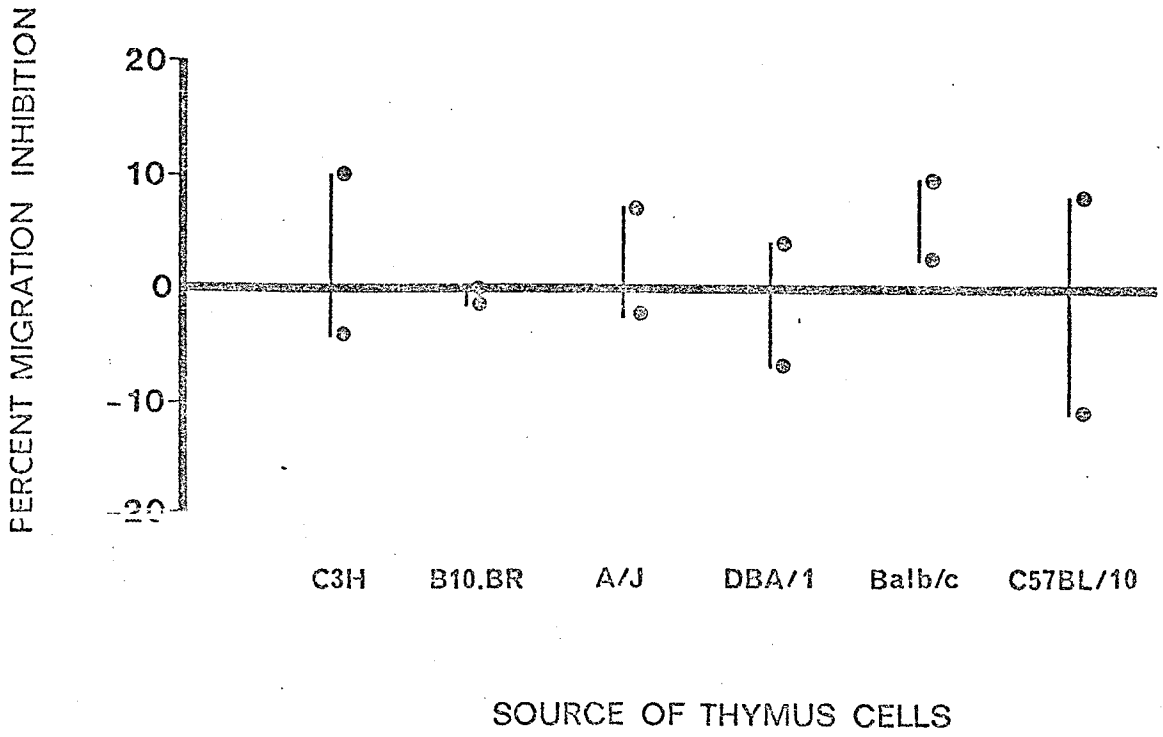

Figure 9

The effect upon the migration of C57BL/10 PEC of the inclusion of 20 per cent of thymus cells from various allogeneic strains.

Table 7

Representation of Presumptive Immunizing Antigens and Those Available for Reaction on Third-Party Cells

Donor	Recipient	Third party	H-2 specificities of the donor	
			H-2K	H-2D
C3H/HeJ ($H\text{-}2^k$)	C57BL/10 ($H\text{-}2^b$)		23* 1,3,8,11,25,45	32* 1,3
			Donor H-2 specificities presented by third party	
		B10.BR ($H\text{-}2^k$)	23 1,3,8,11,25,45	32 1,3
		B10.A ($H\text{-}2^a$)	23 1,3,8,11,25,45	— -,3
		DBA/1 ($H\text{-}2^q$)	— 1,3,-,11,—,45	— -,3
		B10.D2) BALB/c) ($H\text{-}2^d$)	— -,3,8,—,—,—	— -,3
A/J ($H\text{-}2^a$)	C57BL/10 ($H\text{-}2^b$)		23* 1,3,8,11,25,45	4* 3,13,41,42,43,44
			Donor H-2 specificities presented by third party	
		B10.A ($H\text{-}2^a$)	23 1,3,8,11,25,45	4 3,13,41,42,43,44
		B10.D2 ($H\text{-}2^d$)	— -,3,8,—,—,—	4 3,13,41,42,43,44
		B10.BR ($H\text{-}2^k$)	23 1,3,8,11,25,45	- 1,3,—,—,—,—,—
		DBA/1 ($H\text{-}2^q$)	— 1,3,-,11,—,45	3,13,—,—,43,—
		B10.M ($H\text{-}2^f$)	— -,-,8,—,—,—	- -,—,—,—,—,—
B10.M ($H\text{-}2^f$)	C57BL/10 ($H\text{-}2^b$)		9* 8,37	9* 7
			Donor H-2 specificities presented by third party	
		SaI ($H\text{-}2^a$)	- 8,—	- -
C57BL/10 ($H\text{-}2^b$)	B10.D2 ($H\text{-}2^d$)		33* 5,39	2*
			Donor H-2 specificities presented by third party	
		SaI ($H\text{-}2^a$)	— 5,—	-

* denotes a private H-2 specificity and — indicates the specificity is not present. (From the table of Klein and Shreffler, 1971).

Table 8

Specificity of inhibition of migration of C57BL/10 PEC (immune to C3H/HeJ) by antigenic cells from different strains

Experiment	Per cent inhibition of migration (± 1 S.D.) induced by antigenic cells obtained from					
	C3H/HeJ	B10.BR	A/J	BALB/c	DBA/1	C57BL/10
1	30.4 ± 6.9	30.2 ± 6.7	25.3 ± 8.9	9.9 ± 6.0	12.7 ± 6.1	-
2	44.5 ± 2.6	48.9 ± 5.5	40.1 ± 6.2	19.3 ± 4.1	2.0 ± 7.3	6.9 ± 5.4
3	37.9 ± 3.4	37.8 ± 1.4	43.3 ± 2.1	15.0 ± 7.8	9.4 ± 7.7	13.2 ± 2.3
4	49.0 ± 5.0	46.8 ± 3.7	38.7 ± 6.3	5.4 ± 9.6	- 4.2 ± 8.2	- 4.3 ± 8.0

PEC from the graft recipient were mixed 1:4 with thymus cells (antigen) from non-immunized mice prior to cell migration.

This may have been expected, since all the antigens of C3H/HeJ determined by the H-2K region are represented in strain A/J (Table 7). The absence of the D-region antigen H-2.32 of C3H/HeJ did not appear to reduce the inhibition. However, the addition of thymus cells from DBA/1 mice, which have 4 of the 8 H-2 specificities of the graft donor, to the immune PEC resulted in migrations almost identical to those obtained in the presence of C57BL/10 thymus cells, the syngeneic control. Thymus cells from BALB/c mice produced only minimal effects in two experiments when compared to the effects of C57BL/10 thymus cells.

In the second series of experiments, in which A/J was the graft donor, a similar pattern of results was obtained. The ability of B10.A thymus cells to produce inhibitions similar to those caused by cells from A/J mice (Table 9) confirms the dominance of H-2 antigens in this assay, as shown in the former group of experiments. Thymus cells from B10.BR and B10.D2, having all the K- and D-region H-2 antigens of A/J respectively (Table 7), produced inhibitions. However, as before, thymus cells from DBA/1 mice did not inhibit the migration of PEC from immune C57BL/10 mice.

It is apparent from the design of the above migration technique that the sensitivity of the assay may be determined by two limiting parameters: the number of thymus cells in the migrating cell mixture and the number of specifically sensitized cells in the peritoneal exudate population. The first possibility was excluded by the results of an experiment in which antigenic A/J thymus cells were added in varying numbers to PEC from C57BL/6 mice grafted 10

Table 9

Specificity of inhibition of migration of C57BL/10 PEC (immune to A/J) by antigenic cells from different strains

Experiment	Per cent inhibition of migration (± 1 S.D.) induced by antigenic cells from					
	A/J	B10.A	B10.BR	B10.D2	DBA/1	C57BL/10
1	27.4 ± 2.4	25.7 ± 5.7	24.0 ± 6.6	10.9 ± 3.4	4.4 ± 4.3	-3.2 ± 2.7
2	33.5 ± 5.6	27.3 ± 1.9	23.2 ± 7.2	17.6 ±10.3	5.9 ± 7.0	-1.6 ± 5.4
3	19.6 ± 4.9	32.7 ± 6.2	14.7 ± 5.7	12.2 ± 3.6	-1.9 ±10.9	7.8 ± 4.0

PEC from the graft recipient were mixed 1:4 with thymus cells (antigen) from non-immunized mice prior to cell migration.

days earlier with A/J skin. When A/J thymus cells constituted 20, 11, or 5.9 per cent of the migrating cell population, the degrees of inhibition of migration were identical (40 per cent inhibition). Only when the proportion of thymus cells was reduced to 2.0 per cent was decreased inhibition noted (23 per cent inhibition) as considered earlier (p.75). However, an adjustment of the proportion of sensitized cells in the migrating cell mixture, to ensure their presence in non-limiting numbers, was only possible using the modification of the migration technique which involves the use of immune LNC, considered below.

(g) Analysis of the antigen specificity for inhibition of migration by immune LNC

It was of interest to compare the antigen requirements for inhibition of migration in the second form of the migration assay, mediated by LNC, since this test was shown (Figure 8) to have non-limiting numbers of sensitized cells. Also, since it was possible that thymus cells from strains DBA/1 and BALB/c failed to cause inhibition of migration in the thymus cell - PEC form of the assay because of low sensitivity of the test, perhaps due to limiting numbers of sensitized cells in the PEC, such reactions might have been demonstrable with a more sensitive test. Therefore two groups of experiments were performed using the LNC-PEC modification of the migration assay in an attempt to overcome this limitation. In the first of these experiments LNC were obtained from C57BL/10 mice immunized with C3H/HeJ skin grafts and the immune LNC were mixed with PEC from normal mice of several strains having all, part, or

none of the H-2 antigens of the graft donor. Under these conditions the reactivity of the sensitized lymphocytes from C57BL/10 against cells of B10.BR and A/J origin was confirmed (Table 10). Moreover, reactions were observed in every experiment against cells of both DBA/1 and B10.D2 origin which have some donor H-2 specificities but lack identity with either the K or D region of the H-2 chromosome of the donor (Table 7), in marked contrast to the negative or borderline reactions obtained in similar experiments using the previous migration system (Table 8).

The second group of experiments (Table 11) confirmed these findings using the second donor-recipient combination. LNC from C57BL/10 mice grafted with skin from strain A/J mice markedly inhibited the migrations of PEC from all allogeneic strains tested, including PEC from DBA/1 mice, again in contrast with the results obtained in the previous assay using thymus cells as the source of antigen. In this experiment as well, the representation of donor H-2 specificities is incomplete on cells of DBA/1 mice; neither all the K- nor D-region antigens are present on DBA/1 cells (Table 7). PEC from C57BL/10 mice were tested with the immune LNC in each experiment, and provide no evidence for non-specific inhibition of migration.

Table 10

Specificity of inhibition of migration mediated by C57BL/10 LNC (immune to C3H/HeJ) upon PEC from different strains

Experiment	Per cent inhibition of migration (± 1 S.D.) of PEC obtained from					
	B10.BR	A/J	B10.A	B10.D2	DBA/1	C57BL/10
1	44.3 ± 7.2	48.8 ± 6.3	-	16.2 ± 4.9	23.9 ± 10.0	14.1 ± 4.7
2	34.8 ± 3.0	-	-	27.4 ± 4.0	25.5 ± 4.9	11.3 ± 4.5
3	47.5 ± 5.7	-	41.6 ± 3.5	14.7 ± 5.3	50.7 ± 6.7	7.9 ± 7.4
4	-	31.5 ± 3.7	-	8.4 ± 6.0	18.0 ± 8.0	- 0.3 ± 8.2

LNC from the graft recipient were mixed 1:4 with PEC (antigen) from non-immunized mice prior to cell migration.

Table 11

Specificity of inhibition of migration mediated by C57BL/10 LNC (immune to A/J) upon PEC from different strains

Experiment	Per cent inhibition of migration (± 1 S.D.) of PEC obtained from					
	B10.A	A/J	B10.BR	B10.D2	DBA/1	C57BL/10
1	72.5 ± 3.0	77.6 ± 2.0	80.0 ± 4.5	66.4 ± 2.3	53.0 ± 11.4	13.3 ± 4.4
2	70.9 ± 3.5	82.9 ± 3.0	67.6 ± 1.7	72.0 ± 5.2	69.4 ± 7.3	12.8 ± 7.2
3	77.9 ± 2.3	76.3 ± 3.4	67.4 ± 3.2	68.6 ± 1.7	51.0 ± 5.1	8.5 ± 2.2

LNC from the graft recipient were mixed 1:4 with PEC (antigen) from non-immunized mice prior to cell migration.

(h) Effect of anti-theta serum on LNC-mediated inhibitions

An anti-θC3H serum prepared according to the protocol of Reif and Allen (1964) was prepared with Dr. Sabbadini. The cytotoxic titre is represented in the titration curve in Figure 10. To determine the cell type responsible for inhibition of migration mediated by LNC, sensitized LNC were obtained from A/J mice grafted 9-11 days earlier with C57BL/6 skin and were treated with anti-θ serum and complement prior to mixing with antigenic C57BL/6 PEC (Table 12). From the data shown, and from other similar experiments, it is apparent that treatment of LNC with anti-θ serum and complement reduces or abolishes the ability of these cells to mediate inhibition of migration. The activity of the antiserum was removed upon prior absorption with A/J brain homogenates (A/J has the θ-C3H type) but was only slightly reduced upon absorption with AKR brain (possibly a dilution effect).

(i) Studies with an assay of tumor immunity in vivo

The observations of *in vitro* inhibition of migration reactions evoked by third-party cells not having identity with either of the two H-2 regions of the donor suggested that a sensitive and quantitative assay for transplantation immunity *in vivo* might reveal similar reactions. Since prior grafting with A/J skin prevents growth of the A/J tumor SaI in most allogeneic mice, skin grafts from other strains of mice sharing transplantation antigens in common with A/J should suppress tumor growth as well. Therefore mice were immunized with skin grafts from congenic strains (i.e. immunizations confined to H-2 antigens) and after 10 days were inoculated with the tumor. Donors were selected so that in some

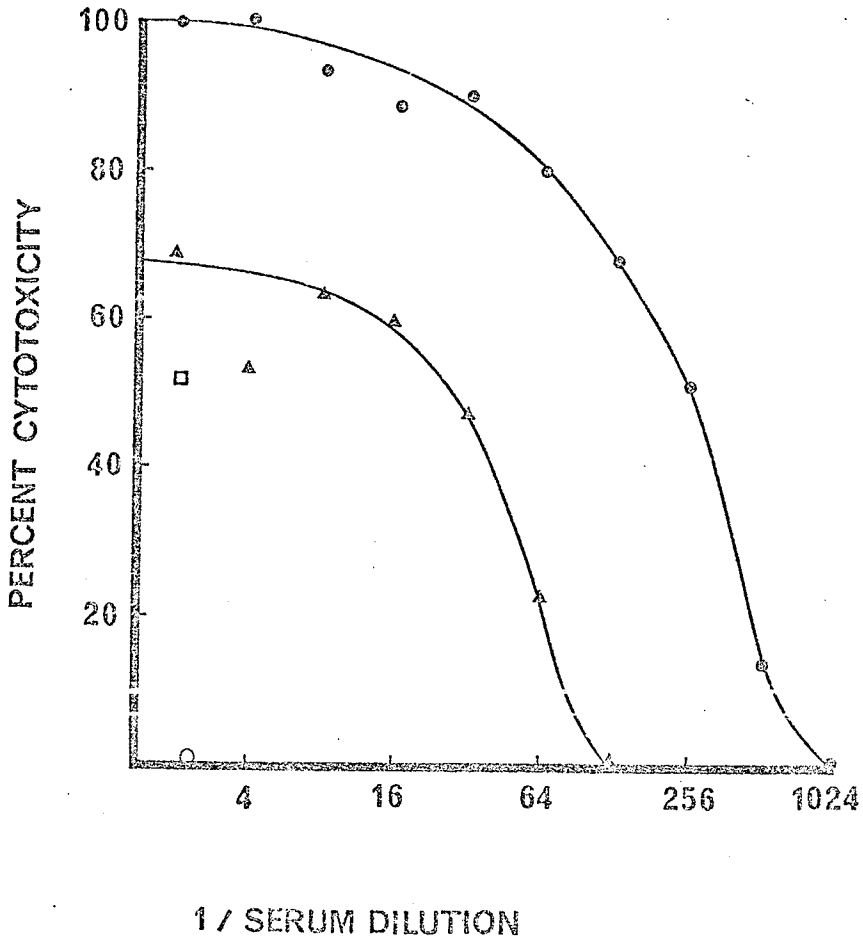

Figure 10

Cytotoxic titration of AKR anti-θC3H serum with thymus cells (●) or lymph node cells (▲) obtained from A/J mice. Each point represents the mean of duplicate determinations. For details see Methods.

Table 12

Abrogation of LNC mediated inhibition of migration with anti-θ serum and complement

Experiment	LNC	Treatment of LNC		Inhibition of migration (per cent ± S.E.)
		Stage 1	Stage 2	
1	Immune	HBSS	HBSS	26.0 ± 2.8
		anti-θ 1:2, 0.4 ml	HBSS	30.6 ± 2.4
		anti-θ 1:2, 0.4 ml	complement	7.0 ± 3.7
		anti-θ[a] 1:2, 0.4 ml	complement	29.7 ± 2.8
		anti-θ[b] 1:2, 0.4 ml	complement	19.3 ± 1.8
	Normal	none	none	8.4 ± 4.7
		anti-θ 1:2, 0.4 ml	complement	- 1.9 ± 2.3
2	Immune	none	none	34.8 ± 3.0
		NMS neat, 0.5 ml	complement	39.7 ± 3.1
		anti-θ neat, 0.5 ml	complement	- 1.0 ± 3.4
		anti-θ 1:2, 0.4 ml	complement	18.6 ± 2.6

PEC from C57BL/6 mice were mixed with LNC from A/J mice which had been grafted with C57BL/6 skin 9 - 11 days earlier. The LNC constituted 7 per cent of the migration cells in experiment 1 and 9.1 per cent in experiment 2.

Anti-θ[a] serum was absorbed with A/J brain.

Anti-θ[b] serum was absorbed with AKR brain.

NMS = normal mouse serum.

in some donor-recipient pairs strain A/J would lack identity with the primary graft donor at both H-2 regions. It is evident (Table 13) that the growth of SaI in mice which had received syngeneic grafts was essentially identical to that in untreated hosts, and that prior immunization of C57BL/10 mice with A/J skin grafts or SaI resulted in animals refractory to SaI growth. Skin grafts from B10.BR prevented tumor growth as well, and this is attributed to the fact that the H-2K region of B10.BR is identical to the H-2K region of A/J (Table 7). Moreover, grafts from B10.M mice reduced tumor growth andincidence, which is notable since in this donor-recipient combination, sharing of a common H-2 region with the B10.M graft donor by the tumor does not occur, and only 1 of 4 of the donor H-2 specificities is represented on cells of SaI (H-2.8).

In a second experiment of similar design, B10.D2 mice served as tumor hosts (Table 13). As before, tumor growth was similar in non-treated mice and in those which had received syngeneic grafts 10 days prior to tumor inoculation, and skin grafts from A/J mice suppressed tumor growth entirely. Grafts from C57BL/10 mice caused a reduction in tumor size when compared with controls and prevented tumor growth in about one-half of the recipients. Neither the H-2K nor H-2D region of C57BL/10 is represented on cells of A/J, and only 1 of 4 donor H-2 specificities (H-2.5) is shared (Table 7). Similarly, skin grafts from B10.M mice caused a reduction of tumor growth. As noted for C57BL/10, B10.M does not have identity with either of the two H-2 regions of A/J; moreover, B10.M shares no known serologically defined H-2 specificities with A/J. Thus, in three donor-recipient combinations, resistance to

Table 13

Growth of SaI ($H\text{-}2^a$) in mice presensitized to different H-2 antigens

Recipient	Primary donor	Takes/no. injected	Mean take size # (mm ± S.D.)	H-2 specificities of primary donor * indicates specificities in common with SaI
C57BL/10 ($H\text{-}2^b$)				
	None	5/5	7.7 ± 4.4	
	C57BL/10 ($H\text{-}2^b$)	5/5	9.2 ± 1.8	None
	B10.M ($H\text{-}2^f$)	2/5	5.0 ± 2.8	7, 8*, 37, 9
	B10.BR ($H\text{-}2^k$)	0/6	-	1*, 3*, 8*, 11*, 25*, 45*, 23*, 32
	A/J ($H\text{-}2^a$)	0/4	-	1*, 3*, 8*, 11*, 25*, 45*, 23*, 13*, 41*, 42*, 43*, 44*, 4*
B10.D2 ($H\text{-}2^d$)				
	None	7/7	3.6 ± 1.8	
	B10.D2 ($H\text{-}2^d$)	8/9@	3.4 ± 1.2	None
	C57BL/10 ($H\text{-}2^b$)	3/8	2.6 ± 1.6	5*, 39, 33, 2
	B10.M ($H\text{-}2^f$)	4/7	1.0 ± 0.0	9, 37, 7
	A/J ($H\text{-}2^a$)	0/8	-	1*, 5*, 11*, 25*, 45*, 23*

Growth attained by SaI on day 7 following inoculation s.c. of 2×10^6 tumor cells. Mean size of takes ± S.D. was calculated from the means of two perpendicular measurements.

@ 9/9 on day 8.

the growth of a third-party tumor was afforded by a primary immunization involving only H-2 antigens and in the absence of shared H-2K or H-2D regions by donor and third-party cells.

CHAPTER 2

MATERIALS AND METHODS

In this section general experimental protocols were identical to those described in Chapter 1, unless otherwise stated.

i. Animals and immunizations

Mice of the highly inbred strains A/J, C57BL/6J, B6AF$_1$ (the F$_1$ hybrid of C57BL/6 and A/J), CBA/J, DBA/2J, 129/J, C3H/HeJ, BALB/cJ, DBA/1J, AKR/J, C57BL/KsJ and the congenic strains C57BL/10, B10.BR, B10.D2 (new), B10.A and B10.M were obtained from the Jackson Laboratory, Bar Harbor, Maine, and were 10 - 14 weeks of age when used as allograft recipients.

Immunizations involved members of one sex only, and target cells were obtained from male mice with the exception of B10.M in which case only females were used. Animals serving as a source of target cells were matched for age and used for this purpose as often as twice monthly, for several months. Single, full-thickness trunk skin grafts of 1.0 cm^2 size were applied as described previously (see Materials and Methods , Chapter 1), while tumor allografting was accomplished by inoculating, intraperitoneally, 2 - 3 x 10^7 viable tumor cells of either Sarcoma I (SaI) or mastocytoma P-815-X2 (a gift of Dr. K. T. Brunner), into appropriate recipients. SaI and P-815-X2 were maintained as ascites tumors by serial passage of 1 x 10^6 viable cells, weekly into female mice of strains A/J and DBA/2, respectively.

ii. Aggressor cells

(a) Spleen cells

Spleens obtained by sterile dissection 9 - 11 days following skin grafting, were teased into fragments in sterile disposable plastic petri dishes in chilled Spinner-modified minimal essential medium (MEM) (GIBCO, Grand Island, N.Y.). The cell suspension was decanted from debris 5 min after transfer to sterile tubes and washed 3 times in MEM with centrifugation at 150 g for 5 min. The cells were finally resuspended in RPMI 1640 medium (GIBCO) pH 7.2, containing 10 per cent heated (56°C for 30 min) fetal calf serum, 0.04 M N-2-hydroxyethyl-piperazine-N'-2 ethanesulphonic acid (HEPES) (Calbiochem, San Diego, California) and antibiotics (penicillin, 100 U/ml and streptomycin, 100 µg/ml). The nucleated cell concentration was determined with Turk solution and adjusted as required.

(b) Non-adherent peritoneal exudate lymphocytes (PEL)

PEL were recovered from mice 9 - 12 days following the i.p. inoculation of tumor cells by injecting, i.p., under sterile conditions, 7 ml of MEM containing heparin (20 U/ml), massaging the abdomen and aspirating the fluid with a syringe. The suspension of cells and debris was placed at 4°C for 30 min to allow for the spontaneous gellation and aggregation of debris. Such aggregates were dispersed with the aid of a vortex mixer. Following this, cell aggregates, debris and some large cells sedimented upon suspending the cell yield of up to 10 mice in 20 ml of HBSS in a conical tube for 1.5 h at 4°C. The upper

portion of the cell suspension, approximately 15 ml, was aspirated, transferred to another tube and centrifuged at 200 g for 10 min. The cell pellet was resuspended (4×10^7 cells per 10 ml) in Medium 199 containing 10 per cent heated fetal calf serum, 0.01 M HEPES and antibiotics (100 U penicillin/ml and 100 µg streptomycin/ml) and transferred to sterile flasks (Falcon Plastics #3024, Oxnard, California) for removal of adherent cells. The flasks were incubated for 45 min at 37°C and then agitated to suspend non-adhering cells. These cells were exposed to the opposite flask surface for an additional 45 min. The non-adherent cells (approximately 33 per cent of the original number) were recovered, washed twice in MEM with centrifugation at 150 g for 5 min, and resuspended at the appropriate concentration in the RPMI 1640 medium.

iii. Target cells

For a given experiment five mice from each strain used to obtain target cells were injected with 1.5 ml of a dextran solution, as described previously (Chapter 1). After 3 days the animals were anesthetized with ether and the PEC were harvested aseptically by injecting 6 - 7 ml of MEM i.p., massaging the abdomen and withdrawing the fluid with a syringe. A macrophage-rich population of cells was prepared from the PEC by exposing a 3.0 ml suspension of the cells to 3 brief centrifugations (30 sec each, maximum g = 100) and discarding the supernatant containing the smaller cells at each step. The cells obtained in this manner were comprised of more than 99 per cent macrophages in all strains of the C57BL/10 background, as defined morpho-

logically and by the ability to adhere to polystyrene and to phagocytose colloidal carbon. The macrophages (10×10^6) were labelled with radioactive chromium upon incubation with 100 µCi of $Na_2[^{51}Cr]O_4$ (specific activity at least 40 Ci/g) in the RPMI medium (above) for 30 min, with agitation at 5 min intervals. The target cells were then washed 5 times in 5.0 ml of MEM and decanted from any aggregates of target cells before being resuspended at the required viable cell concentration (exclusion of 0.2 per cent Trypan blue in saline at 5 min) in the RPMI medium. Mastocytoma P-815-X2 ascites tumor cells were washed twice in MEM and labelled as above for use as target cells.

iv. Cytotoxicity assay

Cytotoxicity was assessed using Microtest II tissue culture plates (Falcon Plastics). Aggressor cells were dispensed in 0.2 ml aliquots in variable concentrations followed by the addition of the target cell suspension (usually 4×10^4 cells) in 0.1 ml. The plates were sealed with a plastic film (Cooke Engineering) and incubated for 12 h at 37°C. The experiment was terminated by agitating the plates to mix the contents of each well, centrifuging the plates at 250 g for 10 min and removing 0.1 ml of the supernatants. Radioactivity in the supernatants was determined with a well-type, auto-gamma crystal scintillation counter (Nuclear Chicago). Cytotoxicity of the target cells was calculated by comparing the amount of radioactivity released into the supernatants during culture with the radioactivity released from a sample of macrophages upon freezing and thawing (3 cycles). Values for spontaneous release were obtained from cultures containing

target cells only. The per cent specific ^{51}Cr release was calculated with the following formula:

$$100 \times \frac{\text{experimental release - spontaneous release}}{\text{maximum release - spontaneous release}}$$

Negative values of release may indicate a protective effect of normal or presumptive aggressor cells but not reutilization of isotope (Canty and Wunderlich, 1970), while values slightly greater than 100 per cent specific release may be obtained as well since freezing and thawing is only an estimate of the maximum release of label.

v. Studies with anti-theta (θ) serum

The anti-θC3H serum was prepared in AKR mice according to Reif and Allen (1964) and used according to Raff (1969) with some modifications of technique. HBSS containing 0.01 M HEPES buffer and 1.5 per cent v/v fetal calf serum was used as the diluent. Non-adherent PEC (8×10^6) was suspended in 2.8 ml of neat anti-θ serum, normal mouse serum (NMS), or HBSS for 30 min at 37°C, with agitation at 5 min intervals. The cells were washed once in the HBSS solution and resuspended in either 1.0 ml of agarose-absorbed guinea pig serum (Cohen and Schlesinger, 1970) at a dilution of 1:9 (as a complement source) or in the HBSS solution for an additional 30 min incubation. The agarose absorption step removes some "natural mouse cytotoxins" in guinea pig serum. The PEC were then washed twice in HBSS and re-suspended in the RPMI medium described above for use in the cytotoxic assay.

RESULTS

1. Macrophages as target cells in the ^{51}Chromium release assay

Preliminary experiments examined the characteristics of dextran-induced, purified, peritoneal macrophages and their suitability to serve as target cells in the cytotoxicity assay. As described more fully in Chapter 1, preliminary experiments with A/J and C57BL/6 mice revealed that, 1) dextran-induced PEC contained the highest proportion of macrophages at 72 hours following injection of the irritant, 2) macrophages were highly vacuolated and large in size (15 - 25 μM) and constituted 75 - 85 per cent of the PEC population, 3) cytological examination of PEC from mice which had received an injection of colloidal carbon 24 hours before cell harvest showed that all of the macrophages contained dense carbon cytoplasmic inclusions indicating the phagocytic ability of these cells, 4) this class of large phagocytic cell readily attached to polystyrene tissue culture flask surfaces when cultured at 37°C in serum-containing media, indicating a propensity for adherence, and 5) such suspensions of PEC sedimented rapidly when stored for 10 - 15 minutes.

Since it is known that large cells can be separated from smaller cells (at 1 g) on the basis of size by "velocity sedimentation" (Miller and Phillips, 1969) PEC suspensions were centrifuged briefly (30 seconds) at 100 g (maximum g) and the larger cells which formed the pellet were recycled through the same procedure two additional times. Cells prepared in this manner from mice of different strains

of the C57BL/10 background rarely comprised fewer than 99 per cent macrophages as defined morphologically.

In preliminary experiments, macrophages purified using the above method were examined for their ability to become labelled with 100 µCi of ^{51}Cr and to release the label spontaneously and upon cell death. Several concentrations of ^{51}Cr (25, 100 and 300 µCi per ml) were used in the labelling media in order to determine the concentration most suited for the labelling of macrophages. As can be seen in Figure 11, a relationship between the amount of label released spontaneously and the concentration of ^{51}Cr used in the labelling procedure was found. The use of 25 µCi resulted in low levels of spontaneous release (21.5 per cent in 12 hours) but was unsuitable for routine use since only 366 c.p.m. were obtained upon freezing and thawing of the cells. The use of 100 µCi per ml ^{51}Cr resulted in only 31.1 per cent spontaneous release and the release of 1331 c.p.m. upon freezing and thawing; this value of c.p.m. allows for high statistical confidence. The use of 300 µCi per ml of ^{51}Cr resulted in high values (61.1 per cent) of spontaneous release in the 12-hour period and was therefore considered unsuitable for use in the assay. Despite these differences found in labelling with the different concentrations of ^{51}Cr, the freeze-thaw values were almost identical, 81.5, 85.8, and 89.5 per cents for 25, 100 and 300 µCi. This indicates that the percent of label released upon cell lysis is not dependent on the total amount of intracellular label as is the case for the percent of label released spontaneously. Table 14 shows, that in general, 45 to 80

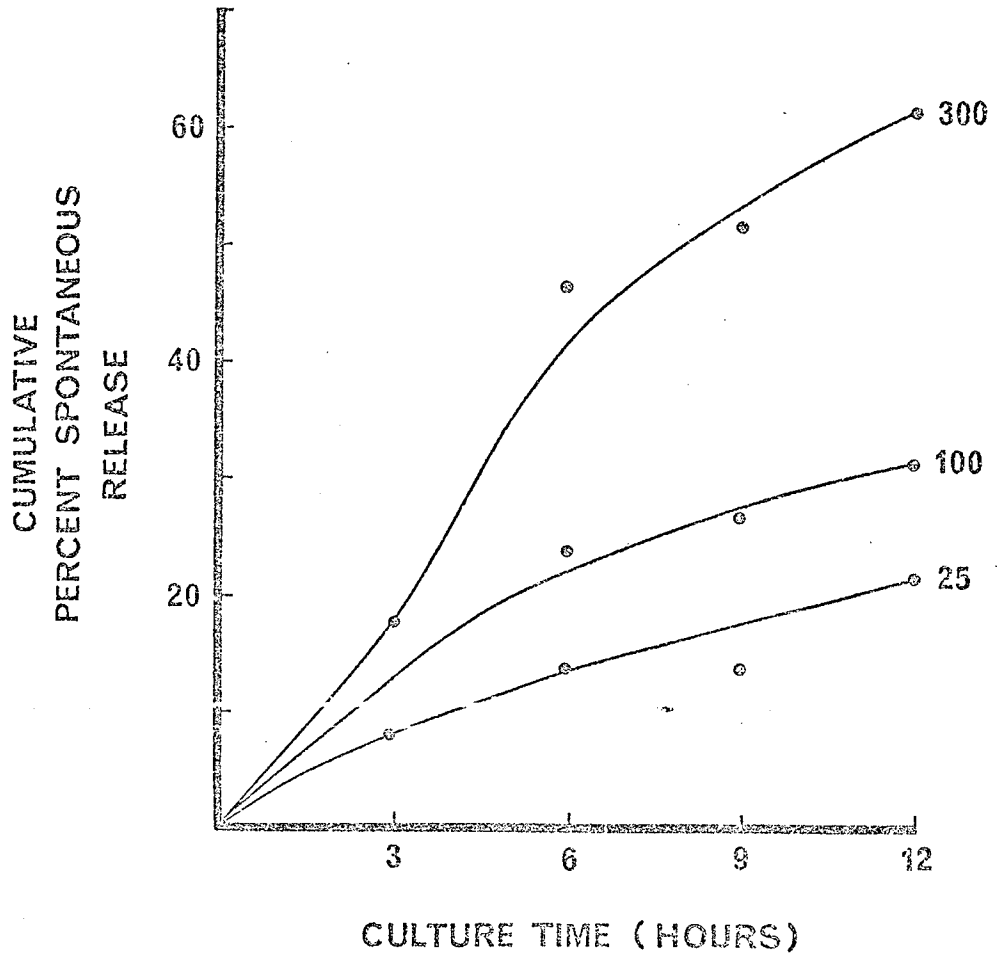

Figure 11

The effect upon spontaneous isotope release of labelling of macrophages with 25, 100 and 300 μCi of ^{51}Cr. Results from a single experiment using cells from one pool of C57BL/10 PEC are shown. Each point represents the mean of triplicate dterminations.

Table 14

Uptake and spontaneous release of ^{51}Cr by macrophages isolated from dextran-induced PEC from different strains

Source of PEC	CPM* present in 0.1 ml of medium supernatant			
	0 h.		12 h.	
	Cell bound + medium	Medium only	Medium only	Freeze-thaw medium only
129	1056	62	263 (30.1)**	729 (67.1)***
C57BL/6	988	59	339 (52.9)	588 (56.9)
C57/L	1074	53	262 (27.6)	810 (74.1)
AKR	778	44	231 (35.3)	574 (72.2)
C58	912	69	406 (54.3)	690 (73.7)
CBA	1677	75	292 (30.0)	798 (45.1)
RF	2432	100	449 (34.4)	1114 (43.5)
C57BL/Ks	1169	56	421 (52.1)	755 (62.9)
BALB/c	553	42	207 (41.9)	436 (77.1)
DBA/2	741	39	183 (32.4)	484 (63.4)

* Mean values from triplicate cultures are shown.

** Values represent the percent of releasable CPM (freeze-thaw) released spontaneously (corrected for CPM present in the medium at 0 h.).

*** Values represent the percent of cell bound CPM released on freezing and thawing (corrected as above).

per cent of the label taken up by the cells was released upon freezing and thawing (three cycles) when followed by an incubation of 12 hours at 37°C. Further, only 30 to 50 per cent of this "releasable" amount of c.p.m. was found in supernatants of living cells which were similarly cultured for 12 hours, presumably released spontaneously by leakage through cell membranes or by cellular attrition. This indicated that a further 50 to 70 per cent of "releasable" label was available for release into the tissue culture medium in the event of cytotoxicity.

As can be seen in Figure 12, the spontaneous release was progressive from 0 to 12 hours, and quite linear from 2 to 12 hours following the short phase of more rapid release.

In the presence of normal allogenenic or syngeneic spleen cells the release of ^{51}Cr usually dropped below values of spontaneous release or remained unchanged. However, upon mixing donor target cells with spleen cells from skin-grafted mice, values of ^{51}Cr release equivalent to almost 100 per cent lysis could be achieved (Table 16), which indicates that macrophages are quite sensitive to cell-mediated cytotoxicity and that the radio-chromium method is suitable to measure this effect.

The exclusion of the colloidal stain, Trypan blue by living cells is a method used to determine cell viability. An experiment was performed to test whether the appearance of ^{51}Cr in the supernatants of cultures containing aggressor lymphocytes and macrophage target cells was due to cell death or, alternatively, to the exocytosis of cytoplasmic or phagocytic contents of viable cells. Non-adherent

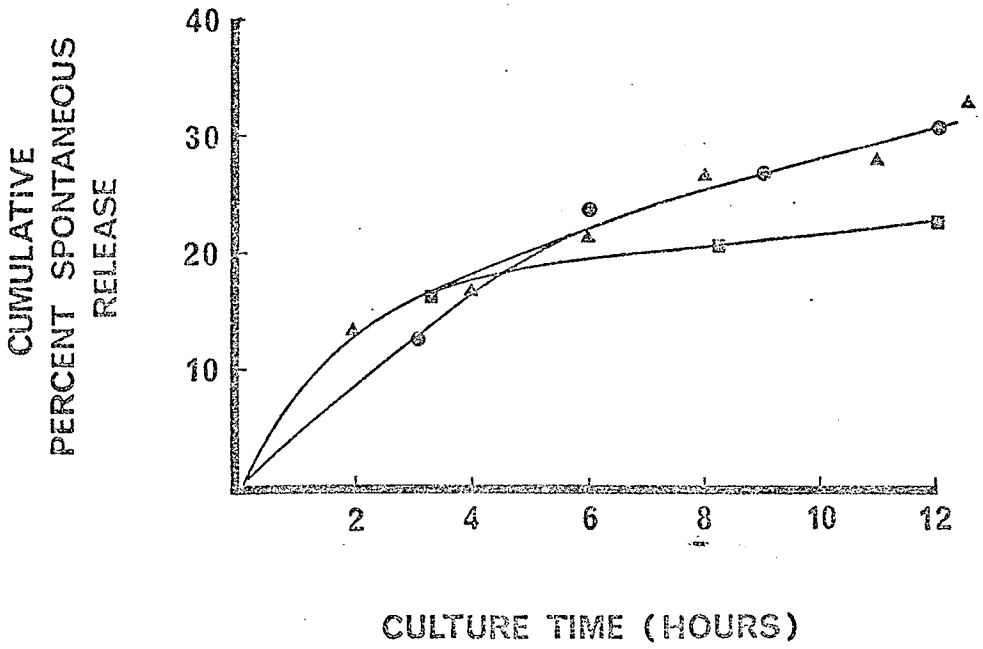

Figure 12

Spontaneous release of ^{51}Cr from labelled C57BL/10 macrophages. Results from three experiments are shown. Each point represents the results of triplicate determinations.

Table 15 and Table 16
(combined)

Cytotoxicity mediated by C57BL/6 anti-DBA/2 spleen cells against allogeneic target cells

	Per cent specific ^{51}Cr released from target cells			
Spleen No.	Macrophage DBA/2 (H-2^d)	Macrophage AKR (H-2^k)	Macrophage C57BL/6 (H-2^b)	Mastocytoma P815-X2 (H-2^d)
1	94.8	10.5	-25.1	99.0
3	88.8	4.0	-14.4	88.1
4	87.7	5.4	-20.3	96.0
5	84.0	14.8	-22.3	98.1
6	78.4	17.6	-19.1	86.6
8	72.1	7.7	-15.8	86.0
10	59.8	13.2	0.6	83.5
11	59.5	5.4	-16.3	80.5
13	46.2	22.0	0.6	81.9
16	16.0	8.8	- 6.4	52.7
17	14.2	8.2	1.9	30.2
Normal Spleen	- 2.4	0.0	- 4.4	13.2

Rank order data from experiments in which 4×10^4 target cells were incubated with 2×10^6 aggressor spleen cells obtained 9 - 13 days following skin grafting.

peritoneal exudate lymphocytes served as aggressor cells. These cells are considered in detail elsewhere (see p. 107) and macrophages, from strain B10.A served as target cells. As can be seen in Figure 13, overlapping curves of percent cytotoxicity of the macrophages were obtained with the ^{51}Cr assay and the dye-exclusion test. This finding supports the view that both tests measure similar phenomena, i.e., an increase in permeability of the cell membrane (Henney, 1973).

The DBA/2 tumor, mastocytoma P-815-X2 has been widely utilized as a ^{51}Cr-labelled target cell in the study of lymphocyte-mediated cytotoxicity (Brunner et al., 1967). It was therefore of interest to compare this neoplastic target cell with dextran-induced target macrophages obtained from the same strain, DBA/2, in a cytotoxic assay. Accordingly, C57BL/6 mice were grafted with skin from strain DBA/2 and recipient spleen cells were used in a cytotoxicity assay with the following ^{51}Cr-labelled target cells: DBA/2 macrophages, DBA/2 tumor P-815-X2 cells, recipient (non-antigenic, control) C57BL/6 macrophages and macrophages from a third-party unrelated strain AKR. The results, (Table 16 and Figure 14) show that an excellent correlation was found between the release of label by DBA/2 macrophages and P-815-X2 tumor cells, with P-815-X2 cells exhibiting a consistent, somewhat greater cytotoxicity than the macrophages. Target cells syngeneic with the aggressor cells, i.e., C57BL/6, were not lysed in the presence of the aggressor cells; the most common result was a decrease of spontaneously released isotope. Target macrophages from the third-party strain, AKR, exhibited variable degrees of release of label in the presence

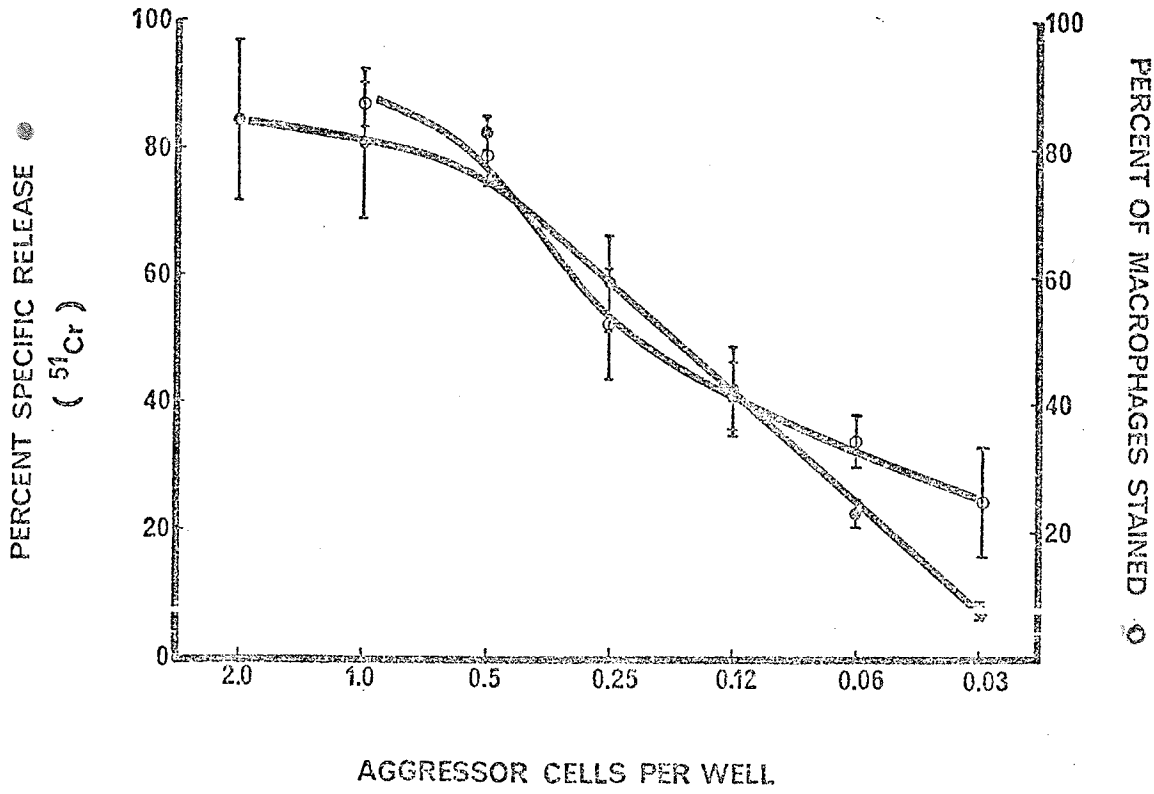

Figure 13

Measurement of cytotoxicity of C57BL/10 target macrophages using the ^{51}Cr release assay and the Trypan blue dye exclusion test. Each point represents the mean of triplicate determinations and the vertical bars indicate 1 standard deviation.

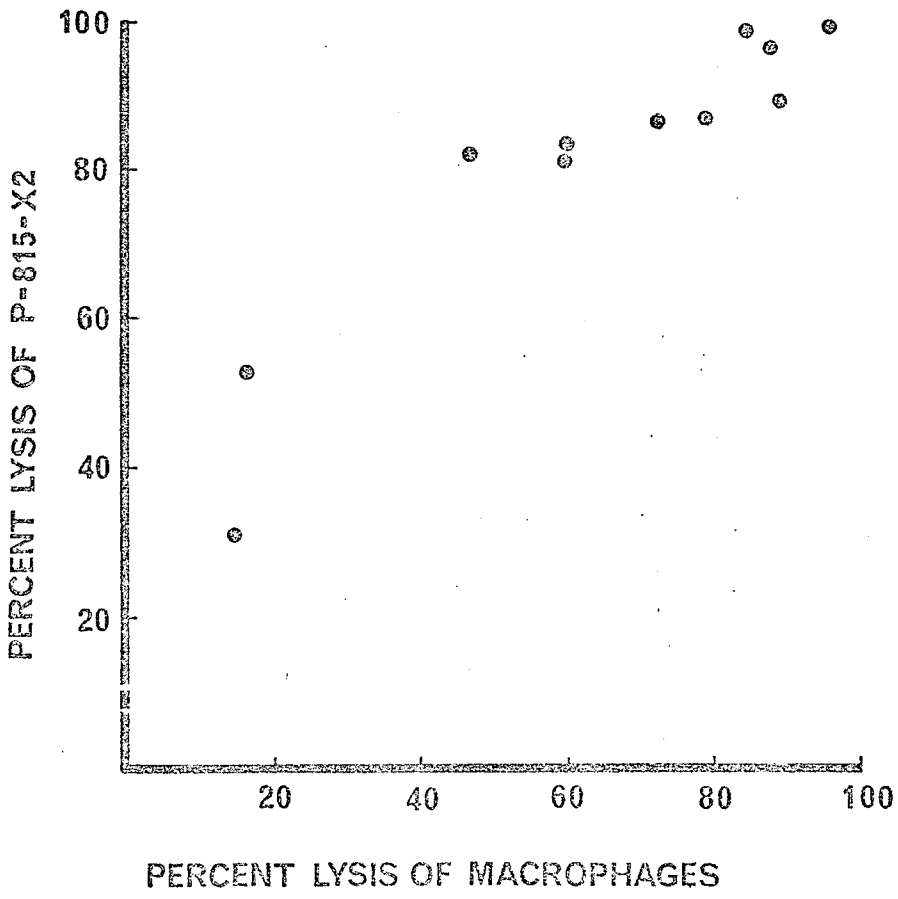

Figure 14

Correlation between the release of label from DBA/2 mastocytoma P-815-X2 tumor cells and from purified DBA/2 peritoneal macrophages by the same population of aggressor cells. A scattergram representing 13 experiments is shown. Detail in Table 16.

of the spleen cells but the amount of label released was not correlated with the release of label from DBA/2 target macrophages. This result is an apparent cross-reaction and is considered further elsewhere.

Another experiment was designed to test whether the arbitrary choice of 12 hours of tissue culture for the cytotoxicity assay was suitable, since the possibility existed that the duration of ^{51}C release was brief and that the prolonged incubation merely served to decrease the sensitivity of the test due to continuing spontaneous release. In such an experiment A/J spleen cells, from mice grafted with C57BL/6 skin, were mixed with C57BL/6 target cells and cultured for 3.25, 8.25, and 12.0 hours. The results of 17.0, 51.8, and 68.1 percents lysis obtained, revealed virtually linear kinetics of cell lysis and indicate that cytotoxic release does not occur transiently at the start of the culture but is progressive with time.

Thus, macrophages obtained from dextran-induced PEC by a simple centrifugation method were shown to behave in the cell-to-cell cytotoxicity assay in a manner similar to other target cells (considered in the Introduction) with respect to, 1) low to moderate levels of spontaneous ^{51}Cr release, 2) high levels of release of label upon cell disruption, and 3) the good correlation of the ^{51}Cr assay of macrophage cytotoxicity with both macrophage death as determined with the Trypan blue exclusion test and with ^{51}Cr release from the widely used target cell P-815-X2.

Additional experiments were performed to examine some other parameters of the cytotoxicity assay. It was of interest to know

whether macrophages behaved as "passive" target cells in the cytotoxicity assay since it is known that under several circumstances macrophages can be activated to behave as killer cells (Alexander and Evans, 1971; Evans and Alexander, 1971). In these experiments aggressor spleen cells from C57BL/6 mice, immunized with DBA/2 skin grafts, were combined with DBA/2 target cells, C3H (third-party) target cells or a 1:1 mixture of both target cells. Two types of target cell mixtures were used. For the first mixture, unlabelled C3H target macrophages were mixed with labelled DBA/2 macrophages (to serve as the positive control) and for the second, labelled C3H macrophages were mixed with unlabelled DBA/2 target cells. The results (Table 17) indicate that using the above conditions no label was released from the third-party target cells despite ongoing cytotoxic interactions among adjacent immune cells and target cells. This observation does not support the view that soluble cytotoxic factors are responsible for lymphocyte-mediated cytotoxicity (Granger and Kolb, 1968) and also argues against the activation of (C3H) macrophages into cytotoxic cells since increased cytotoxity of the DBA/2 target cells was not obtained in the first target cell mixture. The results also illustrate the strict specificity which is obtained with this assay.

ii. Physical and genetic parameters in cell-mediated cytotoxicity

Some experiments were carried out in order to determine the relationship between percent lysis, ratios of immune cells to target cells and numbers of cells in the reaction mixtures. In these experiments spleen cells from A/J mice (immune to C57BL/6) of three fixed

Table 17

Effect on third-party unrelated target macrophages labelled with ^{51}Cr of an ongoing cytotoxic reaction[1]

Number of spleen cells ($\times 10^6$)	Experiment	Per cent specific ^{51}Cr release from target cells			
		DBA/2	C3H	DBA/2+C3H^u	DBA/2^u+C3H
2.0	1	55.4	8.6	54.5	7.7
1.0		43.8	5.7	40.0	13.0
0.5		27.3	10.0	21.4	6.7
0.25		16.5	1.9	7.6	1.0
2.0	2	53.2	3.4	31.1	2.9
1.0		37.5	-2.1	16.2	-6.2
0.5		17.0	0.0	4.7	4.1
0.25		7.8	-2.1	4.7	6.2

[1] Cytotoxicity mediated by C57BL/6 anti-DBA/2 spleen cells. Each well contained 1.5×10^4 target cells; when target cells were mixed, they were combined in a ratio of 1:1. ^{u} Denotes unlabelled target cells.

concentrations, were mixed with target macrophages of C57BL/6 or B6AF mice also prepared in three concentrations. Consequently, ratios of F_1 aggressor cells to target cells ranging from 200:1 to 6.5:1 were studied. As can be seen in Figure 15, parallel semi-logarithmic titration curves of per cent cytotoxicity versus numbers of aggessor cells were obtained. The "horizontal" line drawn through values of percent cytotoxicity using 1×10^6 aggressor cells reveal that using 1×10^6 aggressor cells the percents of lysis of 1×10^4, 2×10^4, and 4×10^4 target cells were almost identical despite a four-fold difference in the ratio of immune cells to target cells. This indicates that in the present system, per cent of lysis is not related to the mere ratio of immune cells to aggressor cells (i.e., numbers of target cells per well) but is related to the numbers of aggressor cells present in each well. This is a favourable finding since in experiments in which target cells from different strains are tested with the same population of sensitized cells, the data will not be affected by small variations in numbers among the different target cells, attributable to experimental variation. Further, it is pertinent to indicate that immune cells are thus "limiting" in only one sense - in the percent of killing - but are not limiting in terms of the numbers of cells which can be killed.

The results from the present experiments also reveal that target cells having one-half the H-2 "allelic doseage" of the graft donor behave as donor-type target cells in reactions with sensitized cytotoxic spleen cells. Thus, essentially identical results were obtained in the two experiments using parental and F_1 macrophage target cells.

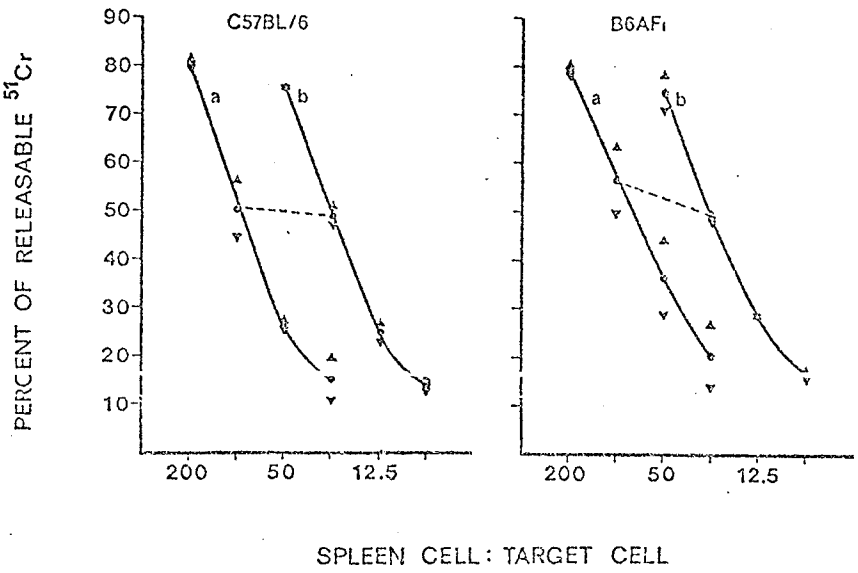

Figure 15

Lysis of C57BL/6 (donor) target macrophages and B6AF$_1$ heterozygous macrophages by immune A/J spleen cells. Aggressor cells of 2.0, 1.0, 0.5 and 0.15 x 10^6 in number were mixed with target cells of 1.0 x 10^4 (curve a) and 4 x 10^4 (curve b). Results of two identical experiments are shown, combined. Closed circles represent the means of the two determinations.

An important reason for the choice of macrophages as target cells is that a similar cell type is available from all inbred strains of mice. The approach desired in the studies which follow was to analyze the nature of antigen recognition on target cells by immune aggressor cells with the use of target macrophages from various strains of mice having known degrees of serological similarity with the graft donor. Target cells of comparable morphological type is considered to be a basic requirement in such studies since it is known that target cells of different tissue origins have varying sensitivities to lysis (Brunner et al., 1970); this would lead to artifactual results and, likely, misinterpretation.

Thus, it was necessary to determine the degree of variability that is obtained using target macrophages from different strains, each having all the immunizing antigens. Great variability, presumably due to other genetic differences, would preclude analyses of the sort considered above, while results indicating the similar lysis of antigenically similar target cells would support this approach. Two groups of experiments were carried out to test this hypothesis. Spleen cells from C57BL/6 (H-2^b) mice immunized with skin grafts from strains DBA/2 (H-2^d) or CBA (H-2^k) were used as aggressor cells against target macrophages from different strains sharing the H-2^k type for the former immunization and against target cells sharing the H-2^d type for the latter immunizations. The results (Table 18) reveal that very similar percents of lysis occur when target cells are obtained from different strains which share common (donor) H-2 types, despite otherwise great

Table 18

Cytotoxicity mediated by sensitized spleen cells against target macrophages from different strains sharing their H-2 type with the graft donor

Immunization	Experiment		Per cent specific ^{51}Cr released from target cells			
		$H\text{-}2^d$:	C57BL/KsJ	BALB/cJ	DBA/2J	
(anti-$H\text{-}2^d$) DBA/2 → C57BL/6J	1*		66.5	74.6	66.7	
	2		42.2	30.1	43.5	
	3		20.8	16.9	17.1	
		$H\text{-}2^k$:	C3H/HeJ	CBA/J	AKR/J	
(anti-$H\text{-}2^k$) CBA/J → C57BL/6	7		15.5	10.8	11.6	
	8		57.3	53.2	61.5	
	9		76.7	68.4	82.3	
		$H\text{-}2^k$:	AKR/J	C58/J	CBA/J	RF/J
(anti-$H\text{-}2^k$) CBA/J → C57BL/6J	4		85.7	72.8	76.0	n.d.
	5		93.3	88.8	79.6	84.9
	6		65.5	81.1	64.9	60.9
(anti-$H\text{-}2^d$)		$H\text{-}2^{(\ )}$	C57BL/6J(b)	AKR/J(k)	C58/J(k)	CBA/J(k)
DBA/2 → C57BL/6J	10*		-12.8	32.3	n.d.	17.3
Normal C57BL/6J			- 2.0	- 0.6	- 2.5	- 3.2

Each well contained 3.0×10^4 target cells and 2×10^6 sensitized spleen cells from 1 or 2 graft recipients. The use of spleen cells from normal mice resulted only in negative values of ^{51}Cr release. Spleen cells were obtained 9 - 12 days following skin grafting. * Cells from the same aggressor pool were used.

disparity in the genetic constitution of the strains. Implicit in these results is the conclusion that H-2 antigens are of paramount importance in the lysis of macrophages by sensitized spleen cells, since the strains used differ widely in their complement of non-H-2 antigens which does not appear to affect the extent of the reactions. Indeed in only one out of six experiments was the cytotoxicity of donor cells (H-2 plus non-H-2 antigens) greater than that of third-party cells (H-2 only), as might have been the expected result.

In a direct attempt to investigate whether cytotoxic reactions against non-H-2 antigens are detectable with the use of sensitized spleen cells A/J ($H\text{-}2^a$) mice were immunized with skin grafts from C57BL/10 ($H\text{-}2^b$) mice. Target cells were obtained from the donor strain, C57BL/10, having all the H-2 and non-H-2 antigens of the donor and from a third-party strain, B10.A ($H\text{-}2^a$), which is congenic with C57BL/10 and shares its H-2 chromosome with A/J but is otherwise genetically identical with C57BL/10. Four spleens were tested individually on day 10 after grafting. C57BL/10 target cells were lysed 29.7, 60.8, 38.3, and 40.2 per cents while B10.A target cells sharing all the non-H-2 antigens of the donor were lysed 2.5, 1.5, 1.0, and 1.0 percents. These results indicated that no reactions are detected against non-H-2 antigens with this system and corroborate the preceding conclusions based on indirect evidence.

Since the direction of these experiments was to develop a quantitative analysis of the specificity of cytotoxic reactions similar to the studies with the migration assay, it was desirable to maximize

the sensitivity of the assay. For this reason, two obvious variables were explored, 1) the donor-recipient combination, and 2) the activity of cytotoxic cells of different tissue origins.

For the first parameter different donor-recipient combinations were studied to examine the contribution genetic background on the immunogenicity of H-2 antigens or on the responsiveness of certain strains to these antigens. In these experiments three different strains sharing the $H\text{-}2^b$ chromosome were sensitized with skin grafts from three strains sharing the $H\text{-}2^k$ chromosome. Skin from a single donor mouse was used to reduce the contribution of antigen variability. The results from these experiments (Table 19) reveal that no donor strain showed a particular propensity to sensitize the $H\text{-}2^b$ mice to $H\text{-}2^k$ antigens nor did any of the recipient strains respond in a consistent manner to different $H\text{-}2^k$ donors, i.e., the variability of results between repeated experiments was as great as that observed among strains in a given experiment. This conclusion was confirmed in two other experiments in which three groups of $H\text{-}2^b$ mice were immunized with A/J skin grafts and individual spleens were examined for aggressor cell activity (Table 20). The results indicate that individual mice may vary greatly in their response to the same antigenic stimulus.

In summary, the strains examined reveal that individual variation in responsiveness is very large even when grafts are obtained from a single donor and, perhaps for this reason, no evidence was obtained to suggest that strains sharing common H-2 types behave differently

Table 19

Attempts to detect donor-recipient combinations of differing immunogenicity or antigenicity

Experiment	Donor	Recipient	Per cent specific ^{51}Cr released from target cells of		
			C3H/HeJ	CBA/J	AKR/J
1	AKR/J	C57BL/6J	17.6	10.8	15.7
	CBA/J		56.1	51.6	47.8
	C3H/HeJ		29.0	21.8	26.5
2	AKR/J	C57BL/6J	65.9	56.3	61.0
	CBA/J		60.9	72.0	66.7
	C3H/HeJ		45.8	61.8	45.7
1	AKR/J	C57/LJ	50.6	51.6	44.0
	CBA/J		36.1	20.0	24.4
	C3H/HeJ		17.6	16.9	17.2
2	AKR/J	C57/LJ	31.0	26.9	32.3
	CBA/J		39.5	33.1	25.8
	C3H/HeJ		19.5	9.3	14.8
1	AKR/J	129/J	15.5	10.8	11.6
	CBA/J		57.3	53.2	61.5
	C3H/H3J		76.7	68.4	82.3
2	AKR/J	129/J	25.4	19.2	18.6
	CBA/J		52.1	55.7	48.4
	C3H/HeJ		13.9	12.2	11.6

Each well contained 4×10^4 target cells and 2×10^6 sensitized spleen cells.
Spleen cells were pooled from two graft recipients.

Table 20

Non-genetic variability in the generation of cytotoxic cells *in vivo*

Immunization	Per cent ^{51}Cr release from A/J macrophages			
	Experiment 1		Experiment 2	
A/J → 129/J	34.1*	47.5	-	-
A/J → C57BL/6J	57.2	68.5	8.3	49.3
A/J → C57/LJ	44.0	59.6	70.0	25.0

Female mice of 9 - 11 weeks of age were used. Experiment 1 and experiment 2 utilized spleens from mice grafted 10 and 11 days earlier, respectively. * Each result represents the mean of triplicate determinations for a single spleen. One graft donor was used for each pair of recipients.

either as donors or responders in the cytotoxicity assay. The data (Table 19) however, provide strong confirmation for the conclusion that target cells sharing the same H-2 types are lysed to the same extent and, as before, indicate no role for non-H-2 antigens in cytotoxicity mediated by spleen cells.

For the second parameter under consideration - the cytotoxic activity of lymphoid cells of different tissue origins - cells were obtained from the lymph nodes draining the graft site (axillary and brachial), the spleen and from the peritoneal cavity 72 hours after the injection of a solution of dextran (as for the induction of PEC). In Table 21, the results from one of two similar studies show that on a cell-for-cell basis, the activity of spleen cells is very comparable to that of lymph node cells; the lymph node cells showing a small but consistent tendency for higher lysis. In another pair of experiments in which the specificity of the reaction was examined as well, it was found that peritoneal cells can be divided into two populations on the basis of differential sedimentation rates. The first population, consisting chiefly of the large cells (used also as target cells) which sediment rapidly, were completely inactive as aggressor cells in the cytotoxicity assay (Table 22). However the supernatant cells, comprising a mixture of lymphocytes and macrophages, were as active as spleen cells in one experiment and more than four times as cytotoxic as spleen cells (obtained from the same pooled donors) in the second experiment. However, the low numbers of cells recoverable from four mice (2×10^7 supernatant aggressor cells) made the use of such aggressor cells

Table 21

A comparison of cytotoxic activity of aggressor cells obtained from lymph nodes or spleen

Immunization	Experiment	Per cent ^{51}Cr release from P-815-X2 target cells	
		Lymph node cells	spleen cells
DBA/2J → C57BL/6J	1	73.5	71.1
	2	83.0	73.2
C3H/HeJ → C57BL/6J	3	14.7	13.5
	4	14.8	4.1
C57BL/6J → C3H/HeJ	5	0.4	4.9
	6	3.7	1.7
*DBA/2J → C57BL/10	7	73.5	71.1
	8	83.0	73.2

In these experiments 2.5×10^6 aggressor cells per well were used, obtained from female mice of 10 - 14 weeks of age. * In this experiment males were used. In experiments 1 - 3 specific lysis was calculated using values of spontaneous release obtained in the presence of syngeneic (DBA/2J) immune cells.

Table 22

Lysis of DBA/2J and C3H/HeJ target macrophages by spleen and peritoneal cells from C57BL/6J mice grafted with skin from C3H/HeJ

Experiment	Concentration of aggressor cells	Type of aggressor	Per cent ^{51}Cr release from macrophasge of			
			DBA/2*	C3H*	DBA/2+C3H*	DBA2*+C3H
			($2x10^4$)	($2x10^4$)	($1x10^4 + 1x10^4$)	($1x10^4 + 1x10^4$)
1	2.0×10^6	spleen	63.6	- 4.4	55.4	7.7
	1.0×10^6		37.7	- 2.1	43.8	13.0
	0.5×10^6		16.8	- 1.8	27.3	6.7
	0.3×10^6		- 0.9	- 2.0	16.5	1.0
	1.0×10^6	PEC supernatant	31.4	5.7	45.5	- 0.5
	0.5×10^6		22.3	0.2	33.9	10.1
	0.3×10^6		10.0	2.5	26.4	4.8
	1.0×10^6	PEC pellet	- 7.7	- 9.4	0.7	-15.4
	0.5×10^6		- 4.5	- 5.0	1.4	-11.1
	0.3×10^6		- 1.8	- 3.7	9.7	- 2.9
2	2.0×10^6	spleen	-	- 8.8	31.1	2.9
	1.0×10^6		-	- 4.8	16.2	- 6.2
	0.5×10^6		-	- 0.4	4.7	4.1
	0.5×10^6	PEC supernatant	33.6	7.4	42.6	12.0
	0.3×10^6		12.1	10.2	22.3	8.3
	1.0×10^6	PEC pellet	- 5.7	-12.2	0.0	-15.8

* Cell population labelled with ^{51}Cr.

impractical. The data show that the cytotoxicity was quite specific.

iii. Specificity of splenic aggressor cells

As considered in the introduction the overall direction of this study was to examine the relationship between serology (cross-reactivity of H-2 antisera or alternatively shared H-2 antigens) and the specificity of cell-mediated reactions directed at strains which share graded numbers of these antigens. For these experiments spleen cells from mice, skin-grafted 9 - 12 days earlier, were assayed for in vitro cytotoxic activity against target macrophages from a panel of strains having all, few or none of the H-2 antigens of the graft donor, as defined serologically. Ten different donor-recipient combinations provided sensitized spleen cells for assay against target cells of 6 H-2 types. Cytotoxicity mediated by the immune spleen cells (Table 23) was evident with target cells of the donor H-2 type in every experiment, while the use of target cells of recipient H-2 type resulted in negative values of specific ^{51}Cr release in 39 of 45 experiments; positive ^{51}Cr release was obtained in only 6 experiments (mean = 3.9 per cent). Reactions of sensitized spleen cells to third-party target cells exhibited a distinct pattern. Only target cells from strains which shared at least one of the two H-2 regions (D or K) with the donor showed definitive cytotoxicity. This occurred for combinations involving H-2^a, H-2^k and H-2^d-type graft donors since the K and D regions of the H-2^a chromosome are identical to the K and D regions of the H-2^k and H-2^d chromosomes, respectively. For example, C57BL/6 (anti-A/J) spleen cells lysed target cells of donor H-2^a type

Table 23

Specificity of ^{51}Cr Release from Target Cells of Different Genotypes by Sensitized Spleen Cells

Immunization		Experiment	Per cent specific ^{51}Cr release from target cells of					
			C57BL/10 ($H\text{-}2^b$)	B10.A ($H\text{-}2^a$)	B10.BR ($H\text{-}2^k$)	B10.D2 ($H\text{-}2^d$)	DBA/1 ($H\text{-}2^q$)	B10.M ($H\text{-}2^f$)
A/J → C57BL/6	($H\text{-}2^a \rightarrow H\text{-}2^b$)	1	-5.3* (0/13)**	29.4 (13/13)	21.3 (7/13)	2.2 (8/13)	0.3 (6/13)	-5.9 (1/13)
		2	-2.5	18.2	40.3	11.7	1.5	3.9
		3	1.3	74.3	76.9	42.6	-25.2	-29.3
$B6AF_1$ → C57BL/6	($H\text{-}2^a \rightarrow H\text{-}2^b$)	4	-26.0	42.0	10.0	23.0	-36.0	-10.0
CBA → C57BL/6	($H\text{-}2^k \rightarrow H\text{-}2^b$)	5	-10.5 (0/8)	82.7 (7/8)	88.2 (8/8)	-15.8 (2/8)	-25.5 (4/8)	12.3 (1/8)
		6	-1.3	13.4	22.6	0.0	2.0	-5.6
		7	-12.0	32.0	38.1	-7.3	-14.1	3.1
		8	3.6	56.1	48.3	-2.7	-2.0	-6.9
CBA → C57BL/10	($H\text{-}2^k \rightarrow H\text{-}2^b$)	9	-17.0	25.0	33.0	--	-7.0	-9.0
CBA → DBA/2	($H\text{-}2^k \rightarrow H\text{-}2^d$)	10	1.3 (0/7)	--	38.0 (7/7)	--	-35.7 (4/7)	-29.3 (0/7)
DBA/2 → C57BL/6	($H\text{-}2^d \rightarrow H\text{-}2^b$)	11	-2.6 (0/10)	22.0 (8/10)	12.3 (2/8)	55.2 (10/10)	7.0 (3/10)	3.6 (1/10)
		12	-6.1	80.9	14.5	67.4	-27.9	-37.5
		13	-13.8	28.5	8.6	30.7	-5.1	3.1
129 → B10.BR	($H\text{-}2^b \rightarrow H\text{-}2^k$)	14	50.0 (10/10)	-2.5 (6/10)	-0.2 (0/10)	0.7 (7/10)	-6.8 (4/10)	3.6 (4/10)
129 → C3H/HeJ	($H\text{-}2^b \rightarrow H\text{-}2^k$)	15	83.9	5.2	-2.2	-0.1	-11.8	-0.4
			C57BL/6	A/J	C3H/HeJ	DBA/2	DBA/1	
C57BL/6 → BALB/c	($H\text{-}2^b \rightarrow H\text{-}2^d$)	16	63.9 (4/4)	4.5 (1/4)	11.1 (1/4)	7.7 (0/4)	0.7 (1/4)	
		17	75.1	7.3	1.6	—	4.7	
C57BL/6 → C3H/HeJ	($H\text{-}2^b \rightarrow H\text{-}2^k$)	18	39.8 (10/10)	-0.3 (6/10)	-3.5 (0/10)	5.7 (7/10)	-2.9 (4/10)	
		19	25.2	3.7	-0.6	4.3	-7.3	

*In this experiment C57BL/6 target cells (4×10^4 per cell) were mixed with 3×10^6 aggressor spleen cells. In all other experiments 2×10^6 spleen cells were mixed with 4×10^4 target cells.

**Fractions in parentheses indicate the number of H-2 specificities of the target cell (strain) in common with the donor/the total specificities of the donor absent in the recipient.

i.e., B10.A, as well as those of B10.BR ($H\text{-}2^k$) and B10.D2 ($H\text{-}2^d$) (Table 23, experiments 1-4). Conversely, when CBA ($H\text{-}2^k$) was the donor, lysis of B10.BR ($H\text{-}2^k$) and B10.A ($H\text{-}2^a$) target cells occurred (experiments 5-9). However, no cytotoxicity was detected in any donor-recipient combination against other third-party target cells which did not share an H-2 region with the graft donor, even when these cells had a substantial representation of donor H-2 specificities (Table 23, experiments, 1-4, 5-9, 10, 14-15, and 18-19). An exception to this pattern, noted in these experiments, occurred when $H\text{-}2^b$ mice were recipients of $H\text{-}2^d$ grafts; a weak reaction of immune $H\text{-}2^b$ spleen cells against $H\text{-}2^k$ target cells was obtained (Table 23, experiments 11-13).

In addition, weak reactions of $H\text{-}2^b$ anti-$H\text{-}2^d$ spleen cells against $H\text{-}2^k$ target cells were detected in previous experiments. Thus, in Tables 16 and 18, C57BL/6 ($H\text{-}2^b$) spleen cells from mice immunized with DBA/2 ($H\text{-}2^d$) skin grafts lysed AKR ($H\text{-}2^k$) target cells and in Table 21 the converse is observed; C57BL/6 spleen cells from mice immune to C3H ($H\text{-}2^k$) lysed the DBA/2 target cell P-815-X2. Since these reactions were very weak it is possible that other cross-reactions were not observed owing to insensitivity of the cytotoxic assay.

IV. Cytotoxicity by peritoneal exudate lymphocytes (PEL)

An aggressor cell population having superior cytotoxic activity than spleen cells, was sought in order to improve the sensitivity of the above study. It was shown earlier (see Results Section II and Table 22) that the smaller cells in dextran-induced PEC of skin-

grafted mice were highly cytotoxic; in one experiment they were found to be four-fold as active as spleen cells from the same animals. Furthermore, Berke, Sullivan and Amos (1972a) showed that non-adherent peritoneal exudate lymphocytes (PEL) from mice which had rejected an intraperitoneal lymphoma allograft were remarkably cytotoxic, consistent with the activity of the smaller peritoneal cells. Therefore a systematic examination of the cytotoxic activity and specificity of peritoneal exudate lymphocytes against target cells from different strains was attempted. To induce the presumptive aggressor peritoneal cells the ascitic tumors sarcoma I (SaI) and mastocytoma P-815-X2 of strains A/J and DBA/2 were used.

The PEL were purified from the mixed cells in the peritoneal cavities of mice of the C57BL/10 congenic background utilizing the property of surface adherence. The PEC were removed 10 days following the i.p. injection of 3×10^7 viable ascites tumor cells. At this time the visibly distended abdomen had begun to decrease in size. Total cell yields from C57BL/10 mice injected with either tumor ranged from $1.4 - 1.8 \times 10^7$ per animal and following the absorption of adherent cells (see Materials and Methods) the useful yield was usually 33 percent of this value i.e., 5×10^6 PEL per mouse. Non-adherent PEL (differential counts revealed that 84 per cent of the non-adherent PEC had lymphocytic morphology), formed as a consequence of the ascites tumor allografts, were indeed found to be highly cytotoxic. PEL from C57BL/10 or B10.D2 mice immunized with SaI, when mixed with target cells from B10.A, which shares the H-2^a type with SaI, resulted

in high values of ^{51}Cr release (Tables 24 and 25). As few as 6.25 x 10^4 immune cells could cause 45 - 75 per cent of specific release of label from 4 x 10^4 target cells of the tumor-donor H-2 type in most experiments (ratio 1.6:1). From the shape of the curves of per cent cytotoxicity versus the numbers of sensitized cells per well (Figures 17 to 21) and from an examination of the data (Tables 24 and 25) it may be seen that maximal cytotoxicity of donor-type target cells was usually achieved with 0.5 x 10^6 aggressor cells. Since the maximum useful cell density per culture was found to be 3 x 10^6 aggressor PEL, cytotoxicity experiments were often conducted using a 6-fold excess of sensitized cells which were adequate to lyse 100 per cent of donor target cells and cytotoxicity was measured over a range of aggressor cell numbers of three orders of magnitude. (The maximum cell density per well of 0.3 ml was determined by the concentration of cells which reduced the pH to below 7.0 after 12 hours of tissue culture.) As for specificity, PEL caused negative values of ^{51}Cr release from syngeneic target cells in 8 of the 9 experiments and only a slight positive release in the remaining experiment, thereby demonstrating that the cytotoxic reaction retains specificity even in situations of gross excess of sensitized cells. This conclusion is further strengthened by the results of an experiment incorporating an irrelevant target cell into the reaction mixture of aggressor PEL and target macrophages. In such an experiment PEL from C57BL/10 mice immunized with the SaI ($H\text{-}2^a$) tumor were added to B10.A target cells or a mixture of B10.A and C57BL/10 target cells (Figure 16). The addition of ^{51}Cr-labelled irrelevant

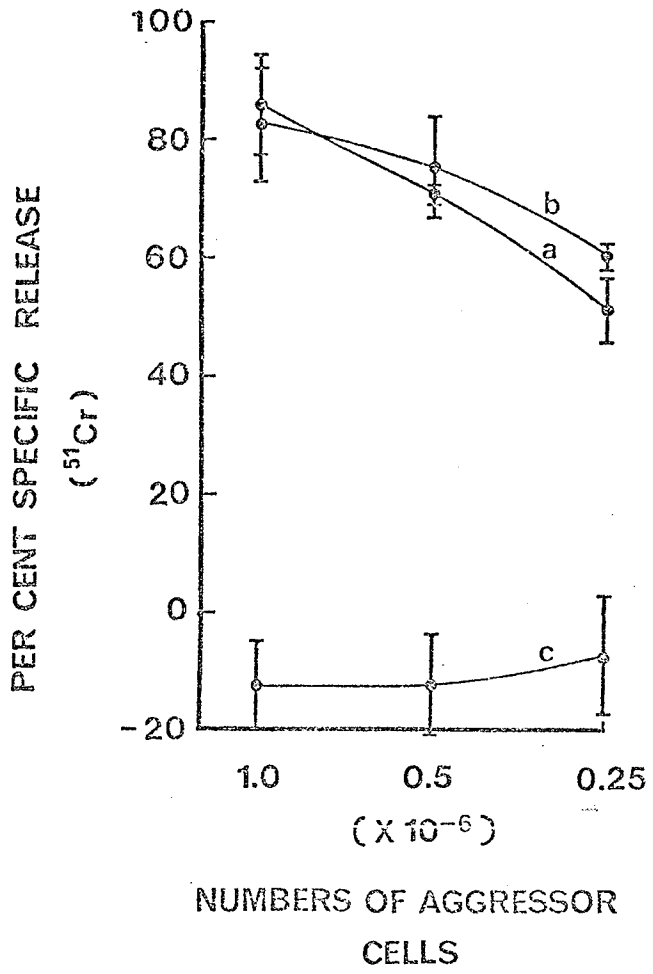

Figure 16

Specific cytotoxicity mediated by C57BL/10 anti-Sa1 (H-2^a) PEL shown by incorporation of irrelevant target cells into the reaction mixture. Aggressor PEL were assessed for cytotoxicity against three target cell preparations: (a) a mixture of unlabelled C57BL/10 + labelled B10.A (H-2^a) target macrophages (2 x 10^4 of each), (b) 4 x 10^4 labelled B10.A macrophages and (c) labelled C57BL/10 + unlabelled B10.A macrophages (2 x 10^4 of each). Vertical bars represent $\pm$ 1 s.d.

syngeneic target cells to wells in which specific cytotoxicity against unlabelled B10.A ($H\text{-}2^a$) target cells was proceeding resulted in no detectable release of label. These results also suggest that dextran-induced macrophages behave as passive target cells and do not become 'armed' aggressor cells (Evans and Grant, 1972) since such arming of syngeneic C57BL/10 target macrophages leading to super-added cytotoxicity of B10.A target cells was not detected. This result is similar to the findings with aggressor spleen cells, considered earlier.

V. Specificity of cytotoxic PEL

Since it was possible that the cytotoxicity mediated by aggressor spleen cells against third-party target cells sharing some donor H-2 specificities was not detected in the previous study involving spleen cells because of limited sensitivity of that assay, experiments were repeated using PEL induced with ascites tumor allografts as a source of more potent cytotoxic cells. In the first series of experiments (Table 24 and an experiment shown graphically in Figure 17), PEL recovered from C57BL/10 mice following the rejection of SaI showed high cytotoxicity against donor H-2 type (B10.A) target cells but no cytotoxicity against syngeneic C57BL/10 target cells. The PEL caused the release of label from B10.D2 and B10.BR target macrophages as expected since these target cells share the H-2D region and H-2K region of the $H\text{-}2^a$ chromosome, respectively.

As considered above, cytotoxic activity of C57BL/10 anti-SaI PEL is evident on both target cells of B10.BR and B10.D2 as may be expected, but in all experiments more cytotoxicity was directed

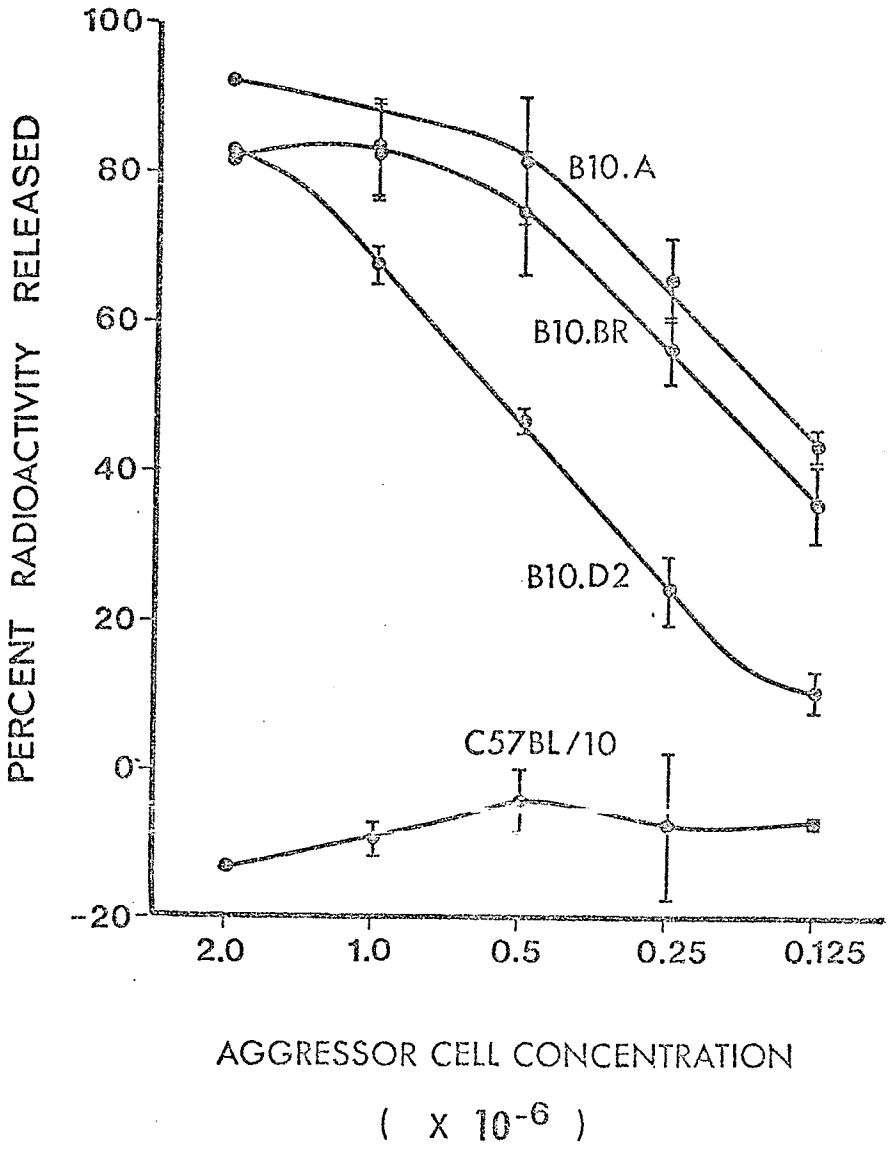

Figure 17

Lysis of target macrophages by PEL obtained from C57BL/10 mice immunized with SaI (A/J). Vertical bars represent $\pm$ 1 s.d. and circles represent the means of triplicate determinations.

Table 24

Specificity of ^{51}Cr Release from Target Cells of Different H-2 Types by C57BL/10 Anti-SaI ($H\text{-}2^a$) PEL

Experiment Number	Aggressor Cell ($\times 10^{-6}$)	Per cent[1] specific ^{51}Cr release from target cells of					
		B10.A	C57BL/10	B10.D2	B10.BR	B10.M	DBA/1
1	3.0	108.5 ± 12.5	-53.2 ± 6.2	103.0 ± 5.3	94.4 ± 6.6	84.9 ± 1.7	75.2 ± 9.9
	2.0	112.6 ± 14.4	-39.1 ± 2.9	108.5 ± 10.7	94.9 ± 9.0	63.5 ± 15.3	73.3 ± 2.3
	1.0	----	-33.3 ± 4.7	110.0 ± 6.5	83.6 ± 1.3	56.1 ± 3.5	51.1 ± 3.0
	0.5	103.1 ± 13.7	-16.2 ± 3.4	95.8 ± 5.1	89.3 ± 3.2	34.1 ± 6.4	22.9 ± 3.9
2	2.0	92.2	-12.7	82.2	81.8	0.8 ± 4.2	12.2 ± 4.3
	1.0	83.7 ± 3.9	-9.4 ± 1.4	67.4 ± 1.3	82.6 ± 3.6	-1.3 ± 2.9	4.7 ± 0.8
	0.5	81.5 ± 5.0	----	----	----	----	----
	0.125	43.9 ± 1.3	-7.2 ± 0.6	10.6 ± 1.7	35.7 ± 3.1	1.7 ± 4.8	——
3	3.0	----	----	----	----	96.7 ± 12.2	69.6 ± 13.4
	2.0	----	----	----	----	73.5	49.2 ± 4.7
	1.0	----	-34.8 ± 7.4	93.5 ± 0.3	107.6 ± 5.3	43.8 ± 6.7	21.1 ± 7.2
	0.50	98.4 ± 11.5	----	----	----	----	----
	0.125	84.6 ± 8.2	----	43.0 ± 6.5	93.7 ± 5.5	----	----
	0.032	47.4 ± 3.6	----	17.1 ± 3.8	36.1 ± 4.6	----	----

1 Each value represents the arithmetic mean ± 1 S.E. of triplicate determinations.

towards B10.BR target cells (having the K-region antigens of H-2^a) than those of B10.D2. Moreover, some of the lysis of B10.D2 target cells can be attributed to antigens common to both strains (H-2.3 and H-2.8). This possibility is most significant in Experiment 2, Table 24 where no contribution to D-region antigens at all is necessary to explain the cytotoxicity of the PEL. The similarity of the slopes of cytotoxicity against the three target cells B10.A, B10.D2 and B10.BR in Figure 17 is considered to indicate that the mechanism of destruction of cells bearing different antigen systems is similar (Henney, 1971). In addition, the larger amount of lysis of B10.BR cells compared with that of B10.D2 cells is maintained proportionately over a range of aggressor PEL concentrations. Therefore, in the donor-recipient combination used here, K-region antigens may dominate those of the D-region. This finding may relate to the dominance of the K-end of the H-2 complex (i.e., Ir-region differences).

In addition, strong reactions against DBA/1 target cells in all three experiments and against B10.M target cells in the two experiments with the most active PEL are evident, in contrast with the results obtained using spleen cells as aggressors. However, it was calculated from the data in Table 24 (experiments 1 and 3) and an example shown graphically (Figure 18) that the reaction against B10.M was about 1/32 and against DBA/1 was about 1/64 of that directed at donor-type target cells (on a cell-for-cell basis calculated from interpolated values at 50 per cent ^{51}Cr release). From these experiments it is readily apparent that aggressor cells, having less cytotoxic

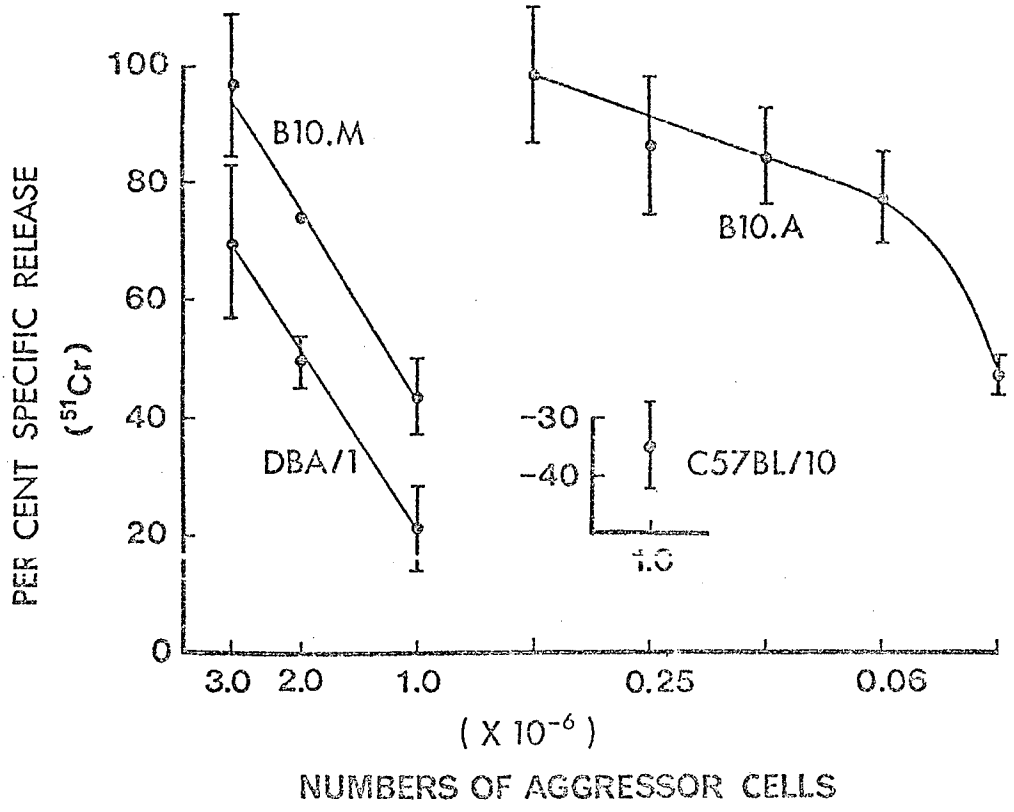

Figure 18

Cytotoxicity mediated by C57BL/10 anti-SaI peritoneal exudate lymphocytes. Results from experiment 3 of Table 24 are shown. Vertical bars represent $\pm$ 1 s.d. Cytotoxicity of donor-type B10.A are third-party B10.M and DBA/1 target macrophages is shown. Syngeneic C57BL/10 were not lysed.

capacity than was required for the release of 100 per cent of the label from donor-type target cells, would not have had a detectable cytotoxic effect on DBA/1 or B10.M target cells (Figure 18). An analysis of available H-2 data in Table 7 reveals that DBA/1 has 6 of the 13 H-2 specificities of the donor cells, SaI ($H\text{-}2^a$), while B10.M has only 1 of the 13 (H-2.8 which is absent in DBA/1). Participation of non-H-2 antigens may be excluded in the lysis of B10.M target cells since this strain is congenic with the graft-recipient strain.

In the second series of experiments (Table 25) PEL from C57BL/10 mice which had rejected the ascites tumor P-815-X2 ($H\text{-}2^d$) were highly cytotoxic to donor-type B10.D2 ($H\text{-}2^d$) target cells and to B10.A ($H\text{-}2^a$) target cells which share the H-2D region with B10.D2 (Table 26). In contrast to results obtained with sensitized spleen cells, cytotoxicity was also observed using target cells from B10.BR which lacks identity with either donor H-2 region (Table 26 and Figure 19) and has only 2 of the 10 H-2 specificities of the donor (H-2.3 and 8). However, the cytotoxicity against B10.BR target cells was only about 1/64 of that directed at donor-type target cells. In one experiment (Table 25), cytotoxicity was also detected against target cells of B10.M having only 1 of the 10 H-2 specificities of the donor (H-2.8) and against target cells of DBA/1 having only 3 H-2 specificities of the donor (H-2.3, 13 and 43).

In the third series of experiments B10.D2 mice served as the source of cytotoxic PEL following an i.p. inoculation of SaI. In these experiments immunization against H-2 antigens was confined to those

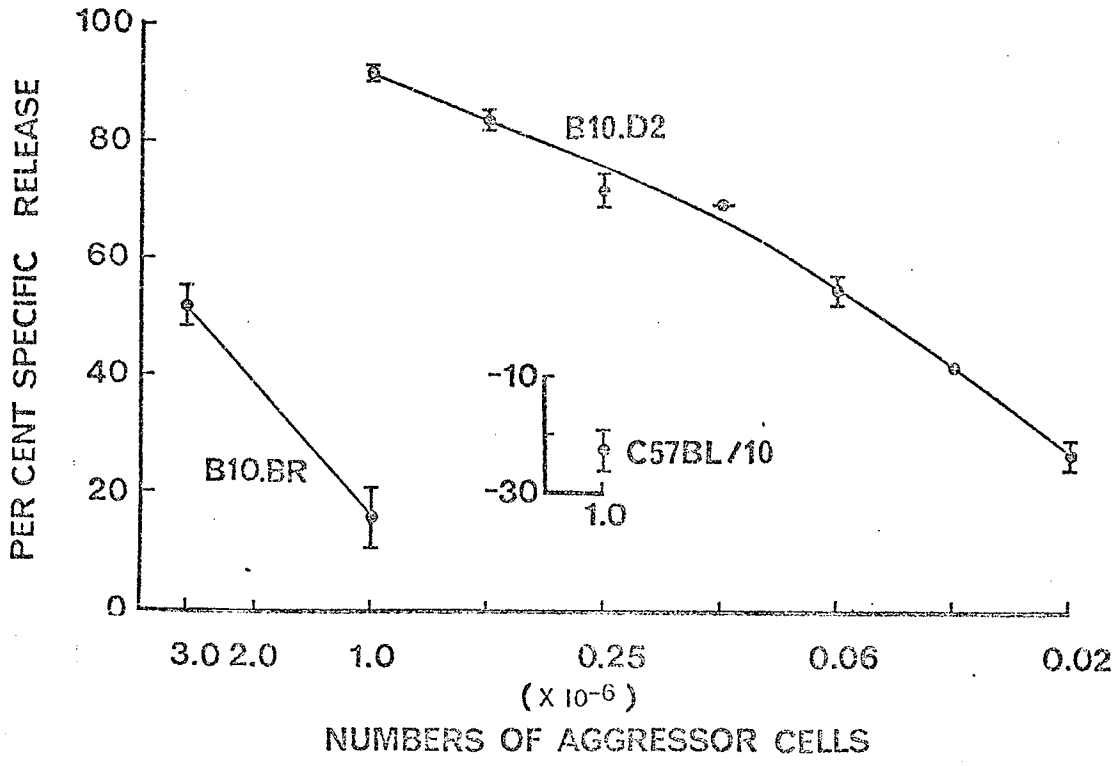

Figure 19

Cytotoxicity mediated by C57BL/10 anti-P-815-X2 PEL against allogeneic target macrophages. Vertical bars represent $\pm$ 1 s.d.

Table 25

Specificity of ^{51}Cr Release from Target Cells of Different H-2 Types by Sensitized PEL

Immunization	Experiment	Aggressor Cell Number ($\times 10^{-6}$)	Per cent[1] specific ^{51}Cr release from target cell of					
			B10.A	C57BL/10	B10.D2	B10.BR	B10.M	DBA/1
P815-X2 → C57BL/10	1	2.0	94.5 ± 10.2	-59.3 ± 3.5	104.6 ± 11.4	38.8 ± 2.3	18.6 ± 7.1	39.3 ± 2.2
		0.50	94.3 ± 2.4	----	----	----	----	----
		0.06	46.2 ± 6.5	----	----	----	----	----
	2	3.0	----	----	----	51.9 ± 3.5	-24.9 ± 0.8	-32.0 ± 20.8
		1.0	----	-22.8 ± 3.6	91.2 ± 1.3	15.6 ± 5.2	-20.5 ± 12.9	-0.3 ± 1.8
		0.50	----	----	83.3 ± 1.8	----	----	----
		0.06	----	----	54.8 ± 2.7	----	----	----
		0.03	----	----	41.6 ± 0.7	----	----	----
SaI → B10.D2	1	2.0	110.8	24.3 ± 2.1	5.1	90.2	51.9 ± 8.5	18.6 ± 0.7
		1.0	102.1 ± 5.7	17.6 ± 2.9	4.4 ± 0.7	90.9 ± 2.6	31.3 ± 1.6	10.5 ± 2.4
		0.5	89.4 ± 3.5	6.6 ± 3.7	-0.4 ± 3.0	88.2 ± 1.1	12.2 ± 11.1	-4.2 ± 5.4
		0.125	58.7 ± 7.1	3.3 ± 1.1	4.8 ± 0.9	----	5.0 ± 4.8	----
	2	1.0	109.5 ± 2.7	57.4 ± 3.4	-10.0 ± 5.5	91.3 ± 1.2	43.6 ± 2.8	42.3 ± 1.4
		0.50	----	----	----	91.7 ± 1.2	----	----
		0.06	----	----	----	67.5 ± 1.2	----	----
		0.03	----	----	----	35.6 ± 4.6	----	----
	3	1.0	100.0 ± 4.8	55.4 ± 3.5	-19.2 ± 2.3	100.4	48.9 ± 0.8	11.4 ± 0.6
		0.50	96.6 ± 4.3	----	----	----	----	----
		0.06	74.5 ± 13.0	----	----	----	----	----

[1] Each value represents the arithmetic mean ± 1 S.E. of triplicate determinations.

Table 26

Representation of Presumptive Immunizing Antigens and Those Available for Reaction on Third-Party Cells

Donor	Recipient	Target Cell	H-2 specificities of the donor	
			H-2K	H-2D
DBA/2 ($H\text{-}2^d$) (P-815-X2)	C57BL/10 ($H\text{-}2^b$)		31* 3,8,34	4* 3,13,41,42,43,44
			Donor H-2 specificities presented by TARGET cell	
		B10.A ($H\text{-}2^a$)	— 3,8,—	4 3,13,41,42,43,44
		DBA/1 ($H\text{-}2^q$)	— 3,-,—	- 3,13,—,—,43,—
		B10.BR ($H\text{-}2^k$)	— 3,8,—	- 3,—,—,—,—,—
		B10.M ($H\text{-}2^f$)	— -,8,—	- -,—,—,—,—,—
A/J ($H\text{-}2^a$) (SaI)	B10.D2 ($H\text{-}2^d$)		23* 1,5,11,25,45	
			Donor H-2 specificities presented by TARGET cell	
		B10.BR ($H\text{-}2^k$)	23 1,5,11,25,45	
		DBA/1 ($H\text{-}2^q$)	— 1,5,11,—,45	
		C57BL/10 ($H\text{-}2^b$)	— -,5,—,—,—	
		B10.M ($H\text{-}2^f$)	— -,-,—,—,—	

* denotes a private H-2 specificity and — indicates the specificity is not present. (From the table of Klein and Shreffler, 1971).

determined by the H-2K and closely associated regions of the $H\text{-}2^a$ chromosome since B10.D2 shares the H-2D region with SaI (Table 26). The PEL had high cytotoxic activity against both B10.A target cells, having the donor H-2 type, and against B10.BR target macrophages which share the H-2K region with $H\text{-}2^a$ (Table 25). Cytotoxicity was also found directed against other third-party target cells lacking identity with either donor H-2 region, i.e., against C57BL/10 (Figures 20 and 21), having only 1 of the 6 donor H-2 specificities (H-2.5), against target cells of DBA/1 having 4 of the 6 donor H-2 specificities (H-2.5, 1, 11 and 45) (Figure 21) and even against target cells of B10.M (Figure 20) having no known donor H-2 antigens (Table 26). This latter finding confirms the earlier observation using the assay of tumor immunity in vivo in which resistance to the growth of SaI was found in B10.D2 mice grafted with skin from strain B10.M. As in the previous experiments, while the levels of ^{51}Cr released from the third-party target cells were quite high, the reactions against third-party cells were 1/32 or less than the reactions directed at target macrophages having identity with a donor H-2 region.

VI. Nature of the cytotoxic cells in the PEL population

PEL from C57BL/10 mice which had rejected the ascites form of SaI were assessed for the presence of the theta allo-antigen by treating the cells with an anti-θC3H serum (as described in the previous Chapter) followed by complement prior to the cytotoxic assay. In view of the high cytotoxic capacity of the PEL, limiting numbers of the aggressor cells were used in the assays against donor-type

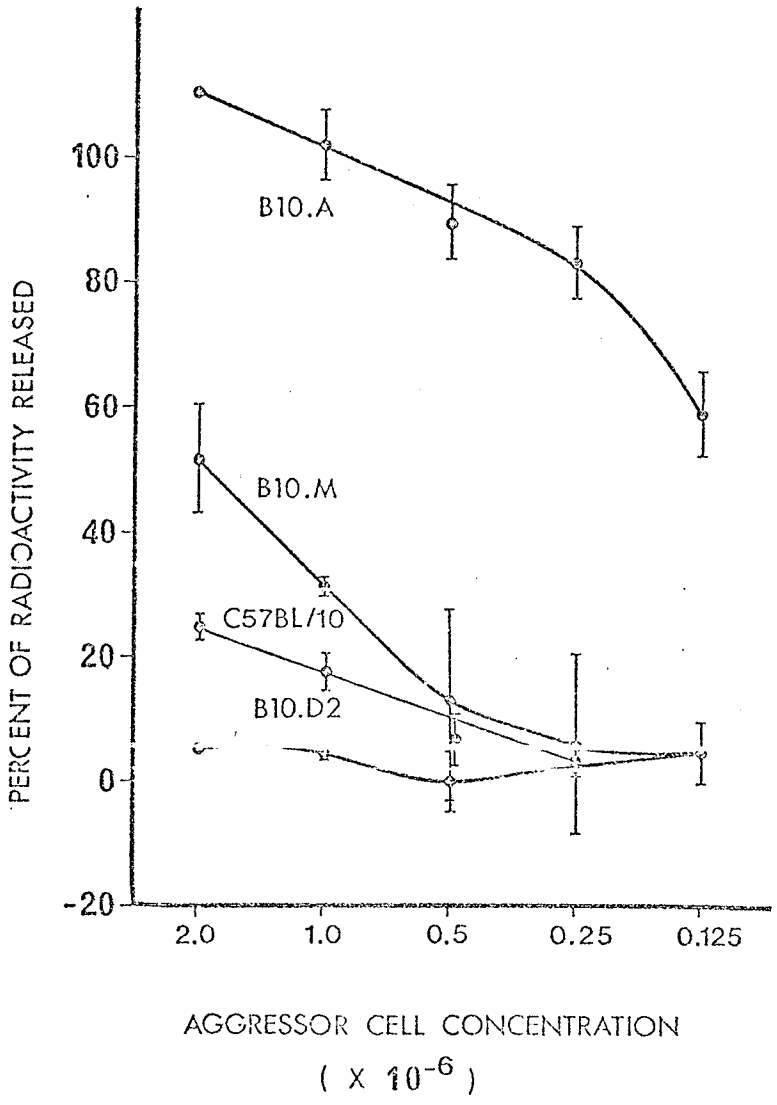

Figure 20

Cytotoxicity mediated by B10.D2 anti-SaI PEL against allogeneic target macrophages. Vertical bars represent $\pm$ 1 s.d.

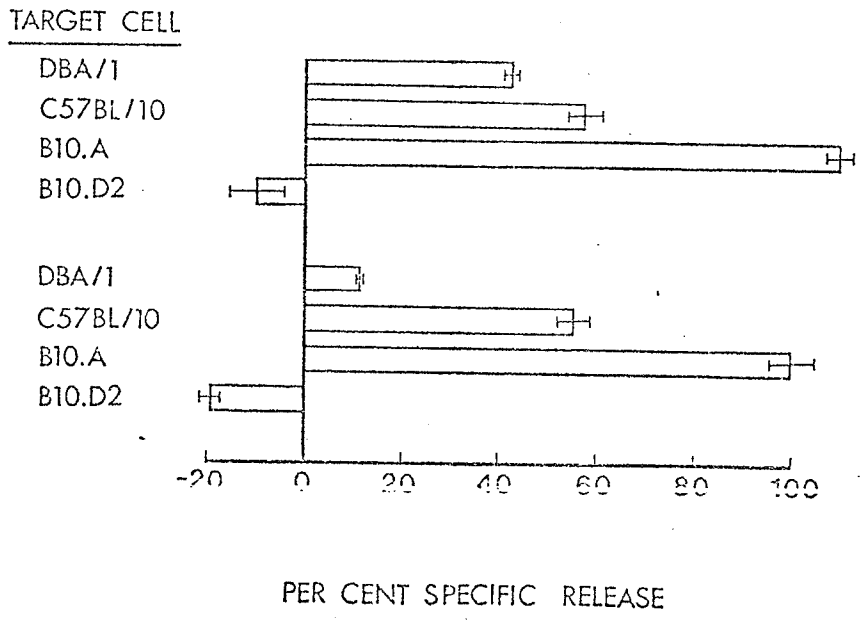

Figure 21

Cytotoxicity mediated by B10.D2 anti-SaI PEL against allogeneic target macrophages. Mean values of triplicate determinations $\pm$ 1 s.d. are shown.

target cells, while larger numbers were used in assays with third-party target cells. It is evident from the results of these experiments (Table 27 and Figure 22) that the lysis of donor-type target cells mediated by PEL in two tumor immunization systems was sensitive to the effects of anti-θ serum and complement. In addition, the cytotoxicity against two types of third-party target cell was abolished by the same treatment.

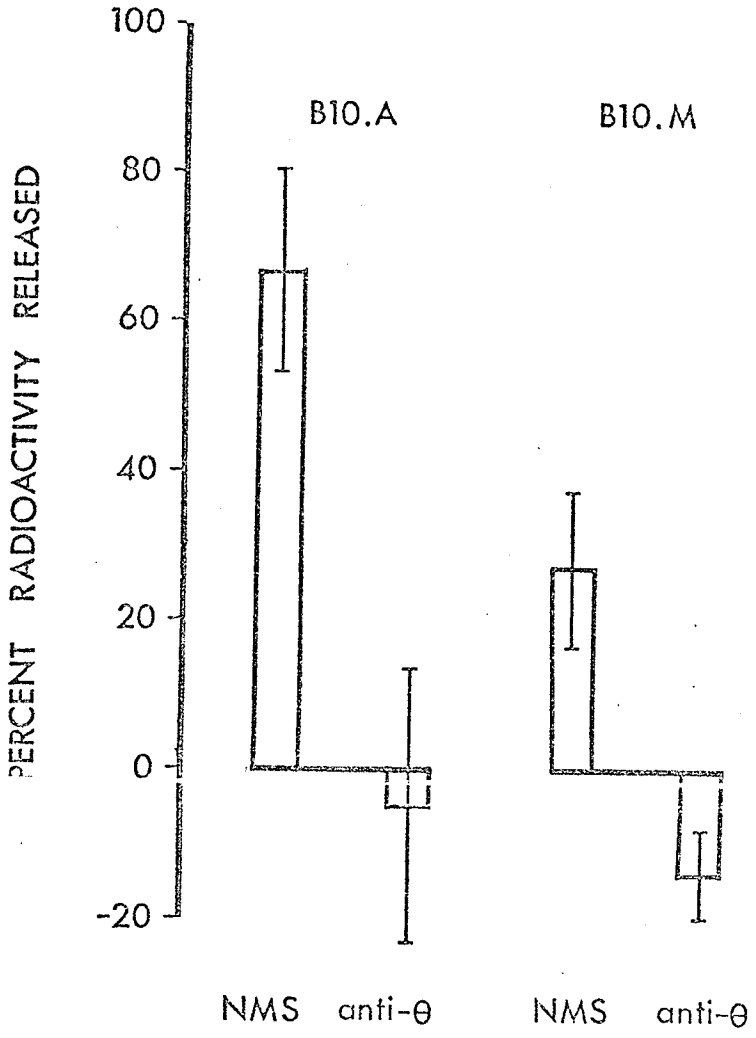

Figure 22

Abolition of the cytotoxicity of B10.D2 PEL with anti-θ serum and complement. Vertical bars represent ± 1 S.E.M. 1 x 10^6 PEL were used as aggressor cells against B10.M target cells while 0.125 x 10^6 PEL were used against B10.A target cells.

Table 27

Abrogation of C57BL/10 PEL Anti-SaI and Anti-P815-X2 Cytotoxicity with Anti-θ Serum and Complement

Experiment	Numbers of PEL	Target Cell	HBSS & HBSS	HBSS & Complement	NMS & Complement	Anti-θ & Complement
	(anti-SaI)		Per cent specific ^{51}Cr released			
1	1.25×10^5	B10.A	45.9 ± 3.3	57.9 ± 4.9	55.0 ± 2.9	-4.3 ± 4.8
	2.50×10^5	B10.A	82.4 ± 1.7	92.6 ± 2.1	87.1 ± 2.0	10.8 ± 3.6
2	1.25×10^5	B10.A	-----	-----	45.7 ± 22.6	1.6 ± 6.3
	2.50×10^5	B10.A	-----	-----	71.3 ± 9.3	12.7 ± 2.5
3	2.0×10^6	DBA/1	-----	-----	20.9 ± 1.8	-23.2 ± 7.9
	(anti-P815-X2)					
4	1.25×10^5	B10.A	-----	-----	66.7 ± 13.8	-4.8 ±18.7

Each value represents the arithmetic mean ± 1 S.E. of triplicate determinations.

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 per cent, 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 genetic characteristics; 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 $4^{o}C$ 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 de-aggregation 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 (Müller, 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 radiolabelled 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 to 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 1×10^7 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, Dray and Grotoff, 1970). Thus, the underlying mechanism of inhibition of migration may include metabolic processes causing intercellular 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 a 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 per cent (range 8 - 14 per cent). 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 per cent (range 21 - 46 per cent). No notable differences were observed regarding the degree of inhibition relative to 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 per cent of C57BL/6J PEC was decreased from 70 to 12 per cent, there resulted a continuous rise in the degree of inhibition (from 34 to 54 percent), as illustrated in Figure 3.

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 of 0 per cent antigenic cell content nor at 100 per cent 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 (Table 11). The results may be interpreted 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 even 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 per cent content of antigenic cells inhibitions do not increase, but are seen to decrease for PEC on 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 analogous 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-? antigens (Winn, 1962), and is quite homogeneous, consisting of lymphocytic elements in excess of 99 per cent. 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, Chaperon and Triplett, 1966). Thus, if inhibitions occurred using these cells as the antigen source, interpretation of the results would be simplified.

Control experiments showed that a 20 per cent content of syngeneic or allogeneic thymus cells in a population of PEC from normal mice had no detectable effect on the migration of such PEC. Using this experimental design (immune PEC mixed with antigentic 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 all the experiments of this modification, PEC from the graft recipients were inhibited in their migration when mixed in the ratio of 4:1 with thymus cells of the donor (Figures 5 and 6, Tables 8 and 9). The use of thymus cells from third-party strains, showed that for detectable inhibition of migration the third-party strain required identity with at least one of the two regions of the H-2 complex which determine serologically defined H-2 antigens of the donor (i.e., either K or D) and thus a representation of all the H-2 specificities of that H-2 region. These results are in keeping with the

independent cell-surface expression of H-2D- and H-2K-region antigens (Neauport-Sautes, Silvestre, Lilly and Kourilsky, 1973). Thymus cells from strains not having identity with the donor at either of the H-2 regions caused marginal or no inhibition.

In some of the experiments, however, PEC from C57BL/10 mice immune to C3H/HeJ appeared to react weakly toward thymus cells of BALB/c mice which lack identity with either H-2 region of the donor and have only 2 of the 8 relevant donor H-2 specificities. As an alternate hypothesis, therefore, it is possible that the sensitivity of the assay was limited, and that the reasons for the failure to detect definitive inhibitions with other third-party antigens may have been quantitative. Although antigenic thymus cells were shown to be present in non-limiting numbers in this assay (see page 75) it was possible that the numbers of specifically sensitized cells in the PEC were limiting. A second modification of the migration assay in which regional LNC from skin-grafted mice were mixed with antigenic PEC from normal mice, ensured the presence of sensitized cells in high numbers.

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 per cent 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 the present study 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 per cent of these lymph node cells were lymphocytes (in agreement with the observations of David).

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 per cent of sensitized LNC were able to detectably inhibit the migration of antigenic PEC, moreover, only a fraction of the 1 - 2 per cent of LNC can be assumed to be specifically sensitized .

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).

v. Specificity of inhibition of migration mediated by LNC

LNC from C57BL/10 mice immune to C3H/HeJ inhibited the migration of PEC of donor H-2 type similar to the results of the previous assay. Moreover, the LNC inhibited the migration of PEC from B10.D2 having only 2 of the 8 relevant donor H-2 specificities and also inhibited the migration of PEC of DBA/1 mice having 4 of the 8 donor H-2 specificities. In both cases neither donor H-2 region is shared by the third-party strains. In the second series of these experiments, reactions to third-party cells were observed as well. Thus, LNC from C57BL/10 mice immune to A/J inhibited the migration of PEC of DBA/1 which lacks identity with both H-2 regions of the donor and has only 6 of the 13 relevant donor H-2 specificities.

Although quantitative comparisons of the inhibitions obtained using donor PEC with the inhibitions obtained using third-party strains were not attempted, the data represented in Figure 8 show that only a large decrease in numbers of LNC resulted in reduced inhibitions and therefore indicate that the reaction of sensitized LNC, on a cell-for-cell basis, was considerably less potent against antigenic PEC lacking complete identity with either of the 2 H-2 regions of the donor than against donor-type PEC (Tables 10 and 11). Participation of non-H-2 antigens can be excluded in the response to the congenic strain B10.D2. However, reactions to non-H-2 antigens cannot be excluded in the reaction of LNC to cells to DBA/1 but are unlikely since attempts to demonstrate non-H-2 immunity (immunizations of the type: 129/J → C57BL/6 and the reciprocal) using recipient LNC and donor PEC were not

successful.

In sum, with a modification of the migration assay involving a mixture of sensitized LNC and antigenic PEC, inhibition of migration was detected in all experiments with antigenic PEC from all third-party strains lacking identity with either H-2 region of the donor and sharing only a few donor H-2 specificities. However, the reactions were weaker than those caused by donor antigens.

It is of interest to note that with similar donor, recipient and third-party strains, absence of cross-reactivity was concluded from results using the method of in vitro cell-mediated cytotoxicity (Brondz and Golberg, 1970). Such conclusions are in apparent agreement with the results obtained with the first modification of the migration assay, but are at variance with the results obtained with the second, and presumably more sensitive modification.

Recently Al-Askari and Lawrence (1972) demonstrated the production of MIF-like material by lymphocytes from skin-grafted mice which parallels the earlier findings of MIF-mediated inhibition of migration in guinea pigs with cellular immunity (Bloom and Bennett, 1966). In view of the selective cytotoxicity of anti-theta serum to lymphoid cells of thymus-derived lineage (Raff, 1969) the abrogation of the capacity of sensitized LNC to inhibit migration following treatment with this antiserum provides direct confirmation for the role of cell-mediated immunity in this in vitro transplantation reaction.

It is unknown if the mechanisms of the two forms of the migration assay presented in this paper are identical, however, two similar assays for cellular immunity in the guinea pig, the first involving the use of immune PEC (Bloom and Bennett, 1966) and the second involving a mixture of normal PEC and immune LNC (David, 1966) were both shown to have MIF-dependent mechanisms. Further, Al-Askari and Lawrence (1972) have demonstrated that MIF but not allo-antibody is produced by cells from immune PEC using an experimental protocol very similar to that described here which suggests that the inhibitions obtained in the first assay (immune PEC plus antigenic thymocytes) are mediated by MIF. In the second migration assay (immune LNC plus antigenic, normal PEC) the dependance of inhibition of migration upon θ-bearing cells is also not compatible with a conventional antibody-mediated mechainsm. The possibility of cell-to-cell cytotoxicity among the migrating cells cannot account for the observed inhibitions since the ratios of cytotoxic cells to target cells, in both forms of the migration assay, were of the order of 100-fold less than required to evidence minimal cytotoxicity in the ^{51}Cr assay (unpublished results). The weight of evidence thus suggests that the two migration assays are similar in mechanism.

vi. 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 radiolabelled cells (considered earlier, p. 12).

With regard to effector mechanisms in rejections of certain transplants, where cell-mediated processes are clearly implicated, such as skin grafts or solid tumor grafts, an accumulation of cellular elements having similar radiolabelling properties to those found in lesions of delayed hypersensitivity has been described, i.e., there is a failure of radiolabelled 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 foreign histocompatibility antigens. These activated cells might then serve in cytopathogenic processes directly (Alexander and Evans, 1971), 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) labelled 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, Bradley and Turk (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.

vii. Studies with an assay of tumor immunity *in vivo*

The reactivity of sensitized cells to third-party antigens was confirmed by *in vivo* transplantation experiments in which the growth of the tumor, SaI was suppressed in mice pre-immunized with skin grafts. B10.D2 mice grafted with C57BL/10 skin were resistant to SaI tumor growth and C57BL/10 mice grafted with B10.M skin were resistant as well. In both cases the third-party strain, A/J (SaI), lacked identity with both H-2 regions of the graft donor and shared only one H-2 specificity with the donor. In addition, B10.M skin grafts conferred some resistance to challenge with SaI in B10.D2 mice as well, despite the lack of identity with either of the donor H-2 regions as in the two previous cases, and the absence of known, shared H-2 specificities with A/J. These unexpected results were confirmed in the *in vitro* cytotoxicity assay which follows, and indicate either that the available serological information is incomplete or that some H-2 antigens evoke cell-mediated reactions but little or no antibody. Since congenic mice were used for immunization, tumor resistance can be attributed only to immunity directed at antigens determined by genes very near or at the H-2 complex. Other studies *in vivo* using skin-grafting techniques in which accelerated graft destruction was detected in mice pre-immunized to H-2 antigens in cases where no H-2 regions was shared (Berrian and Jacobs, 1959 and Klein and Murphy, 1973) are consistent with these results.

It is pertinent to note that in all third-party combinations, some tumor growth did occur, which suggests that, although detectable,

the immunity, i.e., cross-reaction, was quite weak. This aspect could be much less apparent with skin grafting techniques.

The resistance to the growth of SaI *in vivo* in skin-grafted mice may be attributed to cellular immunity since immunological enhancement of SaI, not resistance, is the usual consequence of the presence of iso-antibody (Gorer and Kaliss, 1959).

viii. Summary of data from tumor growth and migration assays

The above results using two assays of cellular immunity, one *in vivo* the other *in vitro*, contrast with the conclusions of Brondz (1968) and Brondz and Golberg (1970) obtained with the method of *in vitro* cell-mediated cytotoxicity and with similar observations by others (Ginsburg, 1968; Mauel, Rudolf, Chapuis and Brunner, 1970; Ax, Koren and Fischer, 1971 and Berke and Levey, 1972; McDonald, Miller and Phillips, 1973b). In all of these studies very little or no immunity was detected against target cells from any third-party strains not sharing a donor H-2 region. As indicated previously, the reactions described in these studies both *in vitro* and *in vivo* to third-party cells lacking H-2 region identity with the donor were demonstrably weaker than the reactions towards donor-type target cells. These quantitative differences suggested that the specificity of *in vitro* cytotoxicity be examined for limitations of sensitivity possibly responsible for the divergent conclusions.

ix. Macrophages as target cells

In these experiments dextran-induced peritoneal exudate macrophages obtained from otherwise untreated mice and labelled with radioactive chromium, served as target cells. In preliminary studies to examine the suitability of macrophages to serve this function, it was found that ^{51}Cr-labelled macrophages exhibited characteristics similar to other target cells used in previous studies (Wilson, 1965; Brunner, Mauel, Rudolf and Chapuis, 1970; Canty and Wunderlich, 1970) with regard to (1) the logarithmic relationship between the numbers of attacking cells and the per cent of cytotoxicity, (2) the low spontaneous release of label and high release upon interaction with aggressor cells, (3) the absence of detectable effects upon incorporating third-party macrophages into cultures with ongoing cytotoxicity, and (4) the correlation of chromium release from DBA/2 macrophages with the lysis of DBA/2 mastocytoma P-815-X2 and with macrophase death as determined with the Trypan blue exclusion test.

For quantitative comparisons of cytotoxicity mediated by one population of aggressor cells against a panel of target cells from strains of different H-2 types, available neoplastic cell lines were excluded on the basis that target cells from different tissue origins do not have the same sensitivity to lysis by allo-immune cells (Brunner et al., 1970). In this respect, it was shown that macrophages from mice which shared their H-2 type with the graft donor but had otherwise disparate genetic backgrounds were subject to equal cytotoxicity as donor target cells - contrary observations would not have permitted

quantitative comparisons of cytotoxicity. In addition, the data did not indicate the participation of non-H-2 antigens in cytotoxicity mediated by sensitized spleen cells.

x. Specificity of cell-mediated cytotoxicity

(a) Aggressor spleen cells

To study the specificity of *in vitro* cell-mediated cytotoxicity, two allo-immune aggressor cell populations, i.e., spleen cells and peritoneal exudate cells non-adherent to polystyrene, were used. In the first series of experiments spleen cells from skin-grafted mice (10 donor-recipient combinations) were assessed for cytotoxicity against target cells from a panel of strains of 6 different H-2 types. The results revealed that splenic aggressor cells were cytotoxic to target cells of the graft-donor strain and to those which had identity with at least one of the 2 H-2 regions (K or D) of the graft donor. However they were not cytotoxic to target cells which shared many donor H-2 specificities but lacked identity with at least one H-2 region. These data confirm the findings of Brondz (1968) and of Brondz and Golberg (1970), and similar results obtained using other *in vitro* cytotoxic assays (Ginsburg, 1968; Mauel, Rudolf, Chapuis and Brunner, 1970; Ax, Koren and Fischer, 1971; and Berke and Levey, 1972).

The above reports were viewed in marked contrast with evidence obtained in other studies of cellular immunity to H-2 antigens. Thus, *in vitro*, using the inhibition of migration assay (Chapter 1), reactions of sensitized cells against antigenic cells sharing only few

H-2 specificities with the graft donor were observed and similar results were obtained in vivo with an assay of resistance to tumor growth. In related in vivo studies accelerated rejection of third-party skin grafts by immunized mice was observed in some donor-recipient combinations (Berrian and Jacobs, 1969) and these observations have been recently confirmed (Klein and Murphy, 1973) as mentioned previously.

(b) Aggressor PEL

The possibility that the contrasting findings were due to different sensitivities of the assays involved was considered. Thus, sensitized aggressor cells cytotoxic to third-party target cells may have been present in numbers insufficient to cause detectable target cell destruction. To test this possibility, non-adherent PEL formed consequent to ascites tumor allografts were used since this cell population was shown to be rich in aggressor cells (Brunner and Cerottini, 1971; Berke, Sullivan and Amos, 1972a). Indeed these cells were shown in the present investigation to be approximately 16 to 32-fold more active than similar numbers of spleen cells from skin-grafted mice. Consequently, the specificity of cell mediated cytotoxicity was studied using numbers of aggressor cells up to 6-fold greater than those required to destroy 100 per cent of donor H-2-type target cells and the cytotoxicity was titrated over a range of three orders of magnitude of aggressor cell numbers. Using the above conditions, cytotoxicity mediated by PEL remained specific in terms of the absence of release of label from syngeneic target cells. As with spleen cells, lysis of target cells having identity with the K or D H-2 regions of the donor

occurred. Moreover, definitive cytotoxicity against target cells from third-party strains which lack identity with either H-2 region of the donor was demonstrated.

Properties of PEL

The exceptional potency of peritoneal exudate lymphocytes (PEL) to mediate in vitro cytotoxicity suggests that this cell population may be enriched with regard to effector T cells involved in reactions of cellular immunity. Indeed evidence is available to support this hypothesis. Data from work involving the transfer of peritoneal cells into X-irradiated mice reveals that even following intraperitoneal immunization with sheep red blood cells no antibody-forming cells (AFC) were detected in this lymphoid compartment which was, however, rich in cells involved in the differentiation of AFC. Such cells could quite possibly be the thymus-derived helper T cells which cooperate in the production of AFC (Claman, Chaperon and Triplett, 1966).

In studies by Koster and McGregor (1971) it was found that a substantial portion of the lymphocyte-like cells in induced PEC are derived from short-lived cells of the thoracic duct. The long-lived thoracic duct lymphocyte does not enter. The results suggest that newly formed cells especially (including effector cells just formed in response to an antigenic stimulus) have a propensity to localize at inflammatory foci. Alternatively, the peritoneal cavity can selectively permit the entry of effector T cells only.

If peritoneal lymphocytes comprise largely effector T cells

related to a recent immunization it might be expected that such cells would be very active in cell-mediated reations. This hypothesis was supported by results which showed that induced, non-adherent peritoneal exudate cells from immune rats were more active than even thoracic duct cells to adoptively protect immunodeficient rats from L. monocytogenes (Koster, McGregor and Mackaness, 1971). PEL have been shown to be far more active than other sources of lymphoid cells to react to antigen (e.g. DNA synthesis). Remarkable stimulation indicies of 50 - 300 were obtained, and in the same study it was shown that the PEL had a 1000-fold greater avidity (optimal antigen concentration) for antigen than LNC (Rosenthal, Rosenstreich, David and Blake, 1972). In favour of the concept of selective entry of T-cells into the peritoneal cavity, it was found in the above study that no antibody producing cells nor even lymphocytes with surface immunoglobulin (B-cells) were detectable among the PEL. The exceptional potency of PEL to manifest effector activity of cellular immunity was also shown in experiments in which PEL were the most potent cell type to release the soluble mediators MIF and SRF (Rick, Krejci and Turk, 1972).

In summary, the results show that in rats and guinea pigs peritoneal lymphocytes are (1) predominantly effector T-cells (with few or no B-cells) which have been recently formed, are (2) rich in cells specific to a recent immunogen and, therefore, extremely reactive in vivo and vitro and (3) are possibly end-stage cells in their maturation to the primary antigen stimulus as may be deduced from their high avidity for antigen.

The exceptional activity of murine PEL in cell-mediated cytotoxity in vitro in the allograft system presented in this chapter may be due to a similar phenomenon. It is possible that (1) mature cytotoxic effector cells enter the peritoneal cavity because of their cell class and attraction of the peritoneal irritation (i.e., the ascites tumor) and/or, (2) immature or precursers of cytotoxic cells enter the cavity and divide became of the antigenic tumor-stimulus. Indeed, Asherson and Ptak (1968) demonstrated that induced PEL were more competent to transfer delayed hypersensitivity in mice than lymph node cells. The high avidity of PEL for antigen can explain the findings that PEL lymphocytes became desensitized (loss of ability to inhibit migration) while lymph node cells remained able to respond (DNA synthesis) to antigen during tolerogenic treatments (Schlossman, Levin, Rocklin, and David, 1971). Since the migration assay accurately correlates with the skin-reactive state of the animal (they were skin-negative) these results also sustain the concept that PEL reflect the true immune effector status of the animal.

The sensitivity of cytotoxic PEL to anti-θ serum and complement is in agreement with similar observations by other workers (Brunner and Cerottini, 1971; Berke, Sullivan and Amos, 1972b) and indicates that the cytotoxicity is mediated by thymus-derived cells. The findings that cytotoxicity to third-party cells was similarly abrogated and that similar slopes of per cent cytotoxicity versus aggressor cell number were obtained using donor or third-party (e.g., DBA/1) target cells (unpublished observations), indicate that both

classes of target cells are lysed by a similar mechanism (Henney, 1971).

xii. Relation of the specificity of cellular reactions to H-2 serology

Analysis of the correlation of cytotoxicity to the presence or absence of individual H-2 specificities is complex and limited by the few H-2 chromosomes suitable for study. In addition, variable concentrations of the same H-2 specificities on cells from different strains (Hilgert and Kristofova, 1971) and the complex behaviour of some H-2 specificities (Snell, Demant and Cherry, 1971) could lead to differing sensitivities to lysis. Despite these limitations, the data from the present study reveal that target cells sharing an H-2 region (K or D) with the donor, and thus all the private, public and perhaps other antigens of that region, are lysed to an extent similar to donor target cells. However, the reaction to third-party cells, sharing all public donor specificities (as many as 4 of 4) but lacking the two private specificities and concomitant identity with either donor H-2 region was, by comparison, extremely weak; from 1/32 to 1/64 of that directed at donor-type target cells. This contrasts with the activity of hyper-immune anti-H-2 sera and with the hemagglutinating and cytotoxic activity of anti-H-2 sera produced in a primary allograft response, since both react with third-party cells (Brondz and Golberg, 1970). Several possibilities may account for this difference. (1) The cell-mediated cytotoxicity considered above reflects a primary immune

response and for this reason may differ markedly with the specificity of the hyper-immune antibody response upon which the H-2 chart is based. Indeed, it is known that 'early' anti-H-2 sera are rich in anti-private antibody and deficient in anti-public activity (Shreffler, personal communication). Since anti-public activity appears only following more rigorous immunization it follows that cytotoxicity should be studied following extended immunization regimens as well. (2) The cytotoxic reactions may be directed at private H-2 specificities as suggested by Thorsby (1971) in analogy with the HL-A system and the weak reactions to third-party target cells may be due to limited cross-reactivity of T-cells (in contradistinction to widely cross-reactive anti-private antisera which artificially define public specificities in this interpretation). Such a hypothesis may be tested with the use of monospecific anti-H-2 sera to block selectively individual H-2 specificities in the system of in vitro cell-mediated cytotoxicity. If the immunizations were to involve a mixture of public and private specificities but'blocking' was effective only with anti-private antisera, this would further sustain the conclusion that public specificities do not participate in cell-mediated reactions relative to private specificities. Indeed, special immunogenetic and immunogenic properties of private H-2 antigens have been proposed (Snell, Cherry and Demant, 1971). They noted the unusually high cytotoxic titre obtained against private specificities and suggested that the private H-2 specificities can be arranged in two allelic series, with each of the two ends of the H-2 complex determining one private specificity. This would allow

for four private H-2 specificities per individual - an exact homology with the HL-A region of man. These allelic antigens would be expected to be the paramount immuno-dominant system and only trace reactions might be expected against public specificities. In this interpretation it may be suggested that public specificities are antigenic determinants of a portion of the H-2 molecule common to all mice but are antigenic owing to their proximity to a private antigen. Cross-reactive antibodies, i.e., antibodies specific for the private determinant, but including some adjacent determinants, are formed which can react with similar public conformational determinants induced by different private H-2 specificities. (3) The specificity of aggressor cells may in part be related to antigens not yet defined serologically, which could explain the cytotoxicity to B10.M cells (having no known donor H-2 specificities) of PEL from B10.D2 mice immune to SaI. Cytotoxicity in the absence of serologically defined differences has been reported (Berke et al., 1972b). However, this concept is not supported by immunogenetic studies (Alter, Schendel, Bach, Bach, Klein and Stimpfling, 1973) and by the ability of anti-H-2 sera to block cell-mediated cytotoxicity (Mauel et al., 1970) although the former test was insensitive and the latter subject to the criticism of steric effects. (4) Private and public H-2 determinants may be independent antigenic moieties, in keeping with traditional interpretations of H-2 serology (Klein and Shreffler, 1971) and cell-mediated reactions are possibly directed at both types of determinants. In this interpretation, however, rather different antigenic properties of public and private antigens

in antibody-mediated and cell-mediated reactions must be postulated to explain the quantitative differences in the cell-mediated reactions reported in this thesis. Thus, it would be necessary to suggest that the determinants reacting as public specificities are detected more readily with allo-antisera than by receptors on T-cells while the converse would be true for private specificities.

SUMMARY AND 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.

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 (1965). It was concluded from the results of experiments in which the two types of PEC were mixed in different ratios that the proportion of specifically

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Asterisk denotes claim to original work by the author.

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 per cent of the migrating cell mixture) was shown to inhibit PEC migration it was concluded that all experiments were performed in conditions of excess of immune cells.

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.

The two forms of the migration assay were used to analyze the cell-mediated cross-reactions between donor and unrelated third-party strains as recognized by the recipient. In the first system, in which thymus cells (antigen) from a panel of strains were mixed with PEC from mice immunized with skin grafts, inhibitions were obtained only when the antigenic cells were of donor origin or were from a third-party strain which shared an H-2 region (K or D) with the graft donor. By contrast, in the second, highly sensitive form of the migration test, in which sensitized LNC were mixed with antigenic PEC from a similar panel of strains, inhibitions were detected also using antigenic cells from third-party strains which did not share an H-2 region with the donor and possessed only few H-2 specificities of the of the donor*. However, the reactions to the third-party, unrelated strains were relatively weak, which may explain their failure to be detected using the first assay. In the above study congenic strain combinations were used to restrict the survey to antigens determined by the H-2 complex. In related experiments it was not possible to demonstrate immunity to non-H-2 antigens.

The relationship of the LNC-PEC migration assay to cell-mediated reactions was shown in experiments in which the ability of

LNC to cause the inhibition of migration was abrogated following treatment with anti-θ serum and complement. This indicates that a cell of thymus-derived lineage is integral in this in vitro reaction*.

A study of cross-reactions in cell-mediated transplantation immunity was devised using an assay of resistance to the measured growth of a third-party sarcoma in mice pre-immunized against selected H-2 antigens. Such immunizations comprised skin grafts among congenic strains. Resistance was found in cases where tumor cells shared an H-2 region with the graft donor and also in cases where no region was shared; the latter reactions were, by comparison relatively weak*.

The apparent discrepancy of all the above findings with published work in the in vitro system of cell-mediated cytotoxicity prompted a new study of the specificity of this assay. ^{51}Chromium-labelled macrophages were used as target cells in a quantitative study of cytotoxicity since cells of identical morphological type are available from all strains. A rapid and simple method was developed for the isolation of such cells from dextran-induced PEC and such cells were shown to be suitable as target cells in the radio-chromium assay*. Spleen cells from skin-grafted mice lysed only target cells of strains which shared at least one H-2 region (K or D) with the donor, in agreement with the reports of others. By contrast, non-adherent peritoneal exudate cells, which had formed as a consequence of ascites tumor allografts, exhibited a cytotoxic potential from 16 to 32-fold greater than spleen cells and were capable to lyse third-party cells of strains having disparity with the donor at both H-2 regions.* The cytotoxicity

mediated by these non-adherent PEL was lost or diminished following anti-θ serum treatment, suggesting that the cytotoxicity is T-cell mediated, in agreement with earlier studies with spleen cells.* Quantitative considerations of the relative proportions of cytotoxic cells present in the spleen and peritoneal exudate suggest that prior failure to detect cytotoxicity of third-party (cross-reacting) strains can be attributed to the use of aggressor cells of limiting cytotoxic potential.

The consistent findings in the present studies which involve the use of two assays in vitro and an assay in vivo reveal that only very weak cell-mediated reactions can be detected against third-party, non-donor tissues. Such weak reactions were obtained despite the use of third-party strains which shared as many as 4 (public) of the 6 (public plus private) H-2 specificities presumably relevant to the immunization. This presumption is based on the simple-complex interpretation of H-2 serology. The present results are considered to be more compatible with a complex-simple interpretation (which places great importance on private H-2 specificities) and indirectly, to support this concept.

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