THE CHARACTERIZATION OF THE CELLS INVOLVED IN THE MIXED LEUKOCYTE REACTION IN THE RABBIT AND ITS RELATION TO ALLOGRAFT REJECTION

BY

PETER MILTHORP

Thesis submitted to the school of graduate studies of the University of Ottawa as partial fulfilment of the requirements for the degree of Doctor of Philosophy in Pathology.

ABSTRACT

The rabbit mixed leukocyte reaction (MLR) was systematically investigated in terms of the properties of the stimulator and responder cells. It was demonstrated that culturing the cells in medium RPMI-1640, supplemented with complement inactivated rabbit serum (final concentration 2.5%), for five days at 37°C, in a humidified atmosphere of air:CO_2 (95:5) provided the optimal condition for the blastogenic response (^{3}H-thymidine incorporation) of cells in the MLR.

The cells of the Peyer's patches and the mesenteric lymph nodes responded to the greatest extent in the MLR. Thymus and bone marrow cells cultured individually or in combination did not respond. The cells of the Peyer's patches, spleen and mesenteric lymph nodes were the most potent stimulating cells. There was no correlation between stimulating capacity and the percentage of stimulator cells bearing surface immunoglobulins.

Whole body irradiation of normal adult rabbits with doses of 600R or more resulted in the consistent elimination of the MLR responder cell activity in the immediate post irradiation period (1 to 2 days). There was no evidence of any preferential recovery of MLR responder activity by cells in any of the different lymphoid organs.

The cells of skin allografted rabbits, following rejection of the allografts, showed accelerated one-way MLR responses specific to stimulator cells from the allograft donors.

ACKNOWLEDGEMENTS

The author is grateful to Dr. Maxwell Richter for helpful discussions, many suggestions and much encouragement during the course of this study. The author is also grateful to Dr. D. Algom, Dr. Diponkar Banerjee and Dr. Y. Behelak for many useful discussions. The author wishes to acknowledge the technical assistance provided by David Steele, Bonnie Graham, Valerie Robb, Teena Walker and Carol-Ann Jodouin-Cauchy and is especially grateful to Richard Belanger, Chief Technician, Animal Care Unit, for skillfully performing surgical procedures. The author also wishes to thank Sandy Fraiberg for the expert preparation of the manuscript.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF TABLES

LIST OF TABLES (CONTINUED)

LIST OF TABLES (CONTINUED)

ABBREVIATIONS

CML	cell mediated lympholysis
Con A	concanavalin A
DNA	deoxyribonucleic acid
GvH	graft versus host (disease)
HBSS	Hank's buffered salt solution
HLA	histocompatibility antigen
HvG	host versus graft (disease)
LD	lymphocyte derived (determinants)
MHC	major histocompatibility complex
MLR	mixed lymphocyte (leukocyte) reaction
PHA	phytohemagglutinin
PWM	pokeweed mitogen
RBC	red blood cell
S.I.	stimulation index
SD	serum derived (antigens)
TCA	trichloroacetic acid

CHAPTER I

INTRODUCTION

The mixed leukocyte reaction (MLR) was first described by Schrek and Donnelly (1961), who observed large blast-like cells in co-cultures of leukocytes from two genetically distinct individuals. This observation, that a blastogenic response occurs upon culturing together two allotypically distinct populations of leukocytes has since been verified by numerous investigators (see below). This response is easily quantitated by the measurement of the incorporation of ^{3}H-thymidine into the newly synthesized DNA of the dividing cells. The MLR has more recently been directly linked to the allograft rejection phenomenon in man and in the mouse. It has been suggested that the blastogenic response in the MLR is an in vitro counterpart of the recognition in vivo of the foreign antigens on an allograft.

The techniques for allografting in the rabbit system have been perfected; nevertheless, the study of the MLR in the rabbit has been virtually ignored. In point of fact, the outbred rabbit constitutes an excellent animal in which to investigate immunological phenomena and to characterize and evaluate the cellular and non-cellular mechanisms recruited in the initiation and maintenance of an immune response. A large number of different lymphoid organs and tissues can be investigated from the same animal. Furthermore, the immune system of the rabbit is more closely related to that of the human than is that of other experimental animals used, especially the mouse and the rat. In these latter animal species, the spleen and lymph nodes

are normally hemotopoietic organs, quite unlike the situation in the human and the rabbit where only the bone marrow in the adult is hematopoietic. Furthermore, the mice and rats used in the majority of experiments are often inbred. Since the results obtained with the animals of one inbred strain are not always observed using animals of a different inbred strain, the investigator is tempted to select animals of that inbred strain which have previously been shown to respond in the desired manner. The rabbits used in this investigation were not inbred and may more realistically reflect the situation in the human. It must be noted, however, that the use of an outbred animal severely limits any investigation of the genetic background of the immune response.

The initial objectives of the research described in this thesis were to define in a systematic fashion several aspects of the MLR response with cells of normal adult rabbits. These were: (i) the identity of the responder and stimulator cells in terms of their organ of localization, (ii) the temporal aspects of this response, and (iii) the recovery of MLR responder cells during regeneration of MLR activity after in vivo irradiation of the rabbit.

On the basis of the results obtained following completion of the initial phase of this investigation, experiments were designed to study whether the MLR responder cells could recognize autologous cells, and whether the allograft rejection reaction

could be reflected by changes in the MLR response. These investigations may provide some insight into the ways in which the homeostatic mechanisms of the immune system of the rabbit function during allograft rejection and related phenomena.

A general review of the literature published on the MLR in the rabbit, the mouse, the rat and the human is presented in chapter II of this thesis. Observations of the work on the rabbit were integrated with those on the other species, even though the rabbit is the subject of this thesis, as the quantity of publications on the rabbit MLR did not warrant a separate discussion. The basic laboratory techniques used are described in detail in chapter III (Methods and Materials). The experimental results (chapter IV) are divided into six sections, each describing the results of a distinct set of experiments. A general discussion of the various aspects of the investigations is presented in chapter V. Chapter VI, Contribution to Knowledge, lists the original contributions made by the candidate. The Vitae lists the background and publications of the author.

CHAPTER II

REVIEW OF THE LITERATURE ON THE MLR

a) The mixed leukocyte reaction
b) The genetics of the MLR
c) The MLR responder cell
d) T-cell memory in the MLR
e) The MLR stimulator cells
f) Suppressor cells and the MLR
g) Other factors influencing the MLR
h) Generation of cytotoxicity in the MLR
i) Autologous stimulation in the MLR and its relation to autoimmunity and immune surveillance
j) The MLR and allograft rejection
k) Summary

a) The mixed leukocyte reaction

When leukocytes from two genetically distinct donors are mixed together and cultured for five to seven days a small proportion of the cells undergo blastogenesis and mitosis. This mixed leukocyte reaction or MLR is now known to be mediated by lymphocytes. Fortunately, the abbreviation MLR is consistent with either a leukocyte or lymphocyte responder cell.

The MLR was first observed by Schrek and Donnelly (1961), who noted blast-like cells in cultures of leukocytes from a

pool of human donors. Leukocytes were responsible for the reaction as plasma, erythrocytes or platelets when added to the allogeneic cells induced no blastogenic response. The blast-like cells observed in the cultures were characterized by their ability to incorporate the radioactive DNA precursor, ^{3}H-thymidine, into their nuclei. The degree of tritium incorporation into the newly synthesized DNA was quantitated by the use of autoradiography. Over the entire period of incubation no significant changes were found in the cell numbers in the control or stimulated cultures. These findings were later confirmed and expanded by Bain, Vas and Lowenstein (1964), who showed that there was no reaction in the MLR between three pairs of monozygotic twins. Reactions did occur between dizygotic twins, but with variable results. The reactions between the leukocytes of unrelated individuals were on the average greater than reactions between dizygotic twins. Hirschorn, Bach, Kolodny, Firschein and Hashem (1963) also made the same findings. These results indicated that the extent of the blastogenic reaction in the MLR was related to specific genetic differences between cell donors. It was then suggested that the degree of transformation of the leukocytes during the MLR might prove to be useful as a measure of histocompatibility and therefore could be a great asset in the determination of allograft compatibility (Bach and Hirschorn, 1964; Bain and Lowenstein, 1964). This observation is elaborated upon in detail in sections 'b' and 'j' of this chapter.

DNA synthesis as measured by the incorporation of ^{3}H-thymidine into cultures has become the standard method of measurement of blastogenenis in the MLR (Dupont, Hansen and Yunis, 1976). The counts per minute (cpm) in the cultures are evaluated using liquid scintillation counting. This methodology has recently been re-reviewed (Thorsby, du Bois, Bondevik, Dupont, Eijsvoogel, Hansen, Jersild, Jorgensen, Kissmeyer-Nielsen, Lamm, Schellekens, Svejgaard and Thomsen, 1974).

To evaluate the immune reaction of a prospective host to an allograft by utilizing the MLR, it is necessary to measure the blastogenic response of the host WBC to stimulation by the WBC of the allograft donor. In the system described above (two-way MLR) the WBC from both the cell donors respond with blastogenesis. Consequently, the final reactivity measured reflects the total proliferation of both cell populations. The response of the cells of only one of the individuals cannot therefore be evaluated. The unidirectional response (one-way MLR) required for the accurate prediction of allograft rejection is obtained by inhibiting the proliferative capacity (DNA synthesis) of one of the cell populations. Under this condition, the quantity of ^{3}H-thymidine incorporated into the cultured cells would reflect the response of the cells of only one of the donors. The one-way MLR permits one to predict the in vivo response toward cells of the potential allograft donor.

The stimulatory capacity of the cells must not be altered by the treatment of the cells with the inhibitors of DNA synthesis. Stimulatory as well as responding capacity is abolished if the leukocytes are freeze-thawed, heated to 56°C for 30 min or mechanically disrupted (Hardy and Ling, 1969; Schellekens and Eijsvoogel, 1970; Dupont, Hansen and Yunis, 1976; Ling and Kay, 1975). Inhibition of the proliferative capacity of the leukocytes without affecting their stimulatory capacity has been produced in two ways. The first method developed was to X-irradiate the cells in vitro (Kasakura and Lowenstein, 1965a). The dose of irradiation required to inhibit DNA synthesis without affecting stimulating capacity was found to be 4000R (Kasakura and Lowenstein, 1968). The great advantage of irradiation is that no extrinsic inhibitory substance likely to cause inhibition of the responding cell populations is introduced into the cultures. However, during the period of culture the irradiated cells are continuously dying and this factor may decrease their efficiency as stimulator cells and may possibly decrease the viability of the responding cells in culture. The second method of inhibiting the proliferative capacity of the stimulating cells is by the use of the drug mitomycin-C which arrests DNA synthesis (Bach and Voynow, 1966). This method is simpler than the use of irradiation but there is a danger of carry-over of the drug into the cultures where it may be eluted from the treated cells as has been suggested by several investigators (Ling and Kay, 1975; Wilson, 1967; Adler, Takiguchi, Marsh and Smith, 1970). This is the method currently used by most investigators.

Another method of measurement of the response of individual populations of cells in the MLR is by using allogeneic cells of donors of different sex. The number of male or female derived cells undergoing blastogenesis can be evaluated in colchicine arrested MLR cultures by the sex typing of the chromosomes in the mitotic cells. The extent of the blastogenic reaction in either direction can then be ascertained without the inherent problems produced by interfering with the DNA synthetic processes of the cell populations (Cepellini, Franceschini, Miggiano and Tridente, 1965; Oppenheim, Whang and Frei, 1965). This method is unfortunately very time consuming and not generally used today.

Mixed lymphocyte reactions occur between allogeneic cells of many species, including monkey (Appelman and Balner, 1972), dog (Main, Cole, Jones and Haire, 1967; Kisken and Swenson, 1969; Rudolph, Hered, Epstein and Thomas, 1969), rabbit (Chapman and Dutton, 1965; Daguillard and Richter, 1969; Harrison, Wei and Ahie, 1971), pig (Bradley, 1974), chicken (Weber, 1970), rat (Wilson, 1967), mouse (Dutton, 1965) and ammocoete (Cooper, 1969). Allogeneic hamster lymphocytes do not stimulate each other, however hamster lymphocytes are stimulated by xenogeneic cells (Fernald and Metzgar, 1976). It seems likely that the MLR is almost universal among the vertebrates.

MLRs have been attained by mixing and culturing leukocytes of closely related species. However, the response of the cells,

in general, decreases as the phylogenetic gap between the species increases (Ling and Kay, 1975). Most of the work on xenogeneic MLRs has been carried out using human and mouse cells (Peck, Alter and Lindahl, 1976). Human lymphocytes will respond to cells of mouse, dog, rat and rabbit in the MLR, and will stimulate mouse and dog lymphocytes (Widmer and Bach, 1972). The MLRs between allogeneic and xenogeneic cells appear to proceed by the same mechanisms, that is by interaction between histocompatibility antigens (Lindahl and Bach, 1976).

The MLR response appears quite early in ontogeny. The first lymphoid cells to appear in mammalian organogenesis are in the yolk sac and liver, and later in the thymus, bone marrow and spleen (Metcalf and Moore, 1971; Lawton, Self, Royal and Cooper, 1972; Solomon, 1971; Owen, 1972). In both man and sheep, fetal liver cells are the first ontogenically to respond in the MLR (Asantila, Vahala and Toivanen, 1974; Asantila and Toivanen, 1976; Granberg, Mamunen and Toivanen, 1976; Carr, Stites and Fudenberg, 1973; Stites, Caldwell, Carr and Fudenberg, 1975). However, this reaction by fetal liver cells in the MLR was found to be clonally non-specific and therefore cannot be attributed to a truly specific immune response.

The specificity of the above reaction was tested by the use in the MLR of the thymidine analogue 5-bromodeoxyuridine (BUdR). If during blastogenesis BUdR is incorporated into the DNA instead of thymidine the cells become sensitive to ultra violet light and

will die if exposed to ultra violet light (Zoschke and Bach, 1971). Using this method fetal liver cells undergoing blastogenesis in an MLR were eliminated from culture. Reexposure of the remaining fetal liver cells to the same stimulating cells, however, provoked another blastogenic response. This could not occur if a clonally preselected population of responder cells from the fetal liver was involved in the reaction as they would have been eliminated from the culture upon exposure to ultra violet light and none would be left to react the second time.

In man, fetal lymphoid cells of the spleen and the thymus have been shown to react with clonal specificity to stimulator cells in the MLR. The cells from these fetal organs responding to certain cellular antigens in the MLR can be eliminated completely using BUdR and ultra violet light. The remaining cells will still respond to other allogeneic cells which are not related to the first set of stimulator cells (Asantila, Vahala and Toivanen, 1974). The same clonal specificity of response in the MLR has been found in fetal spleen and thymus cells of the lamb (Asantila and Toivanen, 1976). The above fetal lymphoid cells are also capable of responding to xenogeneic cells. Neonatal mouse lymphocytes have also been shown to respond in the MLR (Howe, Goldstein and Battisto, 1970), and the mouse liver has been implicated as a source of mouse lymphoid cells (Owen, Cooper and Raff, 1974).

There are many reasons for considering the MLR an immunological reaction related to in vivo phenomena and not an in vitro artifact. Several of these reasons have been suggested by Wilson,

Howard and Nowell (1972) and are listed below. (1) The immunoproliferative behavior of lymphocytes in the MLR and the graft vs. host reaction is similar and these phenomena may represent different manifestations of the same reaction (Wilson and Elkins, 1969). (2) The stimulatory factors in the MLR are determined by the genes of the major histocompatibility loci in man (Bach and Amos, 1967), mouse (Rychlikova and Ivanyi, 1969) and rat (Silvers, Wilson and Palm, 1967; Wilson, 1967). (3) The proliferative activity in the MLR depends on the genetic and immunological status of the cell donors. Cells from immunologically tolerant animals do not respond in the MLR to leukocytes bearing the alloantigens to which the responder animal has been made tolerant, but will respond to third party cells (Virolainen, Hayri and Defendi, 1969; Wilson, Silvers and Nowell, 1967; Wilson and Nowell, 1971). The converse of this phenomenon that cells of a presensitized animal will respond more strongly to a second challenge by the same cells in the MLR is still a source of much controversy, however. (4) The MLR responder cells are thymus derived lymphocytes which are the same class of cells involved in cellular immunity in vivo (Johnston and Wilson, 1970). It is unlikely considering these and other similarities to in vivo immune reactions, which will be amplified later in this chapter, that the MLR is an in vitro artifact.

b) The genetics of the MLR

The major histocompatibility complex (MHC) in all vertebrates studied so far can be described as a genetic system composed of closely linked genes. The MHC controls alloantigens that are the

predominant determinants in allograft or transplantation rejection reactions (Dupont, Hansen and Yunis, 1976). MHC loci are also involved in a multitude of other related functions such as the control of the level of the immune response, disease susceptibility, determination of immune cell interactions (including the MLR), the control of the level of serum complement components, and possibly in the expression of developmental abnormalities (Bach, Bach and Sondel, 1976). In man the MHC is called the HLA system, in rat the AgB system, in mouse the H-2 system and in the rabbit the RL-A system.

HLA differences in man have been defined using two different techniques. The first method utilizes serological assays in which specific antisera are used to detect specific antigenic differences on lymphoid cell surfaces among allogeneic cells. These antisera became available in large quantities when it was discovered that pregnant humans developed high levels of anti-leukocyte antibodies specific for the allotypes contributed to the fetus by the father (van Rood, van Leeuen and Eernisse, 1959; van Rood, Eernisse and van Leeuwen, 1958; Bach and van Rood, 1976a). Extensive reviews on this subject have been published by Dupont, Hansen and Yunis (1976) and Bach and van Rood (1976a, 1976b, 1976c). Antigens identified by this method are called SD or serologically defined antigens. Using family studies the HLA genes have been divided into three loci which specify SD antigens (Dupont, Hansen and Yunis, 1976) and are designated HLA-A,

HLA-B and HLA-C. The existence of these three series of antigens has also been shown by the fact that the antibodies to the determinants for HLA-A, HLA-B and HLA-C cap separately on the lymphocyte membrane and thus the antigens specifying these three HLA groups are separate molecules (Bernoco, Cullen, Scudeller, Trinchieri and Ceppellini, 1973; Solheim, Ek, Thune, Baklein, Bratlie, Rankin, Thorensen and Thorsby, 1976). These SD antigens are present on all lymphoid cells and essentially the cells of all other tissues (Bach and van Rood, 1976a). SD antigens or antigens closely linked to SD antigens are now thought to be the targets of cytotoxic cells which specifically destroy other tissues (graft rejection), or other lymphocytes (cell mediated lympholysis - CML). This function in relation to the MLR will be discussed at length in section 'j' of this chapter.

The second method of measuring MHC differences is by the use of the MLR. As has been mentioned above the reactivity in the MLR is correlated with genetic dissimilarity. In 1967, Bach and Amos showed that the degree of blastogenesis in the MLR between leukocytes from family members corresponded to genetic dissimilarity measured between donor and recipient by the serological identification of leukocyte antigens. Later it was found that mixed lymphocyte reactivity did not always correlate with SD antigen typing (Yunis and Amos, 1971; Amos and Bach, 1968; Plate, Ward and Amos, 1970) as it was found that in some cases

of SD identity, an MLR between the leukocytes of the two individuals still occurred. These MLR determined differences became known as LD or lymphocyte derived differences (Bach, Bach, Sondel and Sundharadas, 1972). While this is a convenient way of expressing MHC differences it has not yet been shown that any antigens corresponding to the LD determined differences do in fact exist (Ling and Kay, 1975). An approach to practical LD typing has been evolved as follows. Theoretically if lymphocytes are obtained from individuals who are homozygous for the MLR locus (known as HLA-D) and these cells are used as stimulators in the MLR, then an individual who shares the same HLA-D allele either in homozygous or heterozygous form as on the homozygous stimulating cell will not respond in the MLR (Bach and van Rood, 1976b; Mempel, Grosse-Wilde, Bauman, Netzel, Steinbauer-Rosenthal, Scholz, Bertrans and Albert, 1973; Thorsby and Piazza, 1975; Dupont, Hansen and Yunis, 1976). By compiling data on MLRs between specific cell donors, the major LD genotypes were classified and this formed the basis for standardized LD typing procedures.

A new method for LD typing has recently been developed in man and mouse, which is based upon the ability of responder cells in the MLR to be restimulated in vitro, and on the specificity of this secondary response. This test is called the primed LD typing test (PLT). It was based on the finding in mice that cells stimulated in the MLR and left for 9 to 14 days in culture will give a very rapid and strong secondary proliferative response if exposed to the original stimulating cell donor (Hayry,

Andersson and Nordling, 1973; Hayry and Andersson, 1975; MacDonald, Engers, Cerottini and Brunner, 1974; Gorczynski and Rittenberg, 1975; Cerottini, Engers, MacDonald and Brunner, 1974; Wagner, Harris and Feldmann, 1972). This type of experiment has been repeated in man by Sheehy, Sondel, Bach, Wank and Bach (1975) and Zier and Bach (1975). It has been found that the LD determinants are primarily responsible for restimulation and that SD antigens are neither essential nor capable of causing a secondary response. It is apparent that cells sensitized by a given cluster of LD determinants will respond in a highly positive manner to cells carrying that same cluster of LD determinants. This strong secondary response indicates LD identity between the first and second set of stimulating cells, and thus permits the easy classification of LD determinants. An advantage of the PLT is that the secondary stimulation can be assayed with radioactive thymidine within 24 hours or even with radioactive uridine at earlier times making the identification of LD types much more rapid than by using the previously described technique (Bach and van Rood, 1976b; Sheehy, Sondel, Bach, Wank and Bach, 1975).

To define the MHC in the mouse the same approach (pre-PLT) to LD and SD typing as was applied to man was used with the added advantage that cogenic inbred strains of mice were available which were genetically identical except in the H-2 loci. Consequently much more extensive studies of the mouse MHC than the human MHC have been possible (Allen, 1955; Amos, Gorer and Mikulsa, 1955; Pizzaro, Hoecker, Rubinstein and Ramos, 1961;

Stimpfling and Richardson, 1965; Shreffler, Amos and Mark, 1966; Stimpfling and Reichert, 1970; Klein, Klein and Shreffler, 1970; Stimpfling, Reichert and Hudson, 1971; Shreffler and David, 1972; Demant, 1973).

The mouse H-2 complex has been divided into five closely linked regions which are referred to, in order of topographical location on the gene, as K, I, S, C and D. The K and D regions control the phenotype of the serologically defined antigens (SD), and the I region controls the phenotypes that are involved in the strongest lymphocyte determined (MLR) responses (Widmer, Peck and Bach, 1973; Demant, 1973; Festenstein and Demant, 1974; Bach, 1976; Bach and van Rood, 1976b; Bach, Bach and Sondel, 1976). There are certain serologically determined antigens found only on restricted cell types (B cells but not T cells) which seem to behave to some extent in an identical fashion to LD determinants. These antigens are designated Ia and are determined by the I region of the H-2 complex (Bach, Bach and Sondel, 1976). On the basis of studying Ia determinants the I region of the H-2 complex can be subdivided into three: I-A; I-B; I-C. In the mouse but not in man there is another less clearly defined locus designated M which is not linked to H-2 but which can stimulate an MLR proliferative response (Festenstein, 1966; Festenstein, Abbasi, Sachs and Oliver, 1972; Singal, Mickey and Teresaki, 1969; Festenstein, 1973; Festenstein and Demant, 1974; Lilliehook, Jacobsson and Blomgren, 1976; Bach and van Rood, 1976b; Bach, Bach and Sondel, 1976).

There are also genetic loci in the mouse which influence the degree of response of the cells in the MLR and other immune responses, these are called immune response genes. The immune response genes controlling the reactions to D-end determinants (SD; allograft rejection) on the stimulating cells map outside the H-2 region (Plate, 1973). The immune response genes controlling the reactions to the I and M determinants (LD; MLR) on the stimulating cells map inside the H-2 region in the I region (Festenstein and Demant, 1974; Demant, 1973; Ling and Kay, 1975; Bach, Bach and Sondel, 1976; Feldmann, 1973; Benacerraf and Dorf, 1974). Immune response genes have also been observed in man.

In the rat it has been clearly shown that LD loci are located within the MHC (Silvers, Wilson and Palm, 1967; Sorensen, 1971). However mapping of the rat MHC has not been as extensive as that in the mouse.

In the rabbit, Tissot and Cohen (1974) first showed that the MLR is linked to the RL-A loci. After typing rabbits for the different SD antigens it was found that animals with identical SD antigens do not respond to each other when their leukocytes are cultured together in the MLR. The data of Tissot and Cohen (1974) also suggest that immune response genes in the rabbit are closely linked or are a part of the RL-A locus. Cohen and Tissot (1974) and Chai and Lerner (1975) have shown that there is a link between SD antigens, LD determinants and skin graft survival in the rabbit although no investigation of the mechanism involved in allograft rejection in the rabbit was attempted.

c) The MLR responder cell

The MLR is a general phenomenon that occurs with allogeneic mixtures of lymphocytes from many and possibly all animal species, and can be mediated with lymphocytes from most of the different lymphoid tissues although there is some species variation (Sorensen, 1972; Ozer, Jr. and Waksman, 1974). The reaction requires direct cell-cell interaction as no reaction takes place when the two cell populations are separated by a Millipore membrane (Chapman and Dutton, 1965). An unusual aspect of the MLR response is that a very high proportion of the cells are initially stimulated to divide (Cunningham, 1975). Wilson, Blyth and Nowell (1968) suggested that 1-2% of the starting population may be activated. This is 10^2 to 10^4 times more than are stimulated by most other kinds of antigen (Cunningham, 1974; Cunningham, 1975). Other experiments have shown that the initial number of cells responding could be as high as 12% (Wilson, Howard and Nowell, 1972), or 20% (Ford, and Atkins, 1972).

The sequence of morphological changes in lymphocytes activated in the MLR is similar to that seen in cultures of lymphocytes activated by mitogens such as phytohemagglutinin (PHA) or concanavalin-A (Con-A). In contrast to mitogen stimulated cells, fewer lymphoblasts are seen in the MLR and the peak of activity is later usually between 5 and 7 days rather than at 3 days (Ling and Kay, 1975; Alling and Kahan, 1969; Wilson, 1967). Most MLR studies using human cells have involved white blood cells (WBC) although

the cells of other organs including thymus are known to respond (Ling and Kay, 1975; Han, Minowada, Subramaman and Sinks, 1976). Most studies with other animals have utilized spleen, lymph node or thymus cells. Thymus cells of mice (Berman, Puryear and Argyris, 1976; von Boehmer and Byrd, 1972; Blomgren and Svedmyr, 1971), of rat (Knight and Thorbecke, 1971), and of man (Schwartz, 1967; Schwartz, 1966) respond well to allogeneic stimulation. In the rabbit, however, thymus cells respond very poorly if at all (Chapman and Dutton, 1965; Ozer, Jr. and Waksman, 1974). Furthermore, the response of WBC in the rabbit MLR is inconsistent in the intensity of the reaction compared to the well defined response of spleen or lymph node cells (Knight, Walker and Ling, 1971; Ozer, Jr. and Waksman, 1974; Ling and Kay, 1975; Sheppard, Jr., Sell, Poler and Redelman, 1977). The response in the MLR is dependent upon the responder and stimulator cell concentrations (Bach and Voynow, 1966; Adler, Takiguchi, Marsh and Smith, 1970; Blomgren and Svedmyr, 1971).

It is now generally believed that the responder cell in the MLR is a T cell rather than a B or null cell in all animal species studied so far (Ling and Kay, 1975; Bach, Bach and Sondel, 1976). T cells are defined in many ways. In the mouse they possess the theta alloantigen and can be lysed using anti-theta serum and complement (Raff, 1971). T cells also lack cell surface immunoglobulin relative to B cells (Raff, 1970). Human T cells form spontaneous rosettes with sheep RBC. T cells compared to B cells

have a slightly longer generation time in vivo (Sprent and Miller, 1972), are slightly larger (Howard, Hunt and Gowans, 1972), more dense and less adherent (Bianco, Patrick and Nussenzweig, 1970) and more negatively charged (Wioland, Sabulovic and Burg, 1972; Nordling, Andersson and Hayry, 1972). Only T cells are able to be stimulated by the mitogens PHA and Con-A (Greaves and Janossy, 1972) although this has been questioned by Chess, MacDermott, Sondel and Schlossman (1974) who found that PHA, Con-A and pokeweed mitogen stimulated T, B, null and unfractionated cells. Null cells were those cells having no surface immunoglobulin, were not able to form E-rosettes, but did form EAC-rosettes. It was noted, however, that the response of B and null cells to mitogens was not as great as that of T cells.

In children with a congenital absence of the thymus there is also a lack of MLR responder cells (Ling and Kay, 1975). This was one of the first indications that T cells were necessary for the MLR response. Mouse lymphocyte populations depleted of T cells using anti-theta serum and complement do not respond in the MLR (Mosier and Cantor, 1971; Tyan and Ness, 1972). Lymphocytes from neonatally thymectomized animals such as rats (Knight, Newey and Ling, 1973; Rieke, 1966; Wilson, Silvers and Nowell, 1967) or chickens (Alm and Peterson, 1970) respond weakly or not at all in the MLR. Chickens without B cells (bursectomized) but with a normal complement of T cells still respond well in the MLR (Alm and Peterson, 1970), indicating that B cells are not required for the response to stimulator cells in the MLR. Further studies in

this area were carried out using immunodeficient animals reconstituted with karyotypically marked thymus and bone marrow grafts. Johnston and Wilson (1970) using thymectomized, X-irradiated rats reconstituted with syngeneic bone marrow and thymus cells with different sex chromosome markers showed that in a unidirectional MLR response to F_1 cells, 90% of the responding cells bore the sex chromosome marker of the thymus cell donor. A similar response was shown with mice (Festenstein, Davies, Leuchars, Wallis and Doehoff, 1969). Nude mice which lack a thymus and have only a few theta positive cells (Raff and Wortis, 1970) do, however, display a weak MLR response (Wagner, 1972). Piguet and Vassalli (1972) prepared T/B radiation chimeras between CBA/Ca and CBA/T_6T_6 mice by transplantation of thymus and bone marrow cells that had been treated with anti-thymus serum and complement. When spleen cells from these chimeras were stimulated in the one-way MLR with F_1 (C57BL/6 x CBA/H-T_6T_6) spleen cells, most mitoses on days 2 and 3 of culture had the karyotype of the thymus graft, but later generations of dividing cells had the karyotype of the bone marrow graft. The authors interpreted these results as indicating that T cells recruited B cells to respond in the MLR. This idea was refuted by Andersson, Nordling and Hayry (1973) who using electrophoretically fractionated mouse T and B cells with distinct sex chromosome markers showed that no more than 5% of the dividing cells had the B cell karyotype when equal numbers of B and T cells were stimulated in the one-way MLR. More recently studies using rabbit WBC have shown that anti-thymus cell serum plus complement will eliminate the ability of

these cells to respond in the one-way MLR (Sheppard, Jr., Sell, Poler and Redelman, 1977). However, the MLR responder cell may not be a T cell as the anti-thymus serum used was absorbed only with rabbit RBC and not with rabbit bone marrow and appendix cells which also carry unique antigens (Colas de la Noue and Richter, 1974; Chou, Cinader and Dubiski, 1977).

Sondel, Chess, MacDermott and Schlossman (1975) observed that human cells retained on columns coated with rabbit anti-human Fab fragments (B cells) did not respond in the MLR. Cell fractionation experiments indicate that the responder cell in the MLR is a T cell. Using rosetting techniques it has been shown that human WBC enriched for T cells (E rosette forming cells) exhibited an enhanced MLR response compared to normal unfractionated cells (Blomgren, 1977; Potter and Moore, 1977; Han and Dadely, 1976; Takasugi, Kiuchi and Opelz, 1977). T cell enriched populations prepared by nylon fibre column absorbtion also responded in an enhanced manner in the MLR (Potter and Moore, 1977). Sephadex columns conjugated with purified rabbit anti-human Fab have been used to separate immunoglobulin and non-immunoglobulin bearing cells, followed by rosetting to produce three purified cell fractions: B cells, which were bound to the Fab columns and also formed EAC rosettes; T cells, which were not bound to the Fab columns and formed E rosettes; null cells, which were not bound to the Fab columns but did form EAC rosettes. Only the T cells responded in the MLR (Chess, MacDermott, Sondel and Schlossman, 1974; MacDermott, Chess and Schlossman, 1975).

It is now well known that during the MLR, cells are generated which are cytotoxic to the stimulator cells (Bach, Bach and Sondel, 1976; Bach, 1976). This cytotoxic cell response will be discussed in section 'j'. Cells responding proliferatively in the MLR have been shown not to be precursors of these MLR generated cytotoxic cells. If lymphocytes that are to be used as responders in a one-way MLR are incubated with a monolayer of cells autologous or syngeneic to the stimulating lymphocytes it is found that no cytotoxic cells are generated in the subsequent MLR. This absorbtion of cells removes only the precursors of cytotoxic cells specific for stimulator cells that are of the same genotype as the absorbing monolayer (Bach, 1976; Wagner, Rollinghoff and Nossal, 1973; Zier and Bach, 1976; Bach, Bach and Sondel, 1976; Bach, Zier and Sondel, 1973). The proliferative (MLR responding) cells are not absorbed.

Hayry, Andersson and Roberts (1976) have shown that MLR responsive cells are not the same cells that respond to PHA or Con-A. In their experiments mouse spleen cells were stimulated by either PHA or Con-A and the resultant blast cells were removed by velocity sedimentation. The remaining non-blast cells did not respond to the mitogens (PHA or Con-A) but did respond normally in the MLR.

Studies using antisera plus complement are now being used to differentiate between different subpopulations of T cells. In the human an antiserum has been developed which in the presence of complement eliminates MLR responsive cells but not those T cells which respond to soluble antigens by blastogenesis as indicated by

thymidine incorporation (Evans, Breard, Lazarus, Schlossman and Chess, 1977). In the mouse the peripheral T cells can be subclassified on the basis of differential expression of cell surface antigens belonging to three Ly systems, Ly-1, Ly-2 and Ly-3 (Cantor and Boyse, 1975a). These antigens are found exclusively on T cells. One subclass bearing all three types of these Ly determinants (Ly-1,2,3$^+$), appears early in neonatal life and is selectively depleted shortly after adult thymectomy. Two other subclasses Ly-1$^+$ and Ly-2,3$^+$ develop later in life and are unaffected shortly after adult thymectomy. Both Ly-1$^+$ and Ly-2,3$^+$ cells respond in the MLR by increased thymidine incorporation, however only Ly-2,3$^+$ cells develop killer cell activity (Kisielow, Hirst, Shiku, Beverley, Hoffman, Boyse and Oettgen, 1975; Cantor and Boyse, 1975b; Cantor and Boyse, 1976).

d) T cell memory in the MLR

Following six to seven days culture with allogeneic cells, mouse lymphocytes revert to small non-dividing cells with loss of cytotoxic activity against the primary stimulating cells (Dupont, Hansen and Yunis, 1976; Andersson and Hayry, 1973; Andersson and Hayry, 1974). When primed mouse cells are rechallenged between 14 and 21 days of culture with fresh stimulating cells identical to those used initially, the rate of the MLR proliferation is accelerated compared to the initial response (Hayry and Andersson, 1973; Hayry and Andersson, 1975; MacDonald, Engers, Cerottini and Brunner, 1974; Wagner, Harris and Feldman, 1972; Fathman, Collavo, Davies and Nabholz, 1977). This is referred to as a secondary MLR response.

Similarly human lymphoid cells also give an accelerated proliferative response after in vitro priming and restimulation on day 14 of culture and this response is of greater magnitude than the primary response (Fradelizi and Dausset, 1975; Sheehy, Sondel, Bach, Wank and Bach, 1975; Zier and Bach, 1975; Singal, 1977). This secondary response will occur against a new stimulating cell only if that cell donor shares an HLA-D (LD) determinant with the donor of the stimulator cell in the primary culture. This is the basis for the PLT test for LD phenotypes, as mentioned previously (Bach, 1976).

These MLR primed lymphocytes can be maintained for at least three or four cycles of rest and proliferation (MacDonald, Engers, Cerottini and Brunner, 1974; Hayry and Andersson, 1975) and thirty-two consecutive cycles have recently been reported (Hayry, Andersson and Roberts, 1976). The cells which respond in the secondary MLR, when isolated by velocity sedimentation, do not respond to the T cell mitogens PHA or Con-A (Hayry and Andersson, 1973; Hayry, Andersson and Roberts, 1976). These MLR primed cells are also relatively long-lived and may be detected for up to twenty weeks after transfer in syngeneic T cell deprived animals (Hayry, Andersson and Roberts, 1976). Gorzynski and Rittenberg (1975) have shown that mouse lymphocytes responding during the secondary MLR have an increased activity for the target LD antigens and also acquire immunoglobulin-like molecules on their surfaces. Lymphocytes primed in the MLR will respond not only to cell bound LD antigens

but also to cell free membrane antigens (LD) which cannot stimulate in the primary MLR (Hayry and Andersson, 1976).

e) MLR stimulator cells

The identity of MLR stimulating cells has not been as firmly established as the identity of the MLR responder cells. Many investigators have suggested that B cells are the most potent stimulators (Lohrmann, Novikovs and Graw, Jr., 1974; Han and Dadely, 1976; van Oers and Zeijlemaker, 1977; Potter and Moore, 1977; Blomgren, 1977; Terasaki, Park, Opelz, Saito and Mickey, 1977). However, others have indicated that B and T cells stimulate equally well (von Boehmer, 1974; Chess, MacDermott, Sondel and Schlossman, 1974). Null cells also seem to be at least as stimulatory as T cells (Chess, MacDermott, Sondel and Schlossman, 1974). It has been found in rabbits that if a population of cells to be used as stimulators in the MLR are first activated by mitogens and then X-irradiated to prevent DNA synthesis, they become much more stimulatory in the MLR than are normal unstimulated cells or normal unstimulated X-irradiated cells (Lowe, 1971; Knight, Hardy and Ling, 1970; Ling and Kay, 1975). The authors concluded that enhanced metabolic activity was responsible for the enhanced stimulation. This enhanced metabolic activity per se is probably not exclusively responsible for the enhanced MLR stimulation as gluteraldehyde-treated mitogen-stimulated cells are also highly stimulatory (Lightbody, Brown and Kong, 1976; Lightbody and Kong, 1976). These cells can be stained with the dye eosin (indicating cell death), and all incorporation of protein precursors, and presumably protein

synthesis has stopped after 24 hours. These cells were, nevertheless, still quite capable of full stimulatory activity in the MLR even after being stored for four weeks at 4°C (Lightbody and Kong, 1976). The stimulatory ability of these cells is related directly to the genetic disparity between them and the responder cells. These cells obviously do not exhibit more metabolic activity than normal, viable, unstimulated cells. However it is possible that during blastogenesis before gluteraldehyde fixation, the stimulatory antigens are either presented in a more optimal manner or that cryptic stimulatory antigens have become exposed, thus increasing the number of stimulating antigens reacting with responder cells. It should be noted that mitogen activated cells will induce some stimulation of autologous responder cells in the MLR, even though these stimulator cells are inactivated by gluteraldehyde or by X-irradiation (Lightbody and Kong, 1976; Ling, Hardy and Steele, 1974).

Cells other than the normal lymphoid variety have been used as MLR stimulator cells. Alter and Bach (1970), Rode and Gordon (1974) and Dyminiski and Argyris (1971) suggested that monocytes could induce an MLR by stimulating responder lymphoid cells. A number of cell types freshly obtained from normal tissues or tumors have been tested as stimulator cells using lymphocytes of normal individuals as responders. Epidermal cells prepared from trypsin-dispersed human skin stimulate human and rat lymphocytes (Ling and Kay, 1975). Other tumor cells such as breast tumor cells, HeLa cells, KB cells, melanoma cells, and oesteogenic sarcoma cells are

inactive as stimulators in the MLR (Han, 1972). Lymphoblast cell lines have been shown to be highly stimulatory in the MLR (Callewaert, Kaplan, Peterson, Jr. and Lightbody, 1975; Ling, Hardy and Steele, 1974; Berstein, Wright and Cohen, 1976; Boyer and Fahey, 1976). Autologous cells modified by chemical groups (Shearer, Lozner, Rehn and Schmitt-Verhulst, 1975), and even autologous mouse spleen cells (Howe, Goldstein and Battisto, 1970; von Boehmer and Byrd, 1972; von Boehmer, 1974) and certain human peripheral blood cells (B, Null, and B plus Null cells; Takasugi, Kiuchi and Opelz, 1977) have all been found to be stimulatory in the MLR. Fibroblasts grown from human skin (cultured cells) do not stimulate in the MLR even though they have HLA antigens on their surfaces (Schellekens and Eijsvoogel, 1970). It has been suggested that the ability or inability of a cell to stimulate in the MLR is a reflection of the pattern of distribution of HLA antigens on the cell surface. It has been suggested that, even though the total number of HLA antigens may be the same on the lymphocyte and fibroblast, the HLA antigens on the fibroblast (LD) may be widely dispersed while those on the lymphocyte may be concentrated in one area and could therefore be much more immunogenic with respect to the responding lymphocytes (Ling and Kay, 1975).

f) Suppressor cells and the MLR

Many instances of suppressor cell activity induced in vivo and in vitro have been observed in respect to immune reactions. Whether all these suppressor cell activities are different

manifestations of the same basic phenomenon has not yet been clearly determined. Therefore several examples of suppression of humoral and cell mediated immunity are included under the above heading in the hope that they bear some relationship to suppression of the MLR.

From the point of view of natural suppression of the normal immune response it is interesting to note that the lymphocytes of healthy human newborns inhibit the mitogenic response of the maternal lymphocytes induced by PHA in vitro. This reaction like almost all known non-cytotoxic suppressor activities is non-specific in that these cells will also suppress the mitogenic response of unrelated adult WBC (Olding and Oldstone, 1974). A pathological lack of suppressor cells occurs in patients suffering from the autoimmune disease idiopathic systemic lupus erythematosus. In this disease T cell suppressors of antibody synthesis are absent when compared to healthy normals (Abdou, Sagawa, Pascual, Hebert and Sadeghee, 1976).

Specific populations of thymus derived (T cells) lymphocytes have been reported to actively suppress almost all T cell functions including the MLR, and also some B cell functions. In some cases specific cytotoxic activity has been inadvertently mistaken for suppression (Fitch, Engers, Cerottini and Brunner, 1976). True suppressive activity is usually attributed to non-cytotoxic mechanisms.

The induction of a T-cell dependent antibody response can be inhibited by Con-A activated spleen cells (Peavy and Pierce, 1974). B cell proliferation in culture is also suppressed by T cells activated by Con-A or PHA in mice (Piguet, Dewey and Vassalli, 1976) and in rabbits (Shek, Chou, Dubiski and Cinader, 1975; Redelman, Scott, Sheppard and Sell, 1976; Bona, Cinader and Dubiski, 1977). Con-A can also induce in culture, cells, that will suppress the human MLR (Hallgren and Yunis, 1977). Heat killed macrophages induce suppression of primary antibody formation in vitro in human cell cultures (Gershon, Eardley and Ptak, 1976) and also in the primary MLR (Gershon, Eardley, Naidorf and Ptak, 1976). This activity is attributed to absorbtion by the macrophage membrane fragments of necessary 'interaction factors' which help or are required for the generation of immune T-cells. Once the immune T-cells are generated no suppression by heat killed macrophages will occur, for example during a secondary MLR. The suppression by macrophage fragments is not an active process and unlike other suppressive processes is not mediated by T-cells.

Other investigators have shown that rabbit spleen cells pre-sensitized in vivo to sRBC are themselves inhibitory during the secondary induction of antibodies to sRBC in vitro (Luzzati and Lafleur, 1976). This inhibition could not be attributed to granulocytes, platelets or erythrocytes. Rabbit appendix cells have been found to suppress the secondary in vitro IgG response of presensitized spleen cells to various antigens (Kamin, Henry

and Fudenburg, 1974). Folch and Waksman (1974) have found that an adherent T-cell has a suppressive effect on the rat MLR. Hodes and Hathcock (1976a; 1976b) have found that nylon wool adherent spleen cells which can be lysed by anti-theta serum plus complement, if cultured alone for 3 to 7 days, can suppress the MLR reaction between fresh spleen cells and allogeneic spleen cells.

Soluble MLR suppressor activity has been found in the mouse. This system requires in vivo priming of an animal followed by in vitro stimulation of lymphoid cells by the same alloantigens. Supernatants of these cultures are suppressive to the mouse MLR but are not cytotoxic (Rich and Rich, 1975; Rich and Rich, 1976; Rich, Chu and Rich, 1977). Nadler and Hodes (1977) have recently compared the MLR suppressive activity induced in vivo (soluble suppressor) with suppressor activity that can be induced in spleen cells by culturing them alone for several days. The latter cultures were found not to exhibit any soluble suppressor activity and the suppressor cells were inactivated by mitomycin-C treatment. It was not determined whether these two populations of suppressor cells were identical. Miller and Phillips (1976) have also investigated a system in which suppressor cells are induced in vivo by injection of mice with semi-allogeneic lymphocytes. The resultant suppressor cells will inhibit the MLR in vitro. Hirano and Nordin (1976a; 1976b) describe a system in mice in which spleen cells taken from a 2 to 3 day MLR culture are suppressive in a fresh MLR if the stimulator cell is of the same H-2 type as used to induce the suppressor cell. This activity occurs even when the suppressor

cells are separated from the MLR culture by a semipermeable membrane, suggesting that the suppression is mediated by a soluble factor. The same system has been found to be operative with human cells (Hirschberg and Thorsby, 1977). Sondel, Jacobson and Bach (1977) using the same protocol (human cells) but with heat killed stimulating cells which will not induce the proliferative MLR (Schellekens and Eijsvogel, 1970) found that MLR suppressor cells are still induced. They conclude that proliferation is not required for the induction of suppressor cells and that no suppressive cytotoxic activity is present in their cultures as primary generation of cytotoxic cells requires initial proliferative activity.

It can be seen that suppressor cells can be induced in several different ways and that their mode of action may vary in each case. Whether these different cell suppressor activities which are characterized (it seems in any random combination) by i) adherence, ii) in vivo induction, iii) in vitro induction, iv) induction in tissue culture in the absence of any other cells, v) requirement to be restimulated in the culture to mediate suppression, vi) production of soluble activity, are in fact the properties of the same cells remains to be seen. Some advance in the classification of suppressor lymphocytes has been made in mice. It is now clear that all suppressor T cells studied in mice as defined by the Ly and antisera typing are Ly-2,3$^+$ while helper T cells are Ly-1$^+$. The precursor of both cell types are Ly-1,2,3$^+$ (Gershon, Eardley, Naidorf and Ptak, 1976b; Cantor and Boyse, 1976; Beverley, Feldmann, Dunkley and McKenzie, 1977).

g) Other factors influencing the MLR

Although helper T cells play a large part in the humoral immune response little evidence has been found for their involvement in the MLR response (Gershon, Eardley, Naidorf and Ptak, 1976). There has only been one example that a lymphoid cell acts as a helper in the MLR. Dyminski and Smith (1975) observed that an immunoglobulin-bearing cell could activate an MLR between mouse cortisone resistant allogeneic thymus cells. This helper cell activity, however, can be replaced by a critical concentration of IgG (Dyminski and Smith, 1977).

The MLR responder cell is itself considered a helper T cell in the sensitization of cytotoxic cell in vitro (Bach and van Rood, 1976a; Bach, Bach and Sondel, 1976). The MLR responder cell is classified in the murine system in the same manner as helper T cells, Ly-1$^+$ (Cantor and Boyse, 1976; Gershon, Eardley, Naidorf and Ptak, 1976b).

Some inhibition of the MLR is mediated by granulocytes, as the removal of this cell population using ficoll-hypaque gradients enhances the human MLR response (Bain and Pshyke, 1972; Mardiney, Block and Chess, 1972; Ling and Kay, 1975). Ragab and Cowan (1973) used velocity sedimentation to separate granulocytes from lymphocytes and found that granulocytes inhibit the MLR. Bach, Bach, Widmer, Oranen and Wolberg (1971) suggested after an extensive study of the blood of healthy university students and hospital patients that granulocytes were responsible for inhibition of the MLR.

Sheppard, Jr., Sell, Poler and Redelman (1977) found that rabbit WBC isolated from heparinized blood, which contain many granulocytes, do not respond as well in the MLR as WBC isolated from defibrinated blood which contains very few granulocytes. This has been confirmed by Linthicum and Sell (1977).

Macrophages also seem to have an effect on the MLR as noted in the section on suppressor cells. In the rabbit, Chapman and Dutton (1965) have found that alveolar macrophages inhibit the MLR. However the number of alveolar macrophages required to inhibit the response was far in excess of the total number of monocytes present in preparations of rabbit peripheral blood. This would imply that the monocytes in normal rabbit WBC do not inhibit the MLR. Wilson (1967) found that macrophages have no effect on the rat MLR. In contrast to these findings it has been shown in man that the macrophage will enhance the MLR (Blomgren, 1977; Alter and Bach, 1970; Hersh and Harris, 1968). Rode and Gordon (1969) found that inactivation of macrophages with high concentrations of sucrose inhibited the MLR. The MLR activity could be restored to these cultures by the addition of irradiated leukocytes which normally did not respond in the MLR. The irradiated leukocytes were considered as a good source of macrophages. Rode and Gordon (1974) had found that macrophages allogeneic to MLR responder cells which had been purified on cotton wool columns (macrophages removed) restored MLR activity to these cells. Neutrophils, on the other hand had no effect. Bach, Alter, Zoschke and Bach (1971) found that MLR responsiveness of macrophage-depleted leukocytes could be

restored in 19 out of 21 cases by the addition of macrophages or supernatants from macrophage cultures to the MLR.

Kasakura and Lowenstein (1965b) have shown that supernatants from MLRs are stimulatory for normal lymphocytes, and it has been suggested that such blastogenic factors may in some way be involved in the stimulation of responding cells in the MLR (von Boehmer, 1974; Croy and Osoba, 1974; Kasakura, 1975; Kennedy and Ekpaha-Mensah, 1973). Kasakura (1977) has recently shown that allogeneic B cells can stimulate T cells to release a blastogenic factor which is mitogenic to both B and T cells. This data can explain the phenomenon of 'back stimulation' where it has been proposed that T cells in the inactivated 'stimulator' population can release a mitogenic agent that will cause B and T cells in the responder population to incorporate thymidine. This would explain the genetically improbable proliferation of F_1 cells in the one-way MLR to parental cells (Kennedy and Ekpaha-Mensah, 1973; Adler, Takaguchi, Marsh and Smith, 1970).

Alloantisera to surface HLA antigens of stimulator cells completely inhibits the MLR (Ceppellini, Bonnard, Coppo, Maggiano, Pespisil, Curtoni and Pellegrino, 1971). This is also true of the rat (Milton, Mowbray, Ruskiewicz and Carpenter, 1973). However, this is not the case with cultured lymphoid cell lines. Anti-HLA allosera which inhibits the stimulatory ability of normal lymphocytes will not inhibit the stimulating ability of lymphoblastoid cells from cell lines even though there are more HLA sites on the cells of the lymphoid cell lines. The author concluded that HLA antigens do not directly participate in lymphocyte activation

(Corley, Dawson and Amos, 1976). Antiserum to specific responder cell surface antigens does not block the MLR.

Heparin, a carbohydrate, has been reported to inhibit the MLR (Currie, 1967), but this has not been confirmed by others (Ling and Key, 1975). Sheppard, Jr., Sell, Poler and Redelman (1977) have noted that rabbit WBC prepared using heparin seem to be poor responders and good stimulators in the MLR compared to WBC isolated by defibrination. Both procedures were followed by gelatin sedimentation to remove the majority of RBC. They found that WBC isolated by defibrination contained 95% lymphocytes compared to those isolated using heparin (50% lymphocytes). This finding has been confirmed by Linthicum and Sell (1977). The explanation for the difference in response between defibrinated and heparinized preparations of rabbit WBC in the MLR is likely due to the difference in granulocyte concentration in each preparation, rather than as a direct result of heparin toxicity in cell culture.

Low concentrations of thiols such as 2-mercaptoethanol, dithiothreitol and cysteamine phosphate increase activity in the MLR in the mouse (Harris, MacDonald, Engers, Fitch and Cerottini, 1976). Thiols also increase the activity in other immune responses. In the presence of these chemicals the number of antibody forming cells generated upon exposure of mouse spleen cells to sRBC is greatly increased (Click, Benck and Alter, 1972), the response of non-adherent (macrophage depleted) cells is restored (Chen and Hirsch, 1972a; Chen and Hirsch, 1976b), and the proliferative response to mitogens

is increased (Betel, van den Berg, Martijnse and van den Berg, 1974; Fanger, Hart, Wells and Nisonoff, 1970). Many others have found 2-ME to enhance the MLR (Soulillou, Carpenter, Lundin and Strom, 1975; Herber-Katz and Click, 1972; Lafferty, Ryan and Misko, 1972). The action of 2-ME is on a T cell, however, the mode of action of the thiol is unknown (Harris, MacDonald, Engers, Fitch and Cerottini, 1976). It has also been found that a synergistic effect occurs when 2-ME is mixed with a synthetic poly nucleotide (poly AU) so that the enhanced response in the murine MLR is greater than when 2-ME alone is added to cultures (Graziano and Mardiney, Jr., 1976).

An enhanced response in the one-way MLR is seen if the responder cells are preincubated with cholinergic agents (acetylcholine or carbamylcholine) but not if the drug is left in the culture (Han, 1976). It is also interesting to note that those agents which elevate the intracellular levels of cyclic GMP enhance the blastogenic response to PHA (Hadden, Hadden, Meetz, Good, Haddox and Goldberg, 1973).

h) Generation of cytotoxicity in the MLR

Following stimulation by normal allogeneic cells or tumor cells lymphocytes proliferate (MLR) and generally acquire a capacity for specific cytotoxicity against the stimulating cells (Cerottini and Brunner, 1974). Some degree of proliferation is required before cytotoxic cells can be detected (Dupont, Hansen

and Yunis, 1976). This cell-mediated cytotoxicity is independent of antibody or complement. Cytotoxic cells are usually only produced when the responder and stimulator cells differ from each other in the histocompatibility complex.

The first report of in vitro generated cytotoxicity was by Hirschorn, Firschein and Bach (1965) who showed that human leukocytes cultured with allogeneic fibroblasts developed a cytotoxic potential towards these cells. Hayry and Defendi (1970) used a mouse one-way MLR to sensitize lymphocytes to allogeneic lymphocytes which were then capable of lysing cell lines syngeneic to the stimulating cells. Target cell death was measured by the release of ^{51}Cr from the target cell. Hardy, Wallin and Ling (1970) performed the same experiment using human cells. Although the cytotoxic effect was shown to be maximal against target cells syngeneic to the original stimulating cells, some cross-reactivity did occur using different lymphoid cell lines as targets (Solliday and Bach, 1970).

Lightbody, Bernoco, Miggiano, and Ceppellini (1971) and Miggiano, Bernoco, Lightbody, Trinchieri and Ceppellini (1972) introduced an inovation that made possible the use of normal lymphocytes,which are normally not lysed efficiently, as target cells. Normal lymphocytes treated with PHA produce blast cells that function as targets just as well as lymphoid cell lines in the ^{51}Cr release assay. This test in which cytotoxic cells generated in vitro in the MLR induce the lysis of specific target cells (PHA blasts), is called cell mediated lympholysis (CML).

Despite the finding that the LD antigens are primarily responsible for activating the proliferative response in the MLR and that cytotoxic lymphocytes are generated in the MLR, the target antigens primarily recognized by cytotoxic lymphocytes are not LD but SD antigens, or phenotypic products of genes very closely linked to SD antigens (Bach, Bach and Sondel, 1976; Bach and von Rood, 1976b; Dupont, Hansen and Yunis, 1976; Eijsvoogel, Schellekens, du Bois and Zeijlemaker, 1976). More recently the target antigens have been referred to as CD (cytotoxically defined) antigens as several differences have been noted between SD antigens and those antigens that act as targets for cytotoxic cells (Bach, Kuperman, Sollinger, Zarling, Sondel, Alter and Bach, 1977; Bach, Bach, Kuperman, Sollinger and Sondel, 1976). The LD-SD collaboration has been confirmed using a three cell system in the MLR in the case of both mouse and human cells. Initially, responder cells are cultured with stimulator cells that have identical SD but different LD antigens. In this case a good MLR proliferative response occurs but no cytotoxicity towards the target cell is generated. A third cell population is then introduced to the culture with identical LD but different SD antigens to the responder cell. The responder cells will not respond in an MLR to this third cell type. Cytotoxicity towards the third cell type however is produced in this third cell culture (Schendel, Alter and Bach, 1973; Eijsvoogel, du Bois, Meinesz, Bierhorst-Eijlander, Zeijlemaker and Schellekens, 1973; Bonnard, Chappuis, Glauser, Mempel, Baumann, Grosse-Wilde and Albert, 1973; Mawas, Sasportes, Christen, Bernard, Dausset, Alter and Bach, 1973). Even M-locus generated MLRs (in which no cytotoxi-

city to the cell bearing the allogeneic M locus is produced) can produce cytotoxic cells in the responder population to a third cell type (Wagner, Gotze and Rollinghoff, 1976). This means that the MLR activating determinant (LD) and the antigens to which the subsequent destruction is directed (SD) do not necessarily have to be present on the same stimulating cell.

The cell responsible for cytotoxicity is a T-cell as it is inactivated by anti-theta serum plus complement and is a T rosetting cell (Goldstein, Blomgren, Svedmyr and Wigzell, 1973; Goldstein, Wigzell, Blomgren and Svedmyr, 1972; Hayry and Andersson, 1973; Bach, Bach and Sondel, 1976; Sondel, Chess, MacDermott and Schlossman, 1975; MacDermott, Chess and Schlossman, 1975). The cytotoxic cell or its proliferative helper are clonally derived as the cytotoxic response to a specific set of alloantigens (LD and SD) can be eliminated without affecting the cytotoxic response to another set of alloantigens (Peavy and Pierce, 1976).

The cytotoxic T-cell and the MLR proliferative T-cell are not the same cell. Cytotoxic cells induced by the MLR or their precursors can be removed from a T-cell population by absorbtion with a monolayer of allogeneic cells either sygeneic or autologous to the MLR stimulator cells. This does not absorb cells capable of a proliferative response to cells syngeneic to the absorbing monolayer (Bach, Bach and Sondel, 1976; Zier and Bach, 1976). Furthermore, studies of Ly antigens in the mouse have shown that the proliferative cell

is Ly-1$^+$ while the cytotoxic cell is Ly-2,3$^+$ (Cantor and Boyse, 1976). It has consequently been shown using genetically different mice that Ly-1$^+$ cells do not differentiate into the Ly-2,3$^+$ cytotoxic cells in the MLR (Cantor and Boyse, 1975b). These studies have led Bach, Bach and Sondel (1976) to propose a model in which the MLR responding (proliferating) cell is a helper cell in the generation of cytotoxic activity in vitro and in vivo.

The cytotoxic activity generated in one-way MLR towards the MLR stimulating cells disappears in 7 to 10 days. Upon subsequent exposure of this culture to the same cells used to stimulate initially, there is a response in which blastogenesis and generation of cytotoxic activity occurs much more rapidly in comparison to the initial response (MacDonald, Engers, Cerottini and Brunner, 1974; Ting and Bonnard, 1976; Hayry and Andersson, 1976; Zier and Bach, 1975). This accelerated appearance of the MLR and cytotoxic cells is called a secondary response. These secondary blastogenic and cytotoxic responses are only generated by stimulator cells with the same LD determinants as the original stimulator cells, however, the secondary cytotoxic response is directed only at the SD antigens present on the original stimulating cells (Zier and Bach, 1975; Alter, Grillot-Courvalin, Zier and Bach, 1976). This means that a secondary cytotoxic response can be generated by a cell that will not necessarily act as a target cell.

Cell mediated lympholysis has not yet been demonstrated in the rabbit.

i) Autologous stimulation in the MLR and its relation to autoimmunity and immune surveillance

Many examples of MLRs between autologous cells have been recorded. The most striking of these is the reaction first noted by Howe, Goldstein and Battisto (1970), who using neonatal thymus cells as responders and syngeneic mitomycin-C treated adult spleen cells as stimulators, noted a significant blastogenic response. This reaction could not be produced when the adult spleen cells were cultured with syngeneic mitomycin-C treated neonatal thymus cells. Adult bone marrow or thymus cells used as stimulators did not provoke a response by the neonatal thymus cells. The ability to recognize syngeneic cells declined within the first week of life and then disappeared in adulthood. Whether the reaction of neonatal thymus cells to syngeneic adult spleen cells is recognition of 'self' antigens was uncertain since spleen cells of adult mice could carry antigens which do not normally occur in neonatal animals and may therefore be recognized as foreign. This point was clarified by von Boehmer and Byrd (1972) who found that adult mouse thymus cells would respond in the MLR to syngeneic adult spleen cells suggesting that recognition of self was indeed occurring. It was later shown that cortisone resistant thymus cells of adult mice would respond to the same mouse stimulator cells (von Boehmer, 1974). It is possible, as von Boehmer (1974) has suggested, that the autologous MLR is the in vivo means of removing autologous reacting clones of cells from the body. Reactions between autologous rat spleen and thymus cells in the two-way MLR have also been noted (Llancer and Uyeki, 1969). In the rabbit, autologous responses of adult cells have

only been noted to PHA pretreated stimulating cells (Ling and Kay, 1975). Takasugi, Kiuchi and Opelz (1977) have recently found that unseparated human lymphocytes, B plus null cells or T enriched cells separated by rosetting techniques will respond in the MLR to autologous null cells or autologous null cells plus B cells. Vande Stouwe, Kunkel, Halper and Weksler (1977) have shown a human autologous one-way MLR between T cells and stimulator B cells or B cell lines. The B lymphoid cell lines were more stimulatory than purified normal B cells. However, no cytotoxic activity could be produced to the stimulating cells in either case. Secondary autologous MLRs (memory) have the same specificity and kinetics as secondary allogeneic MLRs, in experiments using responder human T lymphocytes and target non T cells from peripheral blood (Weksler and Kozak, 1977). The same phenomenon of immunological memory and specificity in autologous MLR has been demonstrated in a reaction between responder rat lymphocytes and autologous stimulator testicular cells or lymph node cells (Werkerle, 1977). The significance of these results becomes apparent if the thymus is considered the exclusive source of cells reacting to self (Burnet, 1968; Jerne, 1971). The above experiments contradict the theory that clones of cells reacting against 'self' exist only for transient periods in the thymus before being eliminated. Instead the above experiments suggest that suppressor cells or soluble mediators play a part in the control of the reaction against 'self' as autologous lymphocytes of peripheral organs can produce an MLR. It is possible that defects in this control mechanism

lead to cell mediated autoimmune diseases (Waldman, Broder, Krakauer, Durm, Coldman, Meade and Strober, 1977).

Autologous stimulation in the MLR in vitro has been suggested as a good model of a possible surveillance mechanism against viral infections and tumors in vivo. In this model tumor cells or virally infected cells have modified surface membranes, consequently they are no longer recognized as self. The individual immune system therefore destroys the offending cells utilizing specific cytotoxic lymphocytes. This reaction does occur in vitro. Lymphocytes taken from rats with Gross-virus induced lymphoma after a secondary MLR in vitro will lyse the autologous lymphoma cells (Bernstein, Wright and Cohen, 1976). The same has been found with lymphocytes taken from mice with plasma cell tumors (Boyer and Fahey, 1976). In other experiments, trinitrophenyl modified mouse lymphocytes will stimulate autologous cells in the one-way MLR, with the subsequent production of cytotoxic cell activity specific for the chemically modified targets (Shearer, Lozner, Rehn and Schmitt-Verhulst, 1975).

The above reaction does not resemble classic allogeneic reactions as no differences in the MHC loci defined LD or SD determinants are known to exist, although tumor specific and viral specific antigens do exist. An attempt to reconcile the classical model of in vitro recognition and production of cytotoxic cells with the above autologous reactions was made by Zarling, Raich, McKeough and Bach (1976) who showed that lymphocytes from a

patient in remission with myelomonocytic leukemia, when cultured with autologous leukemic lymphocytes in an MLR, did not develop cytotoxicity towards the tumor cells. However, in the presence of a third inactivated allogeneic population of cells cytotoxicity developed towards the leukemic population. Presumably the third cell population provided the stimulus (LD antigens) required for the production of proliferative helper cells that initiated a cytotoxic response to the foreign leukemic antigens. This experiment also suggests that MHC loci controlled SD antigens are not the target in this case, or that tumor specific antigens modify the MHC loci controlled SD determinants, or that autologous SD antigens can be recognized and attacked. These results have been confirmed by Sondel, O'Brien, Porter, Schlossman and Chess (1976) and suggests a method for specific sensitization of cytotoxic cells to specific neoplasms. However these experiments do not suggest any reason for the initiation of autologous MLRs between normal cells when no differences in LD determinants are present. It is possible that either virus infected or tumor cells that are successfully eliminated in vivo have specific antigens which modify normal LD determinants, thus initiating a specific immune response to these cells. In cases where abnormal cells are not eliminated it is possible that LD determinants are not modified and consequently the abnormal cell is still recognized as self and is not destroyed.

Zinkernagel and Doherty (1975) have found that effector T cells generated in virus infected mice would only lyse virus infected target cells of the same H-2 type. If the H-2 antigens present on

the target cells are blocked by anti H-2 antisera or if target cells are used which express a fetal antigen instead of the H-2 antigen, no autologous lysis will occur (Zinkernagel and Doherty, 1976). This has suggested a rather unusual model in which the autologous cytotoxic T cell has two specificities, one for the new antigen and one for recognition of self through the H-2 complex (Zinkernagel and Doherty, 1976). Zinkernagel and Doherty (1976) have suggested that cytotoxic cells express a hybrid receptor compatible to an IgG binding site with one variable chain specific for self H-2 and the other for the new antigen. They also suggest that T cells that express two V-genes specific for self would be deleted or suppressed, hybrid expression would be related to surveillance and the expression of two V-genes specific for non-self would be related to alloreactive cells. It could also be possible that cytotoxic cells that recognize one hybrid site composed of part of the H-2 antigen and the viral antigen would need only one recognition site for the resultant complex antigen. It seems likely that a successful virus would not want to eliminate its host and consequently itself, and would therefore stimulate the host's immune system which still allows time for viral multiplication and infection of another host. The most obvious way to do this is for the new viral antigen to be a modification of part of the histocompatibility antigens or closely associated with them. Evidence for the close association of H-2 and viral antigens has been produced by Schrader, Henning, Milner and Edelman (1976) using co-capping

experiments. This proposed system would not require the highly complex hybrid T-cell recognition site as proposed by Zinkernagel and Doherty (1976).

It is interesting to note that cells which are cytotoxic to modified autologous cells have the same mouse Ly markers as do cells cytotoxic to allogeneic cells (Ly-2,3$^+$). Ly-1,2,3$^+$ cells are important as precursors of the Ly-2,3$^+$ cytotoxic cells reacting to modified autologous cells. This is different, however, to production of cytotoxic cells specific for allogeneic cells where Ly-1,2,3$^+$ cells have little part to play (Cantor and Boyse, 1976).

j) The MLR and allograft rejection

Two well documented immunological reactions can occur when tissue transplantation is carried out between allogeneic animals. The most obvious of the two reactions is the rejection of the grafted tissue by the host animal. This is called a host versus graft (HvG) reaction. The second type of reaction occurs when an irradiated (immunologically incompetent) host receives a transplant of allogeneic lymphoid cells. In this case the grafted lymphoid cells attack the host tissue and can cause subsequent death of the host. This is called graft versus host (GvH) disease.

GvH reactions are usually measured by the increased uptake of a radiolabelled DNA precursor into the host spleen (Elkins, Kavathas and Bach, 1973) or splenomegaly (Bach and van Rood, 1976c). This indicates that the transplanted lymphoid cells are

multiplying without restriction in the host's spleen and are attacking the host animal. HvG reactions are measured by observing the integrity of the grafted tissue.

It is likely that HvG and GvH reactions are expressions of the same phenomenon, that is, the reaction of lymphoid cells to allogeneic tissue. The characteristics of GvH and HvG reactions and their relationship to the MLR are described below. It should be noted that some of the characteristics of the GvH reaction are not those of an HvG reaction and visa versa.

GvH disease is a potentially fatal complication of bone marrow transplantation due to a GvH reaction occurring in non-responsive recipients of incompatible bone marrow (Bach and van Rood, 1976c). The results of MLRs between donor and recipient of mouse lymphoid cells correlate well with in vivo GvH reactions (Livnat, Klein and Bach, 1973; Klein and Park, 1973). The T cells responsible for the MLR seem to be the same as the T cells responsible for the GvH reaction (Wagner, Rollinghoff and Shortman, 1974). T cells which do not adhere to allogeneic cell monolayers retain MLR and GvH reactivity but lose specific cytotoxic activity (Mage and McHugh, 1973). It has been shown that LD differences are associated with GvH reactions while SD differences are not (Elkins, Kavathas and Bach, 1973). This is in contrast to HvG reactions in which SD differences play a major role. Mouse spleen cells active in the MLR and GvH reactions separate together in velocity sedimentation or density gradients

(El-Arini and Osoba, 1973; Ling and Kay, 1975). The number of cells responsive to an antigen in the MLR are similar to the number of cells calculated to be responding in the GvH reaction (Nisbet, Simonsen and Zaleski, 1969).

The HvG reaction on the other hand, has been more closely related to the production of specific cytotoxic T cells. For the in vitro production of cytotoxic cells, differences in SD and LD antigens on donor and recipient cells are required (see Section 'h' of this chapter). These are the same conditions required for successful and rapid allograft rejection. Conversely in man, the survival of transplanted tissue is prolonged if donor and recipient cells are identical for either SD or LD antigens (Cochrum, Perkins, Payne, Kountz and Belzer, 1973; Segal, Bach, Bach, Hussey and Uehling, 1975). More recently it has been shown that there is collaboration between the host cells recognizing SD and those recognizing LD determinants during graft rejection in the mouse. Lafferty, Cooley, Woolnough and Walker (1975), transplanted a thyroid lobe under the kidney capsule of recipient allogeneic mice and observed that the allograft was rejected more slowly if it was incompatible only in the H-2K (SD difference) region rather than in both the H-2K and the H-2I (LD difference) loci. On the other hand if, at the time of transplant of a thyroid lobe which differed only in the SD region from the recipient, lymphoid cells which differed from the recipient of the thyroid lobe only in the LD region were

injected into the recipient, a rapid rejection of the graft occurred (Sollinger and Bach, 1976; Bach, 1976; Bach, Bach, Kuperman, Sollinger and Sondel, 1976; Bach, Kuperman, Sollinger, Zarling, Sondel, Alter and Bach, 1977). Mice rendered tolerant to the LD determinants present on the thyroid transplant allowed prolonged survival of the thyroid lobe graft (Bach, Kuperman, Sollinger, Zarling, Sondel, Alter and Bach, 1977). These experiments show that there is a collaboration between cells recognizing LD determinants and SD antigens in vivo, and that LD determinants play major role in transplant rejection.

It is possible that SD antigens play an insignificant role in allograft rejection and that specific cytotoxic T cells are not involved. It has not yet been shown that cytotoxic T cells are active in graft rejection in vivo (Gowans, 1977). It has been observed by several investigators that the majority of cells at the site of a rejected allograft are non-T and therefore could not be specific cytotoxic T cells (Roberts and Hayry, 1977). However, the spleens of these alloimmunized animals are full of specific T killer cells directed against the SD antigens of the allograft donor. Other evidence supports the hypothesis that specific cytotoxic T cells are involved in allograft rejection. Immunologically specific non-T killer cells have not been demonstrated in allograft recipients (Hayry and Roberts, 1977). Also the number of cytotoxic T cells at the site of allograft rejection has been revised. Strom, Tilney, Paradysz, Bancewicz and Carpenter (1977) have demonstrated that T-killer cells are the

major population of cells present at the site of allograft rejection. It should be noted that non-specific killer cells are also present. These results have also been confirmed by Tilney, Strom, Both, Finnegan, Lunden and Carpenter (1977). For the above reasons the production of cytotoxic T cells during the MLR in vitro has been looked upon as a good model for the rejection of allografts in vivo.

Unfortunately it is more difficult to fit the classical GvH reaction into this model. This may be due to the method of assessing the reaction rather than the reaction itself. As has been previously mentioned, SD antigenic differences between donor and recipient seem to play little role in the proliferative GvH reaction in the spleen, and removal of cytotoxic cells or their precursors does not inhibit the proliferative GvH reaction. However, it has been observed that production of cytotoxic T cells occurs during the GvH reaction (Sprent and Miller, 1971; Cerottini, Nordin and Brunner, 1970; Vitale, Jaksic, Matosic, Silobrcic and Tomazic, 1976). Speculation suggests that the classical assays for the GvH reaction (proliferation of grafted cells in the recipient's spleen) may only measure the initial phase of the reaction. The eventual death of the host due to tissue destruction may be due to the recognition of SD antigens by specific cytotoxic T cells. In this case the GvH and HvG reactions could be equated.

Another aspect of the relation between allograft rejection and the MLR is that immunization of an animal by allografting followed by graft rejection modifies the specific MLR between the responder lymphocytes of the allografted animal and the stimulator lymphocytes of the graft donor. The responder lymphocytes of the alloimmunized animal exhibit 'memory' in that their MLR response to cells of the allograft donor occurs more quickly than normal. This accelerated response is specific towards the cells of the allograft donor. Rats after injection of spleen cells (Wilson and Nowell, 1971) or rejection of an allograft (Wilson, Silvers and Nowell, 1968) showed a marked acceleration in the response of their lymphocytes to donor lymphocytes in the MLR. This response has also been shown under similar conditions in mice (Adler, Takiguchi, Marsh and Smith, 1970). Harrison, Wei and Ahie (1971) have found that in rabbits which had multiple skin grafts, the two-way MLR response between recipient and donor cells was greatly enhanced on day 5, which is the normal time for the assay of the rabbit MLR. However, Chapman and Dutton (1965) and Sheppard, Jr., Sell, Poler and Redelman (1977) found no changes in the MLR response of allograft recipient lymphocytes to donor cells after a single allograft in the rabbit. This was probably because the accelerated MLR response had already subsided on the day of the assay (day 5). This was suggested by the findings of Adler, Takiguchi, Marsh and Smith (1970) using mice and Wilson, Silvers and Nowell (1967) using rats, who observed

that the accelerated MLR responsiveness following sensitization in vivo is transient in nature and consequently the kinetics of the post-allograft rejection MLR return to the pre-immunization kinetics after about three weeks. It seems that the MLR memory is of the short term type.

The finding of a 'memory' response in the MLR after in vivo sensitization of animals correlates well with the in vitro model of allograft rejection and the production of memory MLR cells in vitro (see sections 'd' and 'h' of this chapter).

k) Summary

In summary it can be seen that the MLR in mouse and man has been well defined both cytologically and genetically. It has been shown that the MLR responder cell is a classical T cell and that the proliferative response is modulated by histocompatibility differences in LD determinants. The MLR has been shown to be the initial phase in the production of specific cytotoxic cells in vitro and this reaction in turn has been used as a model for allograft rejection in vivo. The MLR is modified by cells and soluble factors, and the T responder cells demonstrate a specific immunological memory for LD determinants. The cells responding in the MLR are also capable of recognizing autologous cells and are implicated in autoimmune disease and immune surveillance.

In the rabbit the MLR has not been investigated as thoroughly as in the human and in the mouse. It has not been unequivocally shown that a T cell responds in the rabbit MLR, that MLR memory

exists or that the rabbit MLR responsive cells can recognize autologous cells. It is also unclear, due to conflicting reports, whether the rabbit MLR response can be modified by presensitization in vivo (allograft rejection). This thesis is designed to elucidate some of these areas in the rabbit MLR response and bring the work on the rabbit MLR more in line with the investigations that are presently being carried out in the human and the mouse.

CHAPTER III

METHODS AND MATERIALS

1. Materials

   a) Chemicals

   b) Apparatus

a) Chemicals:

Tissue Culture Medium - RPMI-1640, NCTC-109, TC-199 and CMRL-1066 (500 ml) were obtained from Microbiological Associates, Bethesda, Maryland, and stored at 4°C until use.

Hank's Balanced Salt Solution (HBSS) - was obtained in 500 ml quantities from Microbiological Associates and stored at 4°C.

Antibiotics - Potassium, penicillin G (5000 units/ml) and streptomycin sulphate (5000 µg/ml) were obtained pre-mixed in 100 ml quantities and stored at -20°C until use.(Microbiological Associates).

Saline Solution - 0.9% in pyrogen free sterile water was obtained in various quantities from Abbott Laboratories Ltd., Montreal, Quebec and stored at room temperature.

Serum - Rabbit serum was obtained from our own stock of rabbits and pooled in 200 ml lots and stored at -20°C until use, or purchased in 500 ml bottles from Miles Laboratories, Kankakee, Illinois (Pentex) and stored at 4°C.

Animals - Outbred, 4-5 lb., New Zealand White (NZW) and New Zealand Black (NZB) rabbits were obtained from Rockland Rabbit Ranch, Rockland, Ontario.

Concanavalin A (Con-A) - was obtained powdered in 250 mg quantities (A-grade) from Calbiochem, Los Angeles, California, and stored at 4°C.

Phytohemagglutinin (PHA) - PHA-P and PHA-M were obtained as a powder from Difco Laboratories, Detroit, Michigan and stored at 4°C.

Pokeweed Mitogen (PWM) - was obtained in powdered form from Grand Island Biological Co., Grand Island, N.Y., and stored at 4°C.

Mitomycin-C - was obtained powdered in 2 mg quantities from Nutritional Biochemical Company, Cleveland, Ohio, and stored in the dark at 4°C. Immediately before use the powder was dissolved in 2 ml of HBSS to give a solution of 1 mg/ml.

Dextran - T-250 was obtained in 500 g lots from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Ficoll 400 - was obtained in 500 g lots from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Heparin (mucosa) sodium, was obtained in concentrations of either 10,000 units/ml or 1,000 units/ml from Allen and Hanburys, Toronto, and stored at 4°C.

Hypaque Sodium (diatriozate) 50% w/v, pH = 6.5-7.7, was obtained from Winthrop Laboratories, Aurora, Ontario. The 30 ml ampules were stored in the dark.

L-Glutamine - 200 mM in isotonic saline was obtained in 100 ml lots from Microbiological Associates and kept at -20°C until use.

HEPES buffer solution (1 molar) in Earle's balanced salt solution was obtained from Microbiological Associates and stored at 4°C.

2-Mercaptoethanol - 98% pure minimum by filtration (100 g in 100 ml) was obtained from Eastman Kodak Company, Rochester, New York. A stock solution of 0.14M was made up in RPMI-1640 and stored at 4°C until use.

Fluorescein Conjugated IgG fraction of sheep anti-rabbit immunoglobulin (IgA, IgG, IgM) lot 8081 was obtained from Cappel Laboratories Inc., Downington, Pennsylvania. The protein was dissolved according to the manufacturer's instructions and stored at -20°C.

^{3}H-Thymidine - (20 µCi/mM) was obtained from New England Nuclear, Boston, Massachusetts. A 0.1 ml aliquot containing 1 µCi in RPMI-1640 was added to each 1 ml culture.

^{51}Cr-Sodium - was obtained (1 mCi/ml) (270-350 Ci/g) from New England Nuclear, Boston, Massachusetts.

Scintillation Fluid - consisted of either a cocktail of 8 g/L butyl-PBD (Beckman, Palo Alto, California) and 10% v/v BBS-3 (Beckman) in toluene (Fisher Scientific), or premixed Beckman GP (Beckman).

b) Apparatus:

Liquid Scintillation Vials were glass and obtained from Fisher Scientific.

Culture Tubes were 17 x 100 mm plastic sterile tubes obtained from Falcon Plastics, Oxnard, California.

Microtest II tissue culture plates and lids were obtained from Falcon Plastics.

Universal Containers (20 ml) were obtained from Sterlin Ltd., Richmond, Surrey, England.

Syringes of all sizes were Plastipak sterile disposable plastic obtained from Becton, Dickinson Co., Canada Ltd., Mississauga, Ontario.

Pipettes - 1, 5 and 10 ml Pyrex disposable serological pipettes were obtained from Corning Glass Works, Corning, New York. A pro-pipette was always used to manipulate fluids.

Incubator - cultures were maintained in a National incubator at 37°C. The interior was humidified by placing a large container of distilled water on the bottom shelf. The atmosphere was maintained at an air to CO_2 ratio of 95:5. Compressed air was passed through a Norgren filter (Littleton, Colorado) to remove suspended oil and water.

2. Methods

a) Preparation of cells

b) Tissue culture medium

c) Preparation of serum

d) Mitomycin-C treatment of cells

e) Detection of cell surface immunoglobulins by direct immunofluorescence

f) Mitogen stimulation of cultures

g) The mixed leukocyte culture reaction (MLR)

h) Whole body irradiation of rabbits

i) Skin allografting

j) ^{3}H-Thymidine incorporation into cultures

k) Liquid scintillation counting of acid precipitable material

l) Cell mediated lympholysis (CML) using ^{51}Cr-release

m) Gamma counting of CML supernatants

n) Calculations

a) Preparation of cells

Dextran sedimentation was used as a standard method for the preparation of leukocytes. Rabbits were anesthetized with nembutal or surital and blood was obtained by cardiac puncture. Surital was the preferred anesthetic as nembutal caused a moderate amount of hemolysis. WBC were prepared for culture as shown in Figure 1. Two parts of blood were mixed with one part of a saline solution of 6% dextran (molecular weight 200,000-250,000) containing 150 units/ml of heparin. The blood was allowed to sediment at 37°C in a 100 ml sterile measuring cylinder. The leukocyte rich supernatant was removed and washed three times (800 g, 10 min) in Hank's balanced salt solution (HBSS). The pellet was resuspended in tissue culture medium and the leukocyte count determined using a model ZBi Coulter counter (Coulter Electronics, Hialeah, Florida). Smears of cells were made using Wright's stain. The type of cell present

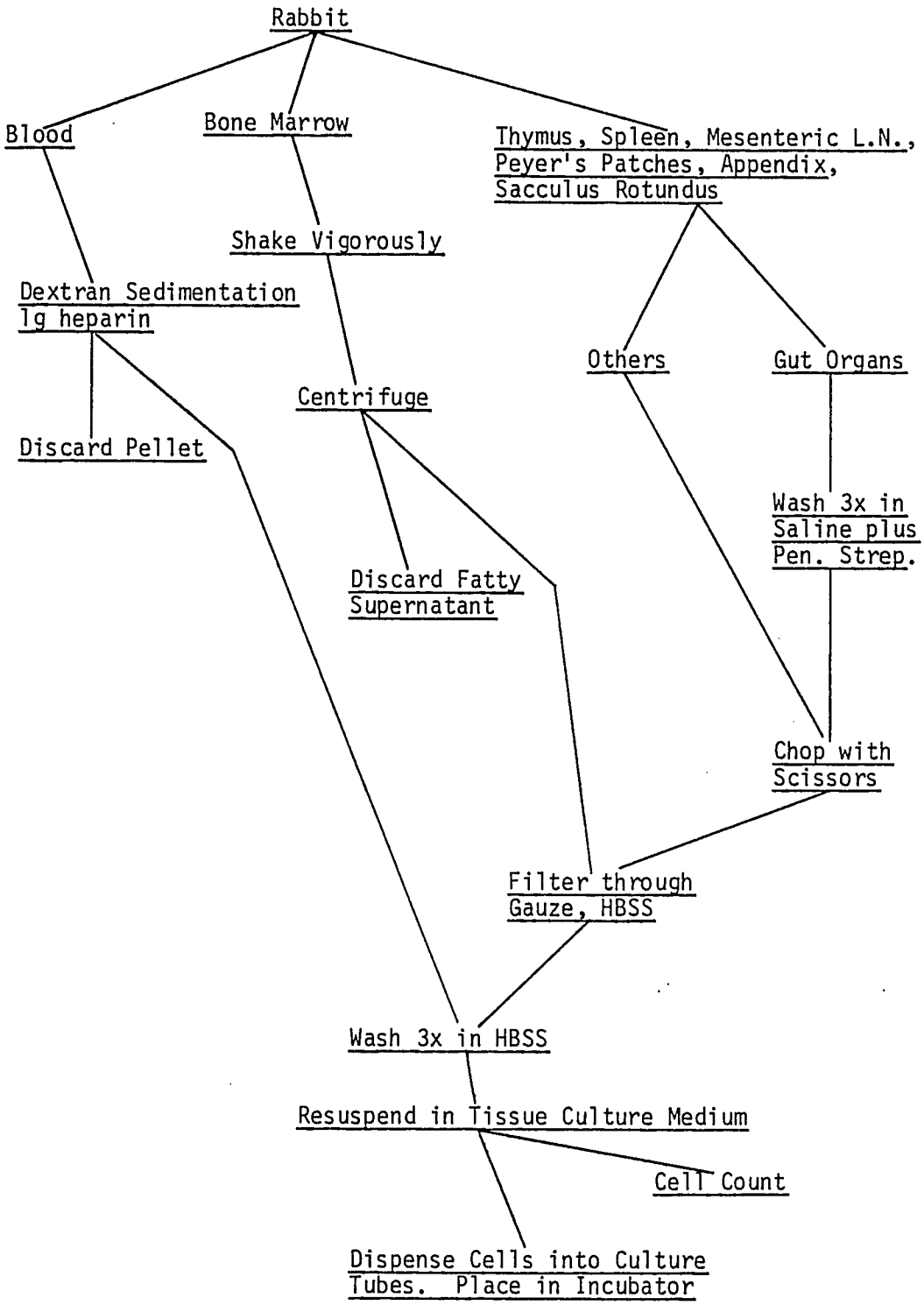


FIGURE 1

PREPARATION OF RABBIT LYMPHOID CELLS FOR TISSUE CULTURE

in the dextran prepared leukocytes reflected the distribution of monocytes, granulocytes and lymphocytes present in whole blood (Table 1). Recovery of leukocytes was approximately 20% of those present in whole blood (Table 2). RBC were reduced to less than 1% of those present in whole blood.

In some cases cells were prepared from heparinized blood using ficoll-hypaque gradient centrifugation. Powdered methyl cellulose 0.6 g was mixed with 40 g of ficoll and dissolved overnight in 500 ml of distilled water. This solution was made up to a density of 1.1 at 18°C by addition of 50% w/v hypaque solution and filtered through a 0.45 μ Millipore filter. The ficoll-hypaque was aliquoted into 100 ml bottles and kept in the dark at 4°C.

Blood mixed with 50 units/ml of heparin was diluted 1:1 with saline. Twenty ml of diluted blood was layered on top of 10 ml of the ficoll-hypaque preparation in a 50 ml glass centrifuge tube. The tubes were spun at 1200 g (2350 rpm) at 18°C for 30 minutes. After centrifugation the interface layer of cells was carefully removed with a pipette. These cells were then washed three times in HBSS and the resultant cells were counted. Smears and cell counts were made on the interface cells before and after washing and on the resultant ficoll-hypaque separated cell pellet. Methyl cellulose was added to the ficoll-hypaque to enhance agglutination of the rabbit RBC.

TABLE 1

DIFFERENTIAL CELL COUNTS OF POPULATIONS OF WBC FROM PREPARATIONS OF WHOLE BLOOD, DEXTRAN SUPERNATANTS AND FICOLL-HYPAQUE INTERFACES

SAMPLE	PERCENTAGE OF TOTAL POPULATION		
	MONOCYTES	GRANULOCYTES	LYMPHOCYTES
WHOLE BLOOD	2.6*± 0.7**	38.3 ± 4.6	59.0 ± 4.3
FICOLL-HYPAQUE INTERFACE	3.8 ± 1.4	8.4 ± 3.9	87.9 ± 4.1
DEXTRAN SUPERNATANT	2.9 ± 0.9	42.1 ± 12.6	54.9 ± 12.7

* The values given are the average of seven samples

** This number represents the standard error of the mean

TABLE 2

THE PERCENTAGE RECOVERY OF CELLS FROM WHOLE BLOOD AFTER ISOLATION OF LYMPHOCYTES BY EITHER DEXTRAN OR FICOLL-HYPAQUE TREATMENT

SAMPLE	PERCENTAGE RECOVERY FROM WHOLE BLOOD				
	TOTAL WBC	MONOCYTES	GRANULOCYTES	LYMPHOCYTES	RBC
DEXTRAN SUPERNATANT	20.73* ± 9.6 **	11.0 ± 3.5	18.4 ± 4.4	16.2 ± 3.6	<1***
FICOLL-HYPAQUE INTERFACE	18.0 ± 3.5	11.6 ± 3.5	1.5 ± 1.0	26.3 ± 4.6	<1

* Each figure is an average of six experiments

** This number represents the standard error of the mean

*** The average ratio of RBC to total WBC recovered in dextran was 10:1 and in ficoll-hypaque 2:1

The proportion of the different cell types present in the ficoll-hypaque interface was not the same as found in whole blood (Table 1). The number of granulocytes was decreased significantly, while the percentage of lymphocytes was increased. The percentage of monocytes reflected the distribution present in whole blood. The total recovery of leukocytes was the same as found with dextran (Table 2). The recovery of lymphocytes was on the average higher than using dextran while the recovery of granulocytes and RBC was significantly less than using dextran.

Bone marrow was removed by cracking the femur and scraping the marrow into a tube containing HBSS. The bone marrow cells were freed from fatty tissues by shaking the tube vigorously until the cells were in suspension. After centrifugation at 1000 g the cell free supernatant, which also contained a layer of fat, was discarded.

The spleen, thymus, appendix, Peyer's patches, sacculus rotundus and mesenteric lymph nodes were rapidly extirpated. The gut associated organs (appendix, Peyer's patches and sacculus rotundus), especially the appendix, were generally contaminated by intestinal contents. These organs were washed vigorously, three times, in a saline solution containing 200 IU/ml of penicillin and 200 µg/ml of streptomycin to minimize chances of introducing infectious agents into the subsequently prepared cell culture. Lymphoid cells were released from spleen, thymus, appendix, Peyer's patches, sacculus rotundus and mesenteric lymph node by chopping the organs in a small volume of HBSS into small

fragments using fine-pointed scissors. The cells were recovered from the tissue fragments by gentle shaking. All of the cell suspensions were filtered through sterile gauze to remove tissue fragments and any fine fragments of bone from the bone marrow cell suspension. The cells were then washed twice (800 g, 10 min) in HBSS, resuspended in tissue culture medium and the white cell count determined using a model ZBi Coulter Counter (Coulter Electronics, Hialeah, Florida; Figure 1).

b) Tissue culture media

The type of media used consisted of the following; RPMI-1640, TC-199, NCTC-109, or CMRL-1066. All media were supplemented with rabbit serum to a final concentration of 2.5% (unless otherwise noted), 100 units/ml of penicillin and 100 µg/ml of streptomycin. Sodium bicarbonate was present in all media purchased and in conjunction with the 5% CO_2 atmosphere used during incubation served as one of the main buffers in the tissue culture medium.

c) Preparation of serum

Rabbit serum was obtained either from Miles Laboratories, or preparation from our own rabbits by cardiac puncture. The blood was left to clot at room temperature for 60 minutes and then placed overnight at 4°C to allow the clot to retract. The serum was separated from the clot by centrifugation (1500 g, 20 min) and pooled in aliquots of 10 ml. All sera was complement inactivated by heating at 56°C for 30 minutes, and stored frozen at -20°C until use.

d) Mitomycin-C treatment of cells

Aliquots of 25 x 10^6 cells in 5 ml of RPMI-1640 containing 25, 50, or 100 µg/ml of mitomycin-C in 20 ml universal containers were incubated at 37°C for 30 minutes in 5% CO_2 in air. HBSS (10 ml) was added to each universal container immediately after incubation. The cells were then centrifuged at 800 g for 10 minutes and the supernatant discarded. This washing procedure was repeated three times following which cells were resuspended in tissue culture medium, counted, and the appropriate number added to culture tubes.

e) Detection of cell surface immunoglobulins by direct immunofluorescence

The lyophilized fluorescein conjugated IgG fraction of sheep antirabbit immunoglobulins (IgG, IgM, IgA) was reconstituted with sterile distilled water and particulate matter was removed by centrifugation. Immediately before use the conjugate was diluted 1:6 with phosphate buffered saline.

Lymphoid cells (4 x 10^6) were washed twice in HBSS (800 g, 10 min) at 4°C. The cell pellet was then resuspended in two drops of diluted fluorescein-conjugated antiserum, incubated at 4° for 30 minutes, washed twice in HBSS (800 g, 10 min) and then resuspended in 0.5 ml of HBSS.

Immediately before counting, an aliquot of the cell preparation was placed on a slide and a coverslip sealed in place with clear nail polish. The total cell count was determined with white transmitted light using a Zeiss Research microscope. Fluorescent

cells were counted using epi-illumination from an HBO-50 high pressure mercury lamp employing an exciter filter of 490 nm and a barrier filter of 515 nm. The percentage of fluorescent cells was then calculated.

f) Mitogen stimulation of cultures

All cultures consisted of 1 x 10^6 cells incubated for three days in the same medium and under the same conditions used in the MLR.

Concanavalin-A (Con-A) was obtained from Calbiochem, Los Angeles, California. The powder was dissolved in RPMI-1640 (50 mg/ml) and stored frozen (-20°C). One tenth ml of a 1/800 dilution added to the 1 ml culture was found to stimulate an optimal blastogenic response of rabbit WBC.

Phytohemagglutinin (PHA-P) (obtained from Difco Laboratories, Detroit, Michigan) was made up as instructed on the vial and stored frozen (-20°C). One tenth ml of a 1/500 dilution added to the 1 ml culture was found to stimulate an optimal blastogenic response of rabbit WBC.

Pokeweed mitogen (PWM) (obtained from Grand Island Biological Company, Grand Island, New York) was made up as indicated on the instruction sheets and stored frozen (-20°C). Previous experiments indicated that the addition of 0.1 ml of a 1/2 dilution added to 1 ml cultures was found to stimulate an optimal blastogenic response of rabbit WBC.

g) The mixed leukocyte culture reaction

Leukocytes were prepared from NZW and NZB rabbits. For the one-way MLR, cells treated with mitomycin-C were designated stimulator cells and untreated cells were designated responder cells. Responder and stimulator leukocyte populations were suspended at a concentration of 1 x 10^6 cells/ml (unless otherwise noted) and 0.5 ml aliquots of each preparation pipetted into 17 x 100 mm plastic sterile tissue culture tubes. The serum concentration and type of media used for the different experiments are noted in the chapter of results. The resultant 1 ml cultures were incubated at 37°C for 3-7 days in a humidified atmosphere of air:CO_2 (95:5). Control cultures consisted of responder cells with the appropriate number of autologous mitomycin-C treated cells.

For the two-way MLR, neither cell population was treated with mitomycin-C prior to culture. Control cultures for the two-way MLR consisted of cells of each rabbit cultured alone.

In cultures designed to generate cytotoxic cells in the MLR responder and stimulator cells were cultured in 5 ml of medium. Each culture contained a total of 1 x 10^7 cells with the ratio of responder to stimulator cells of 1:1. These cultures were harvested after 5 days and assayed for blastogenesis, and identical cultures were harvested after 8 days and assayed for cytotoxic cells. In the CML assay, target cells consisted of WBC taken from the MLR

stimulator rabbit 3 days before the CML assay and cultured with an optimal concentration of PHA-P to produce more susceptible targets (see section '1' for details).

h) Whole body irradiation of rabbits

Rabbits to be irradiated were placed in restraining boxes to prevent undue movement during the irradiation procedure and ensure uniform irradiation of all the rabbits. The rabbits were subjected to between 50 and 800R total body incidence dose irradiation using a Cobalt-60 source at an output of approximately 50R per minute. The rabbits were then returned to their cages and left overnight before any experimental procedures were performed.

i) Skin allografting

The MLR responsiveness of the WBC of the prospective allograft recipient was determined several days prior to allografting. Skin grafts were taken from the rabbits supplying stimulator cells for the MLR and transplanted to the appropriate recipients (responder cell donors). Eight days after allografting and approximately two days after rejection of the grafts, the WBC of the allografted rabbits were assayed for their MLR responsiveness.

The technique used for skin grafting in the rabbit was as follows:- Full thickness skin grafts were taken from the inner surface of the ear of the rabbit anesthetized with surital. Two grafts 2 cm x 1 cm were taken from a single rabbit and placed on

the inner surface of one ear of the appropriate rabbit. Each recipient received one small autograft placed alongside the two allografts. The grafts were secured by finger pressure for a sufficient time (usually less than one minute) to prevent blood from oozing out of the edges of the grafts. The criterion used to determine rejection was the blackening and sloughing off of the allograft (Fig. 17).

j) ^{3}H-Thymidine incorporation into cultures

^{3}H-Thymidine (20 Ci/mM) was diluted in tissue culture medium. A 0.1 ml aliquot containing 1 µCi was added to each culture tube 18 to 22 hours before termination of the culture. The amount of tritium incorporated into DNA was determined by liquid scintillation counting.

k) Liquid scintillation counting of acid precipitable material

After the appropriate period of culture the tubes were removed from the incubator and centrifuged (1000 g, 10 min) at room temperature. The supernatant medium was discarded and the cell pellet was resuspended in 1 ml of trichloroacetic acid (TCA), centrifuged as above, and the supernatant containing acid soluble tritium discarded (Figure 2). This procedure was repeated once. In cultures containing an excessive number of red blood cells (RBC), TCA precipitation resulted in excessive quenching due to retention of hemoglobin in the precipitates. Therefore cultures were initially treated with acetic acid instead of TCA as follows (Figure 3). Ten ml of a 3% acetic acid solution were added

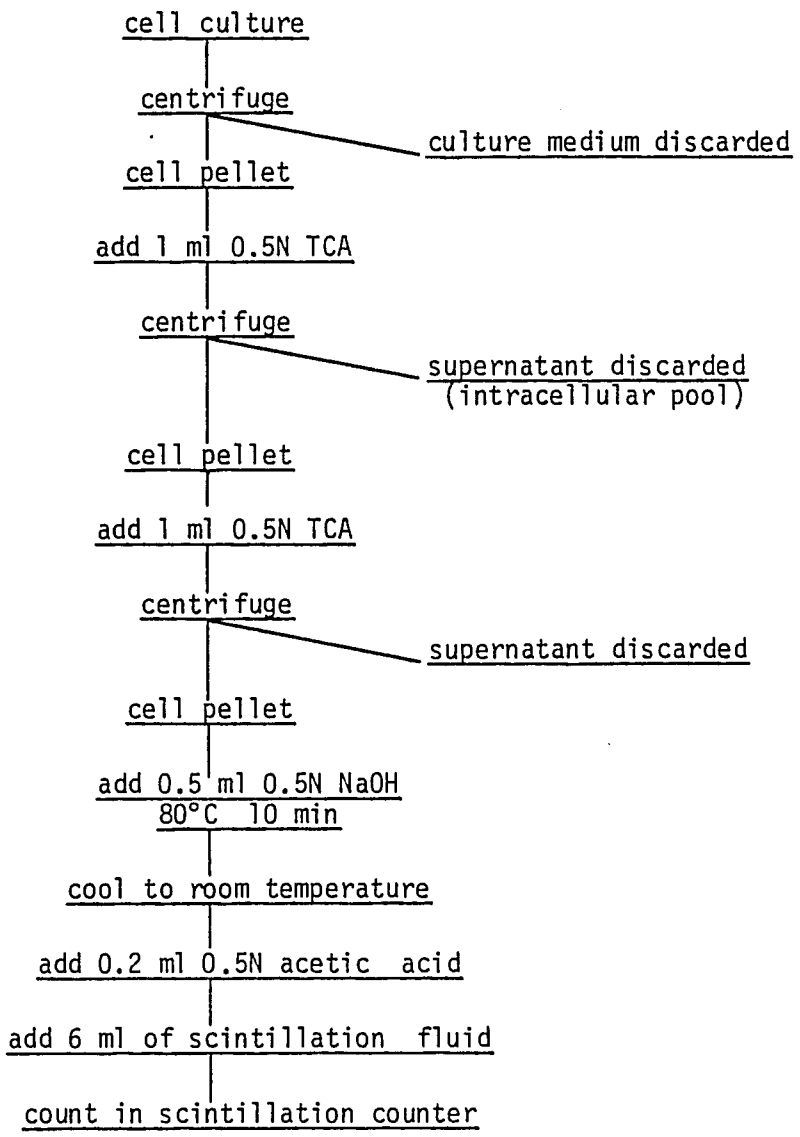


FIGURE 2

MEASUREMENT OF CPM IN DNA USING TCA EXTRACTION

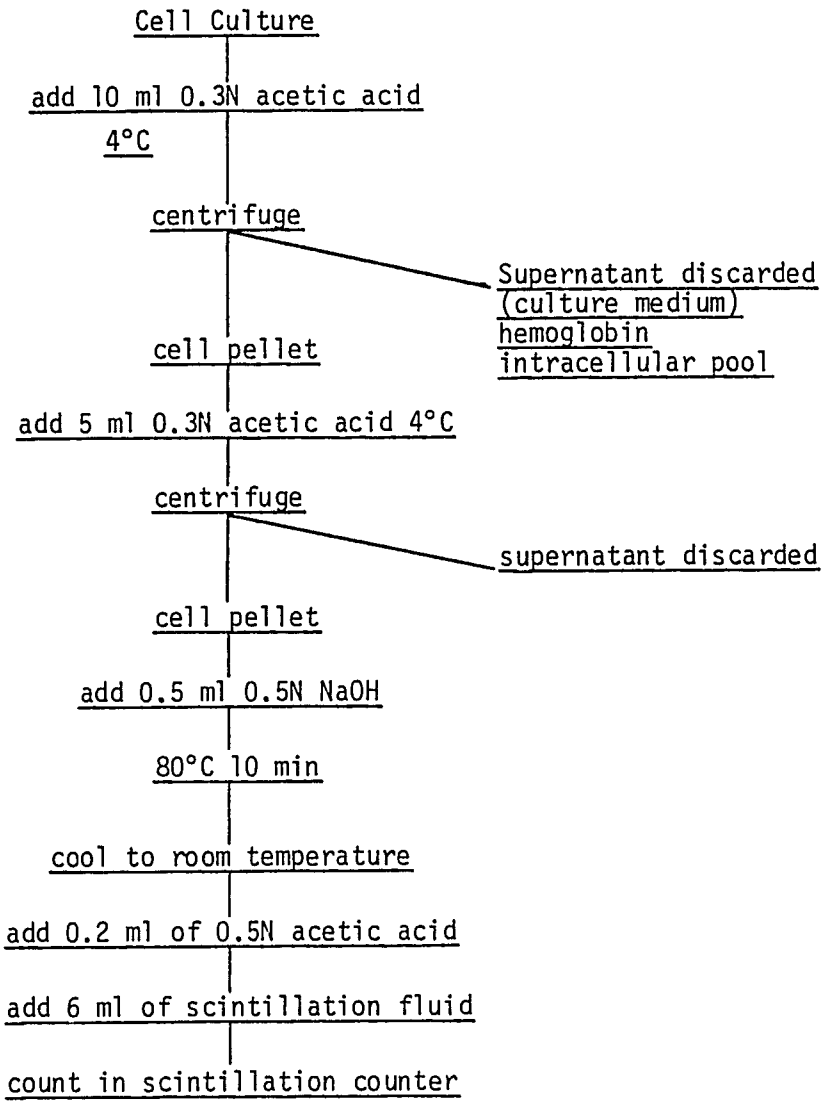


FIGURE 3

MEASUREMENT OF CPM IN DNA USING ACETIC ACID EXTRACTION

immediately to the culture tubes. The tubes were centrifuged as above and the supernatant containing hemoglobin and acid soluble tritium was discarded. This procedure was repeated once. The resultant acid precipitable materials from either of the above procedures were dissolved in 0.5 ml of 0.5 N sodium hydroxide at 80°C for 10 minutes. The solution was left to cool to room temperature and neutralized with 0.2 ml of 5 percent acetic acid. The contents of each tube were then mixed with 6 ml of scintillation fluid and counted at ambient temperature in a Beckman model LSC-230 counter. Counting efficiency was 60% in an unquenched sample.

1) Cell mediated lympholysis (CML) using ^{51}Cr-release

Cells to be used as targets (mesenteric lymph node cells, spleen cells, WBC) for CML were made more susceptible to lysis by culturing them for three days with an optimum concentration of PHA-P to produce blast cells. ^{3}H-Thymidine was added to a small aliquot of these cells for the last 18 hours of culture to determine whether DNA synthesis in response to PHA-P stimulation had occurred. The unlabelled blast cells were washed once in HBSS and resuspended in RPMI-1640 supplemented with 2.5% complement inactivated rabbit serum and 25 mM HEPES buffer. A volume of 0.1 ml of ^{51}Cr (100 µCi) was added to 0.4 ml of cells (2×10^7). These cells were then rotated in an incubator for one hour at 37°C, in an atmosphere of air:CO_2 (95:5). After incubation the cells were washed four times with HBSS. After three washes no further radioactivity

was eluted from the cells. The ^{51}Cr labelled cells were resuspended in the appropriate tissue culture medium at a concentration of 5×10^5 per ml.

WBC to be assayed for cytotoxic activity were taken from eight day MLR cultures or from rabbits that had received skin allografts. Dextran or ficoll-hypaque prepared WBC in the appropriate medium were added to the wells of microculture plates. The highest cell number per well was 5×10^6. Target cells (0.1 ml) were added to doubling dilutions of the cytotoxic cells. The ratio of cytotoxic cells to target cells ranged from 100:1 to 6.25:1. Before incubation the plates were centrifuged at 200 g for five minutes to pellet the cells in the conical wells. The plates were then incubated for 4 hours or 18 hours at 37°C in air:CO_2 (95:5). After incubation the plates were centrifuged at 800 g for ten minutes and 0.1 ml aliquots of the supernatants were removed for gamma counting.

m) Gamma counting of CML supernatants

Aliquots of 0.1 ml were counted for five minutes in a Beckman Biogamma gamma counter.

n) Calculations

The results of an MLR are expressed as either counts per minute (cpm) or as stimulation index (S.I.). The S.I. is the ratio of cpm in the activated culture divided by the cpm in the control culture. Results of cytotoxic activity were expressed as cpm or as the cytotoxic index (C.I.). The C.I. is the ratio of the cpm released by allogeneic cultures minus that of autologous cultures to the total cpm on the target cells minus that released by autologous cultures

and multiplied by 100. All results are based on triplicate cultures unless otherwise noted and all experiments were performed at least three times. All statistical data are based on Student's t-test.

CHAPTER IV

EXPERIMENTAL RESULTS

1. The Establishment of the Optimal Conditions for the MLR

a) Abstract

b) Introduction

c) Results

d) Discussion

i) The optimal culture conditions for the MLR

ii) The inactivation of stimulator cells using mitomycin-C

iii) Other factors affecting the response in the MLR

iv) The identity of the responder cell in the MLR

a) Abstract

Rabbit circulating leukocytes (WBC) have been shown to consistently respond in the allogeneic MLR under the conditions described in this section of results. The medium which permitted the optimum response was RPMI-1640, supplemented with decomplemented rabbit serum (final concentration 2.5 percent). The culture

conditions were 5 percent CO_2 in air at 37°C for 5 to 6 days. The optimum concentration of mitomycin-C required to inactivate the stimulating WBC in the one-way MLR was 50 µg/ml of cells. The results demonstrate that the majority of rabbits possess MLR responsive cells in the circulation, in contrast to the findings of other investigators. The WBC of a minority of the rabbits (18 percent) did not respond in the conventional MLR. Removal of RBC and granulocytes from the responder WBC population enhanced the blastogenic response in the one-way MLR, however RBC alone did not inhibit the response of spleen cells in the one-way MLR. The significance of these findings in relation to the response of rabbit WBC in the MLR is discussed. Whether the MLR responsive cell in the rabbit is a T cell or a T-like cell with B cell properties is still controversial.

b) Introduction

The mixed leucocyte culture reaction (MLR) was first observed by Schrek and Donnelly (1961) and later described in detail by Bain, Vas and Lowenstein (1964) and Hirschorn, Bach, Kolodny, Firschein and Hashem (1963). Blast cell formation and increased DNA synthesis are the parameters used to measure the extent of an MLR following the culture of two allogeneic populations of lymphoid cells. The one-way MLR was introduced by Bach and Voynow (1966) who used mitomycin-C to effectively block DNA synthesis in one of the allogeneic cell populations, thus permitting an accurate evaluation of the MLR responsiveness

of each cell population. It is therefore possible to correlate the MLR with transplantation rejection phenomena in a more accurate manner.

Many findings related to transplantation genetics have been made during subsequent experiments with animals, especially mice (Bach and van Rood, 1976), where T-cells but not B-cells have been shown to respond in the MLR (Bach, Bach and Sondel, 1976). In the rabbit, the spleen and lymph nodes respond well (Chapman and Dutton, 1965; Ozer, Jr., and Waksman, 1974); however, the response of rabbit circulating leukocytes in the MLR has generally been found to be absent or very low (Cohen and Tissot, 1974; Harrison, Wei and Ahie, 1971; Ozer, Jr. and Waksman, 1974; Tissot and Cohen, 1974). This has led to difficulty in the interpretation of results of experiments designed to correlate MLR with allograft histocompatibility in the rabbit. It has consequently been suggested that the circulating leukocytes of the rabbit consist mainly of cells equivalent to the B-cells of mice and are therefore unable to respond in an MLR (Ozer, Jr., and Waksman, 1974).

The purpose of this investigation was to demonstrate that, in general, rabbit circulating leukocytes do indeed respond in the MLR. It should be noted that a minority of allogeneic combinations showed no MLR and this may explain the negative findings of other investigators. The possible effect of rabbit granulocytes on the MLR is also discussed.

c) Results

Table 3 shows the results of MLRs carried out in a number of different media. All cultures were supplemented with 2.5 percent heat inactivated rabbit serum and were incubated for 5 days. This concentration of rabbit serum was found to facilitate an optimal MLR (see below). NCTC 109 and CRML 1066 were markedly inferior in their ability to support an MLR compared to TC 199 and RPMI 1640. In 10 experiments the nutritional qualities of TC 199 and RPMI 1640 seemed equal but a statistical analysis showed a slight superiority of RPMI 1640 (Confidence level >95%)*. Table 4 shows the cpm in the control cultures of three of the experiments presented in Table 3. It can be seen that cells cultured in RPMI 1640 undergo significantly more mitoses than those cultured in the other media. This may be due to activation of cells by RPMI 1640 or greater viability of the cells in this medium. On the basis of these results it was decided to use RPMI 1640 in all subsequent experiments.

The next set of experiments was designed to determine the effect of varying the concentration of rabbit serum on the five day MLR. Table 5 shows that the optimal S.I. is attained at a serum concentration of between one and ten percent. The optimum S.I. was obtained with 2.5 percent serum. There is a marked drop in the S.I. at a serum concentration greater than 10 percent (Table 5)**. Table 6 shows the cpm incorporated into control cultures supplemented with different concentrations of serum.

*Wilcoxon non-parametric test for matched pairs ($z = 1.68$).

**2.5% serum was superior to 1% (Confidence level >95%; $z = 1.82$), and to 5% serum (Confidence level >95%; $z = 2.05$).

TABLE 3

THE MLR USING CIRCULATING RABBIT WHITE BLOOD CELLS. A COMPARISON OF DIFFERENT MEDIA IN THE TWO-WAY MLR

EXP. NO.*	MEDIA RPMI 1640	TC 199	NCTC 109	CRML 1066
1	25**	17	1.7	1.0
2	3	2.5	1.6	1.9
3	31	26	5.0	2.0
4	27	3.3		1.5
5	62	37		3.7
6	24	18		2.1
7	40	14		1.7
8	53	76		
9	25	1.6		
10	18	29		

* The counts per minute of the control cultures of Experiments 1 to 3 are presented in Table 4.

** All the values represent the S.I.s obtained.

TABLE 4

^{3}H-THYMIDINE INCORPORATION INTO THE UNSTIMULATED CONTROL CULTURES OF THE TWO-WAY MLR (EXPERIMENTS 1 TO 3 IN TABLE 3) SUPPLEMENTED WITH DIFFERENT MEDIA

EXP. NO.	MEDIA			
	RPMI 1640	TC 199	NCTC 109	CRML 1066
1*	625**	425	517	568
	3190	922	390	523
2	1786	815	812	694
	816	1068	529	619
3	1206	778	931	702
	667	562	584	648
AVG.	1382	762	627	625

* The two values shown for each experiment represent the cpm of the cells of each participant in the two-way MLR cultured alone.

** Counts per minute (cpm).

TABLE 5

THE EFFECT OF VARYING THE CONCENTRATION OF

RABBIT SERUM ON THE TWO-WAY MLR

EXP. NO.	PERCENT SERUM IN THE CULTURE MEDIUM						
	0.1	1	2.5	5	10	20	30
1	12.0*	15.0	17.0		2.0	2.2	2.0
2	4.5	5.1	3.4		1.2	2.1	1.9
3	10.0	35.0	45.0		39.0	11.0	8.9
4	3.9	6.5	11.0		6.3	2.7	
5		4.9	7.2	4.0			
6		7.4	8.4	5.1			
7		9.6	3.3	3.1			
8			5.9	1.8			
9			75.0	56.0			
10			1.2	1.3			
11			20.0	34.0			

* All the values represent the S.I.s obtained.

TABLE 6

^{3}H-THYMIDINE INCORPORATION INTO THE UNSTIMULATED CONTROL CULTURES OF THE TWO-WAY MLR (EXPERIMENTS 1 TO 3 IN TABLE 5) SUPPLEMENTED WITH SERUM IN DIFFERENT CONCENTRATIONS

EXP. NO.	% SERUM					
	0.1	1.0	2.5	10	20	30
1*	687**	763	297	772	497	616
	722	390	553	364	410	405
2	1852	1072	1505	880	524	644
	2502	1761	1296	1004	438	433
3	918	739	924	718	485	822
	1618	732	720	583	1054	1123
AVG.	1383	909	932	720	568	674

* The two values shown for each experiment represent the cpm of the cells of each participant in the two-way MLR cultured alone.

** Counts per minute (cpm)

These results show on the average a general decrease in cpm as the concentration of serum is increased. The decrease in cpm in control cultures may be due to competitors of ^{3}H-thymidine in the serum diluting the pool of available tritium (Milthorp and Forsdyke, 1973).

The next set of experiments was designed to determine whether pooled serum from our own rabbits supported cultures as well as a commercially available serum (Pentex). Both sera were decomplemented. In 12 experiments comparing the two serum batches under the optimum conditions (medium RPMI 1640, 2.5 percent serum) as described above, pooled serum from our rabbits (mean S.I. = 27) proved significantly better than the commercial preparation (mean S.I. = 12)*.

Table 7 shows the results of 5 time-course experiments covering 3, 4, 5 and 6 days of culture. In three experiments, the MLR had begun to decline by 6 days whereas in two experiments the highest response occurred at 6 days. It was decided to use 5 days as a standard culture period for the MLR.

In order to determine the number of responding cells required for an optimal response in the MLR, it was necessary to establish the conditions for the one-way MLR so that the response of each of the individual allogeneic populations could be analyzed. As mitomycin-C was used to inactivate the stimulator cells, it was first necessary to determine the optimum concentration of mitomycin-C

*Confidence level >95%; Wilcoxon non-parametric test for matched pairs; z = 2.36.

TABLE 7

THE TIME COURSE OF THE RESPONSE OF CIRCULATING RABBIT WHITE BLOOD CELLS IN THE TWO-WAY MLR.

EXP.	TIME (DAYS) 3	4	5	6
1	0.9*	2.0	3.8	1.7
2	1.2	1.5	1.9	2.4
3	1.5	2.3	6.3	13.0
4	6.3	23.0	28.0	9.2
5	10.0	21.0	28.0	3.3

*S.I.

required to treat the cells. Sufficient mitomycin-C must be used to eliminate or effectively limit DNA synthesis but an excess must be avoided as it could be introduced into the culture by the stimulator cells, be released into the medium, and lead to inhibition of the responder cells (Wilson, 1967).

Preparations of lymphoid cells in RPMI-1640 were treated with 25, 50, or 100 µg/ml of mitomycin-C. Aliquots of these cells were stimulated with three different concentrations of the mitogen concanavalin A (Con-A) to determine whether DNA synthesis had been blocked. Cultures were harvested after 3 days. Figure 4 shows that cells not treated with mitomycin-C responded well to Con-A. The best response was observed with 6.25 µg/ml of Con-A; concentrations of 12.5 and 25 µg/ml were only slightly less stimulatory. In cultures containing cells treated with 25 µg/ml of mitomycin-C, incorporation of ^{3}H-thymidine was reduced by 75 to 90 percent, thus indicating that up to 25 percent of the cells were still capable of DNA synthesis. Cultures containing cells treated with 50 or 100 µg/ml of mitomycin-C exhibited almost negligible ^{3}H-thymidine incorporation. These results indicate the cells exposed to 25 µg/ml of mitomycin-C are not satisfactory for use as stimulator cells in the one-way rabbit MLR as they are still capable of incorporating ^{3}H-thymidine to a moderately high level. To determine which preparation of mitomycin-C treated cells was the best in the one-way MLR, cultures were set up using the same cells shown in Figure 4 as stimulators (Bm). The results of this experiment are shown in Figure 5. These results are

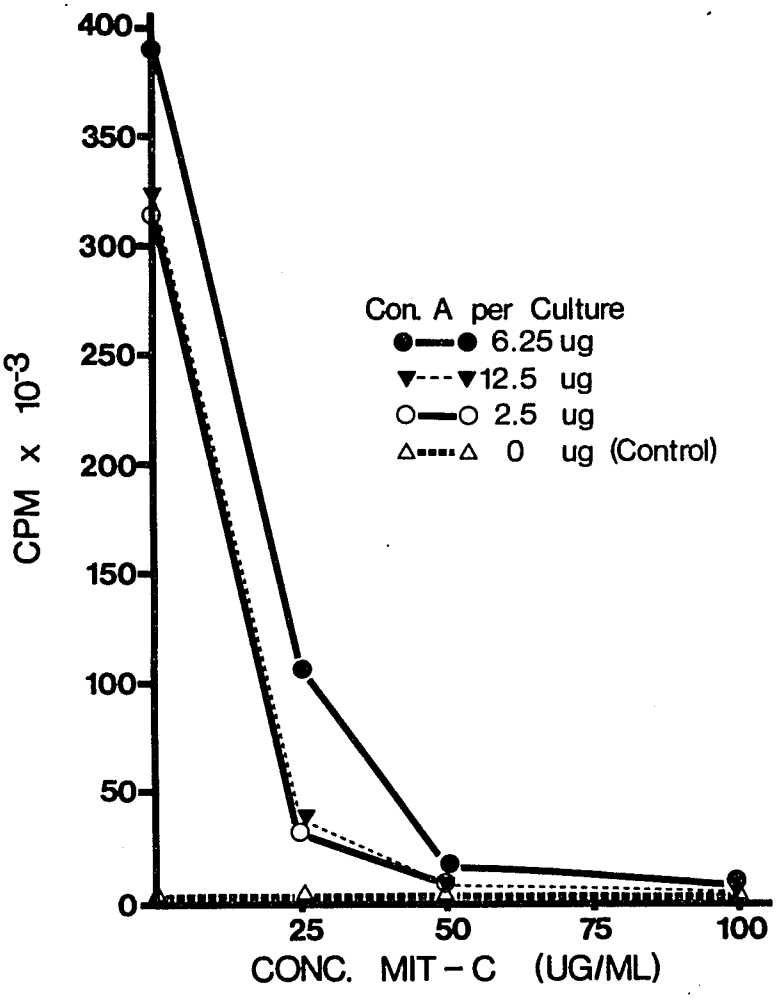


FIGURE 4

THE MITOGENIC RESPONSE OF MITOMYCIN-C TREATED CELLS TO STIMULATION WITH THREE DIFFERENT CONCENTRATIONS OF CONCANAVALIN-A

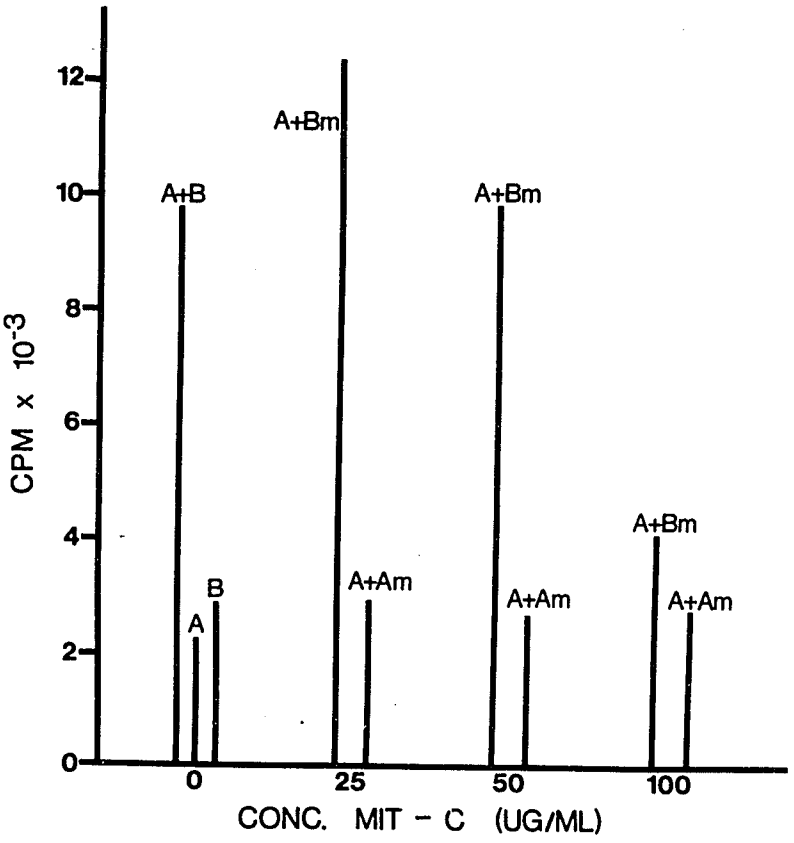


FIGURE 5

THE EFFECT ON THE MLR OF TREATING STIMULATING CELLS WITH DIFFERENT CONCENTRATIONS OF MITOMYCIN-C.

A represents responder cells; Am and Bm represent mitomycin-C treated cells.

representative of four experiments. There appears to be a progressive decrease in the intensity of the one-way MLR with increasing concentrations of mitomycin-C used to treat the stimulator cells. It was observed that, in 3 out of 4 experiments, treatment of the stimulating cells with the low concentration of mitomycin-C (25 µg/ml) seemed to enhance or stimulate the one-way MLR as the cells incorporated significantly more ^{3}H-thymidine than in the two-way MLR using the same cells not treated with mitomycin-C (the controls were not significantly different). The degree of stimulation was greatly reduced in cultures containing stimulator cells treated with 100 µg/ml of mitomycin-C compared with cells treated with 50 µg/ml of mitomycin-C. In control cultures where both cell populations were treated with 50 µg/ml of mitomycin-C, ^{3}H-thymidine incorporation was negligible (200 cpm or less). It is therefore possible that, in cultures using cells treated with 100 µg/ml of mitomycin-C, a residual amount may remain on the cells after washing and leak into the cultures, inhibiting the responder cells (Wilson, 1967). To further verify that treatment of stimulator cells with 100 µg/ml of mitomycin-C inhibited the MLR, a series of experiments were set up using stimulator cells treated with either 50 or 100 µg/ml of mitomycin-C. Table 8 depicts the results of 18 experiments of which 16 are reciprocal experiments in which the cells of each rabbit served as stimulator and responder with respect to the cells of the other. In the majority of the experiments, stimulation of responder cells (A) was higher when incubated with allogeneic cells treated with 50 µg/ml than with 100 µg/ml mitomycin-C. In several experiments, the differences between the S.I.s using the two concentrations of

TABLE 8 THE EFFECT ON THE MLR OF TREATING THE STIMULATOR CELLS WITH DIFFERENT CONCENTRATIONS OF MITOMYCIN-C.

EXP. NO.	Responder Rabbit	Stimulator Rabbit	Two-way MLR		One-way MLR					
					CONC. OF Mit. C(ug/ml)					
					50			100		
			A + B	SI	*A + Am	A + Bm	SI	A + Am	A + Bm	SI
1	1	2	3589**	5.9	204	1044	5.1	294	423	1.4
2	3	4	45717	13.0	2645	6163	2.4	3136	7087	2.2
3	5	6	102898	38.0	2051	114313	56.0	2520	84572	34.0
4	6	5			2249	15530	6.9	1872	13375	7.1
5	7	8	91343	17.0	6245	114265	18.0	7963	50419	6.3
6	8	7			6261	6539	1.0	4800	5262	1.1
7	9	10	78844	28.0	3263	118194	36.0	4704	57071	12.0
8	10	9			3829	44904	12.0	3696	17251	4.7
9	11	12	133551	18.0	1349	4041	3.0	863	659	0.8
10	12	11			829	49643	60.0	1105	15568	14.0
11	13	14	31398	2.0	7356	20079	2.7	7349	15491	2.1
12	14	13			15618	40113	2.6	14701	37252	2.5
13	15	16	75750	7.1	2789	25287	9.1	3740	11308	3.0
14	16	15			2998	26774	8.9	2964	11956	4.0
15	17	18	57320	17.0	3107	10442	3.4	2974	6153	2.0
16	18	17			3196	63897	20.0	3741	48631	13.0
17	19	20	9221	2.2	2735	7227	2.6	3152	2542	0.8
18	20	19			2312	4972	2.2	2371	4076	1.7

* A and B represent the responder and stimulator cells, respectively, used in the MLR. Am and Bm represent mitomycin-C treated cells.

** cpm.

mitomycin-C were slight. It was therefore decided that 50 µg/ml would be used in all further experiments*. It is of interest to note (Table 8) that in a large number of these reciprocal experiments, the response in one direction greatly exceeded the response in the other direction (for example, compare experiments 5 and 6 in Table 8). There was only one experiment in which responses in both directions were equal. In some cases the response in the one-way MLR equalled or exceeded that in the two-way reaction, using the cells from the same rabbits.

Figure 6 shows the results of a typical responder cell dose-response experiment. As the number of responding cells is increased, the ^{3}H-thymidine incorporation increases in a linear fashion. Although a plateau was usually observed with 25×10^5 responder WBC per culture tube, this condition could not usually be attained due to the low yield of leukocytes obtained (10-25%). It was therefore decided to compromise and use 10×10^5 responder cells in subsequent experiments as this number of cells resulted in a response 60 to 80 percent of the maximum. A total of 79 one-way MLRs were performed; 63 percent gave an S.I. greater than 5, and 82 percent gave an S.I. greater than 2. The average S.I. in those experiments showing S.I.s greater than 5 was 40.5. The S.I. used by most investigators to indicate a positive MLR in the rabbit is 2 (Cohen and Tissot, 1974; Ozer, Jr. and Waksman, 1974; Tissot and Cohen, 1974).

*Statistical analysis showed 50 µg/ml of mitomycin-C gave significantly higher S.I.s than 100 µg/ml of mitomycin-C. (Confidence level >95%; $z = 3.57$).

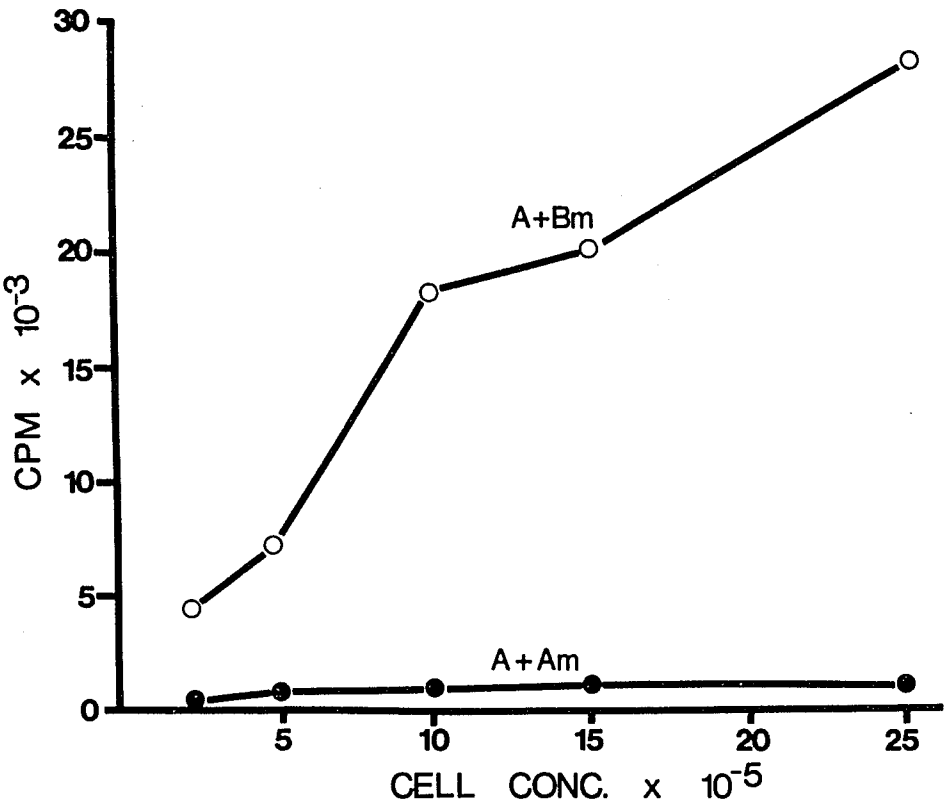


FIGURE 6

THE DOSE RESPONSE CURVE OF CIRCULATING WBC RESPONDING CELLS.

A represents responder cells; Am and Bm represent mitomycin-C treated cells. The stimulating cell concentration is constant at 5 x 10^5 cells.

To determine whether RBC found in the WBC preparations were inhibiting the blastogenic response in the rabbit MLR an experiment was set up in which increasing numbers of RBC were added to a one-way MLR between 5×10^5 responder spleen cells and 5×10^5 mitomycin-C treated allogeneic stimulator spleen cells (Table 9). Spleen cells normally respond well and contain many less RBC than the dextran prepared WBC population. The RBC were autologous to the responder spleen cells and prepared by dextran sedimentation. The number of WBC in the RBC preparation were negligible and were two orders of magnitude lower than the number of spleen responder cells in the cultures. No MLR were recorded when the RBC preparations were incubated with the mitomycin-C treated allogeneic spleen cells. It can be seen that the addition of RBC to the MLRs had little effect on the S.I.s.

In addition responder WBC were prepared with most of the contaminating RBC removed. Table 10 illustrates the results of MLRs using responder cells isolated from dextran supernatants or from ficoll-hypaque interfaces. The stimulator cells were mitomycin-C treated allogeneic spleen cells. The maximum S.I. in the MLR was found using responder cells prepared from the ficoll-hypaque interface. It should be noted that ficoll-hypaque preparation also removes granulocytes from the WBC preparation.

TABLE 9

THE EFFECT OF AUTOLOGOUS RBC ON THE RESPONSE OF SPLEEN CELLS IN THE ONE-WAY MLR

RBC added to culture x 10^7**	S.I.*	Control cpm
0	35	313
2.4	22	263
4.8	22	274
9.6	37	311

* The MLR consisted of 5 x 10^5 responder spleen cells (including 1.2 x 10^6 RBC) and 5 x 10^5 mitomycin-C treated allogeneic stimulator spleen cells (including 1 x 10^6 RBC) in a one ml culture.

** The numbers of RBC used were equivalent to the number present in preparations of 1, 2 and 4 x 10^6 WBC isolated using dextran sedimentation.

TABLE 10

THE RESPONSE OF DEXTRAN PREPARED AND FICOLL-HYPAQUE PREPARED RESPONDER CELLS TO SPLEEN CELLS IN THE ONE-WAY MLR.

Responder cell concentration x 10^5*	MLR response (S.I.) Stimulator Cells**	
	Dextran preparation	Ficoll-hypaque preparation
2.5	1.1	3.2
5.0	2.0	6.0
10.0	2.5	1.2
12.5	2.3	0.5

* The stimulator cell concentration was 1 x 10^6 cell per ml. Stimulator cells were treated with mitomycin-C.

** Each number represents a triplicate sample.

TABLE 11

THE DIFFERENTIAL CELL COUNT IN PREPARATIONS OF WHOLE BLOOD, DEXTRAN SEPARATED LEUKOCYTES AND FICOLL-HYPAQUE INTERFACE LYMPHOCYTES

Preparation	Percentage*		
	monocytes	granulocytes	lymphocytes
Whole blood	3.2	39.5	57.3
Dextran separation	1	16.4	83.3
Ficoll-hypaque interface	2	1	98.4

* These differential cell counts were performed on the cell preparations used in the experiment illustrated in Table 10.

The differential cell counts in preparations of whole blood, dextran supernatants and cells from the ficoll-hypaque interface (Wright's stain) prepared from the same rabbit are presented in Table 11. These cell preparations were utilized in the experiment shown in Table 10. The ficoll-hypaque preparation had far fewer granulocytes than the dextran preparation, and although not always the case, this particular preparation of dextran isolated cells had a higher concentration of lymphocytes and a lower number of granulocytes than did the whole blood. In most cases the differential cell count in preparations of whole blood and dextran isolated leukocytes are equivalent (Table 1).

d) Discussion

The purpose of this investigation was to demonstrate the responsiveness of circulating rabbit WBC in the MLR. The results indicate that, under the conditions used in these experiments, rabbit peripheral blood contains cells capable of responding with blastogenesis in the MLR and these responses are comparable to those observed with human and mouse cells.

i) The optimal culture conditions for the MLR

It was found that medium RPMI-1640 was the best for sustaining the MLR for a five day period (Table 3). This medium also permitted maximum incorporation of ^{3}H-thymidine into unstimulated control cultures. It is possible that the

components of RPMI-1640 increase the viability of the cells and thus enhance the MLR, that this medium stimulates cells leading to mitosis, or that this medium may contain fewer competitors of ^{3}H-thymidine than the other media (Milthorp and Forsdyke, 1973). It should be noted that the latter two possibilities would increase apparent blastogenesis in both stimulated and control cultures resulting in S.I.s no greater than found using other media. It is likely therefore that RPMI-1640 increases the viability of the cultured cells compared to the other media.

The final serum concentration (2.5 percent) used in the culture medium was substantially lower than that used by other investigators (Cohen and Tissot, 1974; Harrison, Wei and Ahie, 1971; Lindh, 1973; Tissot and Cohen, 1974) who employed 15 to 30 percent serum in their cultures. These higher concentrations of serum were found to be inhibitory in our cultures (Table 5) and this might explain some of the negative results obtained by other investigators. Some commercial preparations of rabbit serum were found to be toxic to the rabbit WBC even after heat inactivation, and some preparations of heat inactivated fetal calf serum were found to support the rabbit MLR as effectively as our own preparations of rabbit serum.

The observation that the optimal time of response in the MLR was between 5 and 6 days is similar to that found in man (Schrek and Donnelly, 1961).

ii) The inactivation of stimulator cells using mitomycin-C

The findings concerning the effects of varying the concentration of mitomycin-C used to inactivate the rabbit MLR stimulator WBC, are in agreement with those of Etheredge, Shons, Hohenthaner and Najarian (1973) using human cells. The pretreatment of rabbit WBC with a concentration of 25 µg/ml of mitomycin-C was not adequate to suppress the mitosis of rabbit cells in the presence of the mitogen concanavalin A (Con-A; Figure 4). The use of excess mitomycin-C (100 µg/ml) to treat the MLR stimulating cells suppressed the MLR response (Figure 5). This is in agreement with the findings of Wilson (1967) who, using F_1 cells as stimulators in the MLR, suggested that the mitomycin-C treatment inhibited the rat MLR due to possible leakage of the drug into the culture medium.

The observed "enhancing" effect of 25 µg/ml of mitomycin-C which led to cpm in the one-way cultures being greater than in the two-way cultures (Figure 5) may be similar to the phenomenon observed by Etheredge, Shons, Hohenthaner and Najarian (1973), who observed, in human MLR cultures, that the addition of mitomycin-C treated cells autologous to the responder cells in control MLR cultures resulted in a substantial increase of ^{3}H-thymidine incorporation. It was suggested that mitomycin-C induced the secretion of mediators from the treated cells stimulating mitosis in the untreated cells. This may be occurring in rabbit MLRs using stimulator cells pretreated with 25 µg/ml of mitomycin-C.

It is also possible that, in our stimulated cultures the low concentrations of mitomycin-C preferentially inhibited the functioning of suppressor cells compared to those cells undergoing blastogenesis thus allowing a more vigorous response.

Another explanation for the superior blastogenic response in the one-way MLR using stimulator cells pretreated with low concentrations of mitomycin-C may be related to the generation of specific cytotoxic cells in culture. Cytotoxic cells have been found to be generated in the MLR (Fitch, Engers, Cerottini and Brunner, 1976). In a two-way MLR reaction, cytotoxic cells could be produced to both responding populations, limiting the blastogenic response as some of the responding cells would be eliminated from culture. In contrast, in the one-way reaction in mice few or no cytotoxic cells are generated in the mitomycin-C (25 μg/ml) pretreated stimulating cell populations in the MLR (Cantor and Jandinski, 1974); thus the responding cells would not be eliminated from the culture. This may be occurring in the rabbit MLR, allowing greater blastogenesis in the one-way reaction. However, the destruction of the stimulating cell population in the MLR (which would also include responder cells in the two-way MLR), has not been found to be mediated by previously uncultured cells (Fitch, Engers, Cerottini and Brunner, 1976) and therefore this mechanism cannot be considered as likely to apply to the MLR rabbit system utilized in this study.

iii) Other factors affecting the response in the MLR

It was noted that large differences occur in the intensity of the response (S.I.) from one set of rabbits to the next, and also between the same two rabbits in the one-way MLR depending on which is used as the donor of responder cells (Table 8). These findings are undoubtedly due to genetic disparity (Chai and Lerner, 1975), and also indicate that the polarity of the experiment is important in the interpretation of the results.

Eighteen percent of the MLRs performed using rabbit WBC resulted in no blastogenic response. It is unlikely that 18 percent of our outbred rabbits are genetically compatible. In most cases this lack of response is evident only in the WBC as cells of the other organs in the rabbit, such as the spleen, respond well in the MLR. This is in agreement with the findings of Ozer, Jr. and Waksman (1974). To possibly explain this lack of response in the MLR of the WBC from the minority of rabbits tested, the effect on the MLR of RBC and granulocytes was investigated.

It is unlikely that the large number of RBC found in the WBC preparations isolated by dextran sedimentation inhibit the MLR response as this is not the case in the one-way MLR using spleen responder cells (Table 9). The human MLR is also not inhibited by RBC (Bain and Pshysk, 1972).

It is possible that some rabbits have an excess number of MLR suppressor cells in the WBC population. It has been demonstrated that suppressor cells capable of inhibiting the rabbit

humoral immune response (Luzzati and Lafleur, 1976; Kamin, Henry and Fudenberg, 1974), and the rabbit mitogenic response (Shek, Chou, Dubiski and Cinader, 1975; Chou, Cinader and Dubiski, 1977) are found in the rabbit WBC population. Table 10 shows that ficoll-hypaque isolated lymphocytes respond to a greater extent in the MLR than do dextran prepared cells. This enhanced response was not due to the higher number of lymphocytes present in the ficoll-hypaque cell preparation as dose response experiments showed that increasing the number of dextran prepared responder cells did not produce a blastogenic response approaching the optimum response given by ficoll-hypaque prepared cells. One of the major differences between these two cell preparations is that the ficoll-hypaque isolated cells contain not only far fewer RBC but also a much lower number of granulocytes (Table 11). It is possible that the granulocytes inhibit the rabbit MLR. This is in agreement with the findings of Chapman and Dutton (1965) who have shown that large numbers of alveolar macrophages added to the rabbit MLR inhibit the blastogenic response. Using human leukocytes, Bain and Pshysk (1972) have shown that the removal of neutrophils (granulocytes) on a ficoll-hypaque gradient greatly enhances the blastogenic response in the MLR. The same observation has been made by Ragab and Cowan (1973) and Mardiney, Block and Chess (1972).

Alternately, unresponsive rabbit WBC populations may lack a helper cell required for the MLR. In mice, cortisone resistant thymus cells will only respond in the MLR when supplemented by a B-like helper cell from peripheral organs (Dyminski and Smith, 1975). Thymus cells have been shown to act as helper cells in the mouse MLR when added to responder cells from the lymph nodes (Howe and Cohen, 1975). However, no evidence for helper cells participating in the rabbit MLR has been found.

iv) The identity of the responder cell in the MLR

Some investigators have claimed that rabbit WBC give a poor or negligible MLR (Cohen and Tissot, 1974; Harrison, Wei and Ahie, 1971; Ozer, Jr. and Waksman, 1974; Knight, Walker and Ling, 1971; Sheppard, Jr., Sell, Poler and Redelman, 1977). It has been suggested that rabbit peripheral blood does not contain a cell capable of responding in the MLR (a T cell) as compared to human, mouse and most other species, and that rabbit WBC are B-like (in an analogy to mice) in nature (Ozer, Jr. and Waksman, 1974). B cells in the mouse and in man do not respond in the MLR (Bach, Bach and Sondel, 1976). In support of this theory that T cells are absent in the rabbit circulatory cells, it has been claimed that WBC are devoid of the T cell specific antigen characteristics of thymus cells. Colas de la Nöue, Koperstych and Richter (1972) observed that less than one percent of WBC could be lysed in the presence of specific anti rabbit thymocyte serum and complement. In subsequent investigations, it was demonstrated that 20 to 40

percent of the WBC are lysed by bone marrow specific antiserum and complement (Colas de la Noue and Richter, 1974a) and more than 50 percent of the WBC are lysed by appendix specific antiserum and complement (Colas de la Noue and Richter, 1974b). Thus from an antigenic point of view the WBC in the rabbit seem to be mainly composed of cells possessing bone marrow and appendix derived antigens, and very few cells bearing thymus derived antigens (Richter, Colas de la Noue and Hamdy, 1975).

In contrast to the findings of Richter's group, however, Fujiwara, Armstrong and Cinader (1974) and Cavaillon, Bona, Cazenave and Cinader (1977) have claimed, using anti-rabbit thymocyte serum and complement, that approximately 50 percent of rabbit WBC possess thymus derived cell surface antigens. The dichotomy between the findings of the two groups of investigators may be explained by the fact that they were using different preparations of anti-thymocyte sera. To add to the confusion, it has been suggested that the majority of rabbit WBC are T-cells, as they respond with blastogenesis to the T-cell mitogen PHA, but also bear surface immunoglobulins giving the appearance of B cells (Sell and Sheppard, 1973).

From a functional point of view it is quite obvious that rabbit WBC do contain cells capable of responding in the MLR in contrast to the previous claim of Ozer, Jr. and Waksman (1974). It is also interesting to note that Sheppard, Jr., Sell, Poler

and Redelman (1977) using anti-rabbit thymocyte serum and complement found that the response of WBC in the MLR was eliminated. This suggests that the rabbit MLR responder cell has thymus derived antigens on its surface, and that cells without this antigen do not respond in the MLR. However, this finding must be evaluated with caution as no indication was given by the authors that the anti-thymocyte sera had been absorbed with anything other than red blood cells. It is possible that this serum is active against bone marrow and appendix derived cells. In conclusion it can only be said that the classification of cells into B and T cells in the rabbit may be quite different compared to that in the human or the mouse.

2. The Distribution of the MLR Responder Cells Among the Different Lymphoid Organs

a) Abstract

b) Introduction

c) Results

d) Discussion

i) The lack of response of bone marrow and thymus cells in the MLR.

ii) The response of the cells of the remaining lymphoid organs in the MLR.

iii) Helper and suppressor cells in the MLR.

iv) Cytotoxic cells in the MLR.

v) The influence of the duration of the cell cycle on the MLR response.

vi) The ability of cells to detect stimulatory determinants in the MLR.

vii) The classification of rabbit MLR responder cells.

viii) The autologous MLR response, and the spontaneous incorporation of ^{3}H-thymidine by rabbit lymphoid cells.

a) Abstract

The organ distribution and kinetics of response in the one-way MLR of the cells of the various rabbit lymphoid organs were investigated. Mitomycin-C treated rabbit bone-marrow cells or white blood cells were used as a source of stimulating cells. The culture conditions used were those shown to be optimal for the response of WBC in the MLR. It was found that the cells of all organs responded optimally on day 5 of culture. Cells of the thymus and bone marrow cultured individually or in combination did not respond at all. The optimal response of cells responding in the MLR was dependent on the concentration of these cells. Peyer's patches and mesenteric lymph node cells responded optimally at 2.5 to 5 x 10^5 cells per culture; spleen cells and WBC at 10 to 15 x 10^5, and sacculus rotundus and appendix cells at 20 to 25 x 10^5. The greatest magnitude of response was attained with cells of the Peyer's patches and mesenteric lymph nodes. The lowest response was found using cells of the appendix.

b) Introduction

Numerous studies of the responder activities of lymphoid cells in the rabbit MLR have been carried out (Chapman and Dutton, 1965; Knight, Walker and Ling, 1971; Ozer, Jr. and Waksman, 1974). However, these results are difficult to evaluate as no systematic study of responder cell activity utilizing time course and dose response analyses have been performed. In the human and the mouse

the MLR responder cell has been identified as a T-cell (Bach and van Rood, 1976; Bach, Bach and Sondel, 1976). It is likely that the rabbit responder cell in the MLR is a T-cell as the MLR response of rabbit WBC has been abrogated using anti-thymus cell serum and complement (Sheppard, Jr., Sell, Poler and Redelman, 1977).

The experiments reported here were designed to characterize the responder activities of the cells of each of the different lymphoid organs (WBC, thymus, spleen, bone marrow, mesenteric lymph nodes, sacculus rotundus, appendix and Peyer's patches). These results will provide a background for more extensive studies of these lymphoid organs as sources and/or reservoirs and/or sites of maturation of the MLR responder cells, and also for the elucidation of migration pathways of responder cells during the allograft rejection reaction. These experiments also constitute the basis for an extensive study of MLR-associated cytotoxicity in the rabbit and the relation of the MLR to the generation of specific cytotoxic cells in the allograft rejection reaction.

c) Results

Table 12 shows results of five separate one-way MLR experiments. Each experiment measured the effect of varying the responder cell concentration in the 5-day MLR. The organs analyzed were spleen, bone marrow, thymus, Peyer's patches and appendix. Each of the

TABLE 12

THE EFFECT OF VARYING THE RESPONDER CELL CONCENTRATION ON THE ONE-WAY MLR RESPONSE (S.I.) TO MITOMYCIN-C TREATED ALLOGENEIC WBC ON DAY 5 OF CULTURE.

ORGAN	EXPT. NO.	CELL CONCENTRATION X 10^5				
		2.5	5.0	10	25	50
Spleen	1	13.0	44.0	59.0	22.0	8.0
Bone Marrow	2	1.2	0.9	0.7	0.9	1.2
Peyer's patches	3	93.0	51.0	19.0	3.9	2.1
Appendix	4	0.6	2.9	8.4	26.0	21.0
Thymus	5	1.1	1.2	1.1	1.3	1.6

experiments shown was repeated at least three times. The stimulating cells in all cases were allogeneic circulating white blood cells (5×10^5 per culture) treated with 50 µg/ml of mitomycin-C, a concentration shown previously to optimally facilitate the one-way MLR with rabbit cells. Each organ exhibited a unique dose-response curve. The number of spleen responder cells required for an optimal MLR (Table 1) was 10×10^5. Thymus and bone marrow cells did not respond at all. Peyer's patches cells exhibited a maximal S.I. at the lowest number of responder cells used (2.5×10^5), whereas the optimal MLR response with responder appendix cells was obtained at a responder cell concentration of 25×10^5 cells. Table 13 shows the responder cell concentration required for the optimal response of WBC, spleen, appendix and Peyer's patches cells from several different experiments. There is some variation in the concentration of the responder cells required to give an optimal MLR response. However, the variation is minimal considering the fact that the rabbits are outbred.

Table 14 shows the cpm recorded for the experiments outlined in Table 12. Results are shown for the allogeneic culture, the autologous control, and the responder cells alone. In the case of the responder spleen, the optimal S.I. (Table 12) corresponds to the cell concentration with the highest cpm in the allogeneic culture (Table 14). The cpm incorporated into the autologous control (A + Am) increased only minimally over a 20 fold increase in responder cell concentration. However, with responder spleen cells

TABLE 13

VARIATION AMONG DIFFERENT RABBITS WITH RESPECT TO THE CONCENTRATION OF RESPONDER CELLS REQUIRED FOR OPTIMAL ONE-WAY MLR RESPONSE TO MITOMYCIN-C TREATED WBC ON DAY 5 OF CULTURE

CELLS OF ORGAN TESTED*	CONCENTRATION OF RESPONDER CELLS X 10^5				
	2.5	5.0	10	25	50
WBC		1**		2	
Spleen			2	1	
Peyer's patches	2	1			
Appendix			1	2	

* Bone marrow and thymus do not respond to stimulation with allogeneic mitomycin-C treated WBC.

** Each number represents the number of experiments which gave an optimal MLR response (S.I.) at the concentration indicated.

TABLE 14

THE EFFECT OF VARYING THE RESPONDER CELL CONCENTRATION ON THE ONE-WAY MLR RESPONSE (CPM) TO MITOMYCIN-C TREATED ALLOGENEIC WBC ON DAY 5 OF CULTURE*.

ORGAN	EXPT. NO.	CELLS CULTURED	CELL CONCENTRATION X 10^5				
			2.5	5.0	10	25	50
Spleen	1	A+Bm	8685	24134	35895	16643	6604
		A+Am	685	552	611	740	831
		A alone	925	1198	1746	2431	2011
Bone marrow	2	A+Bm	3325	6465	5957	4621	2842
		A+Am	2832	7418	8164	4953	2287
		A alone	2879	5260	6752	10552	3683
Peyer's patches	3	A+Bm	70915	98160	145955	159513	136295
		A+Am	762	1908	7500	40830	65132
		A alone	148	195	348	4487	31483
Appendix	4	A+Bm	112	286	1466	34735	67449
		A+Am	174	100	175	1348	3178
		A alone	139	348	156	1143	8056

* cpm represent the data of experiments 1 to 4 presented in Table 1.

** A and B represent the responder and stimulator cells, respectively. Bm and Am represent mitomycin-C treated WBCs.

A+Bm represents the allogeneic culture.

A+Am represents the autologous control.

A alone represents responder cells with no mitomycin-C treated cells added.

cultured alone, there is a two-fold increase in cpm as the concentration of cells was increased from 2.5 x 10^5 to 50 x 10^5.

Bone marrow cells (Table 14) showed very little difference in thymidine incorporation on day 5 in either allogeneic or autologous cultures. Responder cells alone seemed to incorporate approximately the same cpm as cells incubated with mitomycin-C treated stimulators.

The maximum S.I. for Peyer's patches cells was found at the lowest cell concentration used (Table 12); however, this was not equivalent to the highest cpm incorporated in the allogeneic MLR (Table 14). In the case of both the autologous MLR (Peyer's patches cells plus autologous WBC) and Peyer's patches cells cultured alone, there was a dramatic increase in the spontaneous incorporation of tritium when the cell concentration was increased above 10 x 10^5 per culture.

With respect to the appendix cells (Table 14) there was little increase in cpm in the allogeneic MLR until a concentration of 25 to 50 x 10^5 cells per culture was reached. The autologous control MLR and responder cells alone also showed an increase in cpm but this did not generally occur until a concentration greater than 10 x 10^5 responder cells per culture was attained.

Table 15 shows the cpm in "responder" thymus cells on day 5 of culture in three different experiments, using three different concentrations of thymus cells. There is a slight response in

TABLE 15

THE RESPONSE OF THYMUS CELLS, AT DIFFERENT CONCENTRATIONS, IN THE ONE-WAY MLR ON DAY 5 OF CULTURE.

EXPERIMENT NO.	CELL CONCENTRATION X 10^{5}*					
	5		25		50	
	A+Am**	A+Bm	A+Am	A+Bm	A+Am	A+Bm
1	556	738	956	2771	1632	4182
2	269	316	972	1213	1533	2378
3	872	500	626	663	861	2819

* Stimulating cells are mitomycin-C treated circulating WBC.

** A and B represent the responder and stimulator cells, respectively. Am and Bm represent mitomycin-C treated WBC.

A+Bm represents the allogeneic culture.

A+Am represents the autologous control.

the MLR at the highest concentration of thymus cells used (50×10^5 per tube), but the S.I. is three or less. The cpm in the control cultures are quite low. Furthermore, there was no significant response by thymus cells in the MLR on day 3 or day 7 of culture.

Figure 7 shows the cpm in "responder" bone marrow cells (10×10^5 per culture) at days 3, 5 and 7 of an MLR culture. It can be seen that no allogeneic MLR response is evident. Maximum incorporation of ^{3}H-thymidine occurs on day 3.

Figure 8 shows cpm in responder spleen cells (10×10^5 responder cells per culture) at days 3, 5 and 7 of an MLR culture. It can be seen that the maximum incorporation of tritium into the allogeneic MLR occurred on day 5 and that by day 7 the tritium incorporation had dropped almost to the level of the controls. In this experiment, the tritium incorporated into the responder cells alone (A) was greater than that incorporated into the autologous MLR cultures (A + Am). There was a general decrease in the incorporation of tritium into control cultures over the time period studied.

Figure 9 shows a typical MLR time course experiment with responder appendix cells (25×10^5 cells per culture). There is a linear increase in cpm in the allogeneic MLR (A + Bm) during the time period studied. In other experiments a plateau in the incorporation of tritium was found between days 5 and 7.

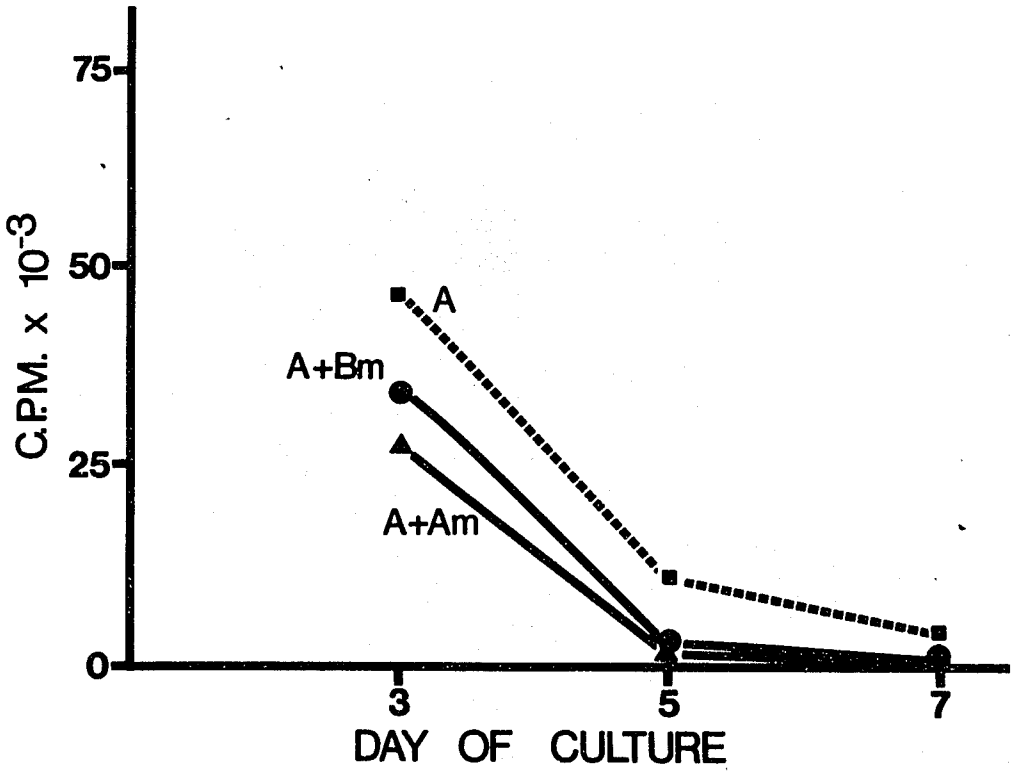


FIGURE 7

THE INCORPORATION OF ^{3}H-THYMIDINE BY RESPONDER BONE MARROW CELLS IN THE ONE-WAY MLR AT DAYS 3, 5 AND 7 OF CULTURE.

A represents responder cells; Am and Bm represent mitomycin-C treated cells.

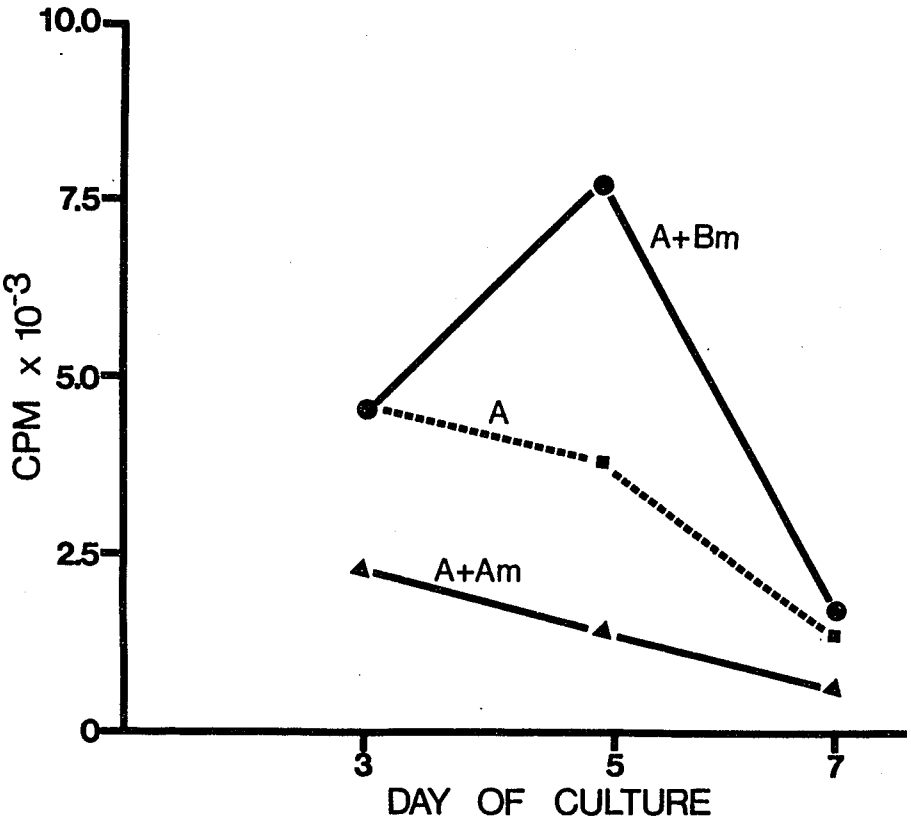


FIGURE 8

THE INCORPORATION OF ^{3}H-THYMIDINE BY RESPONDER SPLEEN CELLS IN THE ONE-WAY MLR AT DAYS 3, 5 AND 7 OF CULTURE.

A represents responder cells; Am and Bm represent mitomycin-C treated cells.

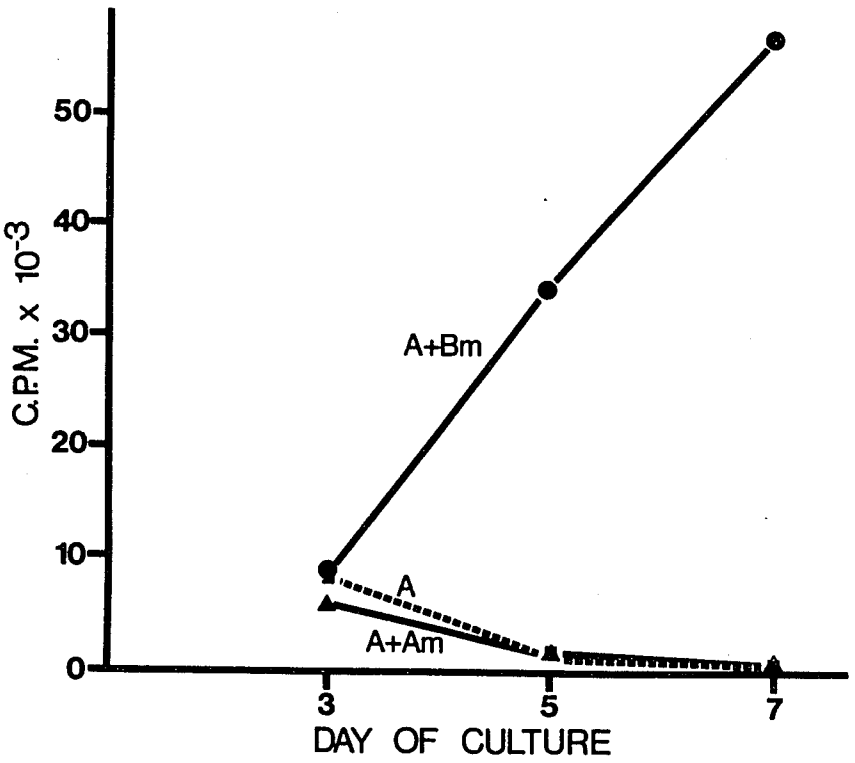


FIGURE 9

THE INCORPORATION OF ^{3}H-THYMIDINE BY RESPONDER APPENDIX CELLS IN THE ONE-WAY MLR AT DAYS 3, 5 AND 7 OF CULTURE.

A represents responder cells; Am and Bm represent mitomycin-C treated cells.

It can be seen that the cpm in control cultures gradually decreased between days 3 and 7 of culture and that there was little difference between responder cells alone (A) and responder cells in the presence of autologous mitomycin-C treated circulating white blood cells (A + Am).

Figure 10 shows the cpm in an MLR time-course experiment with responder Peyer's patches cells (5×10^5 cells per culture). It should be noted that very little spontaneous incorporation of tritium by control cultures occurs at this cell concentration (Table 14). The optimal incorporation into Peyer's patches cells in the MLR occurred on day 5 (A + Bm). By day 7 the amount of tritium incorporated had dropped by 50 percent. There was little difference in cpm between responder cells alone (A) and responder cells in the presence of autologous mitomycin-C treated circulating white blood cells (A + Am).

In all of the experiments described above, mitomycin-C treated WBCs were used as the stimulating cells. However, the yield of WBCs was never sufficient to permit the analysis of responder cell activity of more than one organ at a time. In order to compare the responder cell activity of all the organs from a single rabbit, bone marrow cells (available in large quantities from individual rabbits) were used as stimulating cells. It has been found that bone marrow cells are as stimulatory as circulating WBCs at the same cell concentration (5×10^5 cells per culture) used in the one-way MLR. (See Section 3 of this chapter).

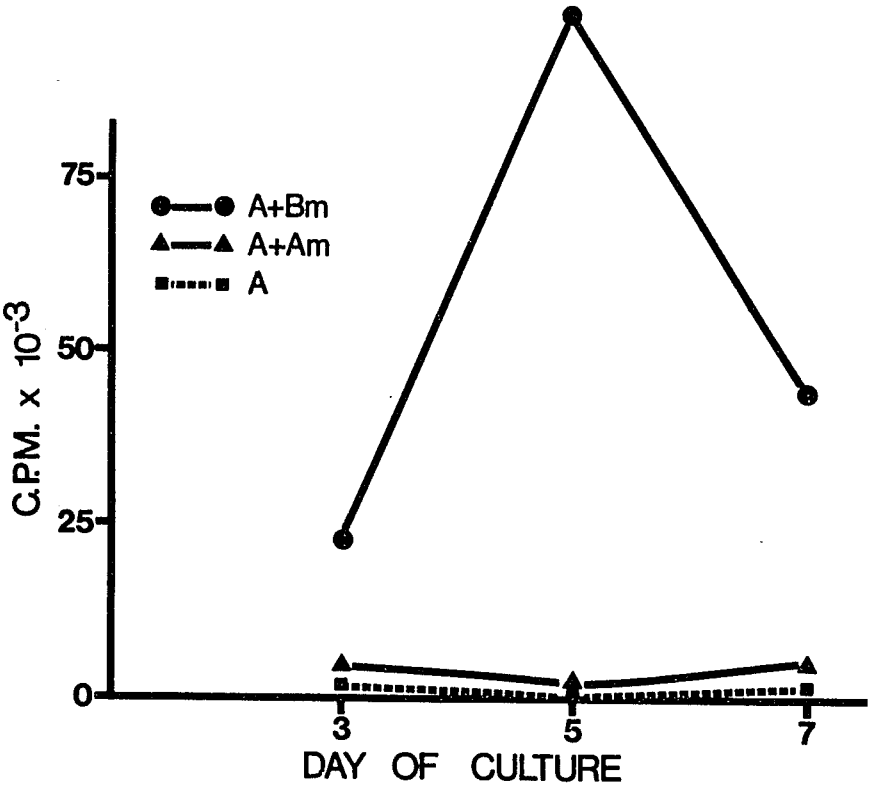


FIGURE 10

THE INCORPORATION OF ^{3}H-THYMIDINE BY PEYER'S PATCHES CELLS IN THE ONE-WAY MLR AT DAYS 3, 5 AND 7 OF CULTURE. A represents responder cells; Am and Bm represent mitomycin-C treated cells.

Table 16 depicts the average yield of white cell separated from the bone marrow obtained from two femurs in 12 experiments ($5.3 \pm 21 \times 10^8$ cells) as compared to the yield of WBC obtained from 70 to 80 ml of whole rabbit blood in 16 experiments ($1.5 \pm 0.4 \times 10^8$ cells). The recovery of white cells using dextran is quite low (between 10-25%). It can be seen that, on the average, the bone marrow yields 4 to 5 times the number of white cells that can be obtained from the blood. For all experiments involving MLRs in which cells from a number of organs were tested simultaneously, bone marrow was used as the source of stimulator cells.

Figure 11 shows the results of an MLR experiment using various concentrations of spleen, WBC, mesenteric lymph node, sacculus rotundus, Peyer's patches and appendix cells on day 5 using mitomycin-C treated allogeneic bone marrow as the source of stimulator cells. The results for the thymus and bone marrow responder cells are not included as no response to mitomycin-C treated bone marrow stimulator cells was observed. Popliteal lymph node cells were not included in this experiment as the yield of cells from any one rabbit was inadequate for this type of experiment. However, this organ does respond in the MLR (Ozer, Jr. and Waksman, 1974). The WBC, spleen, Peyer's patches and appendix cells responded to bone marrow stimulator cells in a manner similar to their response to stimulator mitomycin-C treated allogeneic WBCs (Table 12). Sacculus rotundus and mesenteric lymph node cells (not tested previously

TABLE 16

THE YIELD OF NUCLEATED CELLS
FROM BONE MARROW AND PERIPHERAL BLOOD

ORGAN	NO. OF EXPTS.	AVERAGE CELL YIELD ± S.E.M.
Bone Marrow*	12	$5.3 \pm 2.1 \times 10^8$
Blood** (dextran)	16	$1.5 \pm 0.4 \times 10^8$

* Marrow was removed from two femurs.

** Leukocytes were removed from 70ml of blood by dextran separation.

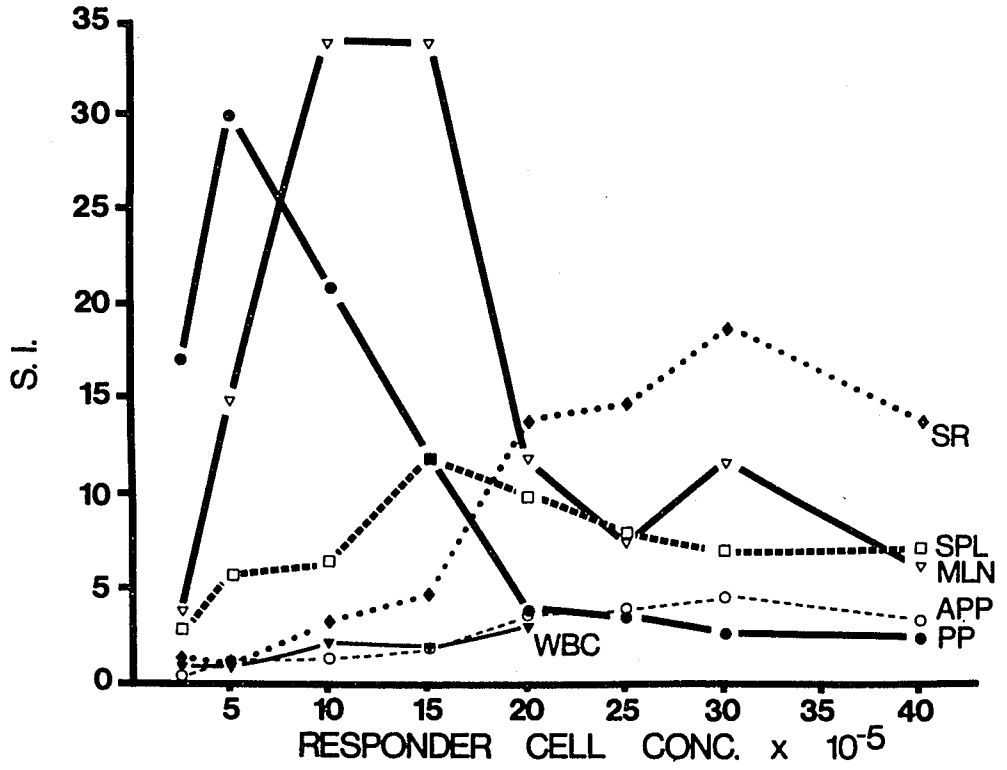


FIGURE 11

A COMPARISON OF RESPONDER CELL ACTIVITY IN THE ONE-WAY MLR OF LYMPHOID CELLS OF THE DIFFERENT LYMPHOID ORGANS OF THE SAME RABBIT.

The stimulating cells were mitomycin-C treated allogeneic bone marrow cells (5×10^5 cells per culture).

against stimulator WBCs) showed their own characteristic dose response curves. The mesenteric lymph node cells show an optimal S.I. at 10 to 15 x 10^5 responder cells per culture whereas the sacculus rotundus cells showed optimal responses at 25 to 40 x 10^5 responder cells per culture. In most experiments the magnitude of the response (S.I.) of mesenteric lymph node cells was quite large compared to that of the other lymphoid organs.

Table 17 shows the cpm in the cells used in the experiment shown in Figure 5. As with WBCs as stimulator cells (Table 14), control cultures of Peyer's patches cells show increased spontaneous thymidine incorporation at a cell concentration greater than 5 x 10^5 cells per culture. A similar but less marked spontaneous DNA synthesis can be noted with mesenteric lymph node cells. Sacculus rotundus cells appear to react in a manner similar to responder appendix cells in their incorporation of tritium into control cultures at responder cell concentrations greater than 20 x 10^5 cells per culture. There is a disproportionate rise in cpm in relation to increasing cell numbers.

Maximum incorporation of ^{3}H-thymidine into stimulated cultures occurred with Peyer's patches cells at a responder cell concentration of 25 x 10^5 per culture; however, this was not at the maximum S.I. (Figure 11). With the three gut associated organs - sacculus rotundus, appendix and Peyer's patches, and the

TABLE 17

THE EFFECT OF VARYING THE RESPONDER CELL CONCENTRATION ON THE ONE-WAY MLR RESPONSE (CPM) TO MITOMYCIN-C TREATED ALLOGENEIC BONE MARROW CELLS ON DAY 5 OF CULTURE*.

ORGAN	CULTURE	RESPONDER CELL CONCENTRATION X 10^5 (PER CULTURE)							
		2.5	5.0	10	15	20	25	30	40
WBC	A+Am	1790	1609	1518	1554	1156	N.D.**	N.D.	N.D.
	A+Bm	1570	1422	3392	3147	3549			
Spleen	A+Am	1589	2573	3857	4531	3710	3912	4112	3677
	A+Bm	4498	15064	25150	52096	37749	31748	29633	27456
Mesenteric L.N.	A+Am	1013	1290	2393	4708	17457	25103	20980	38975
	A+Bm	3645	19190	80566	159481	211743	189632	250940	244044
Sacculus rotundus	A+Am	477	521	766	1473	2719	4276	5144	9036
	A+Bm	621	486	2513	7136	38457	62498	97578	125109
Appendix	A+Am	1433	951	1474	2506	3692	5325	6957	14934
	A+Bm	479	1124	1601	4609	13769	21503	32863	50194
Peyer's patches	A+Am	1482	3103	9573	21310	65705	81809	88748	110381
	A+Bm	24472	93221	197556	252930	263884	295208	241453	264217

* cpm represent the data of the same experiments presented in Fig. 6.

** N.D. = Not done.

mesenteric lymph node, the maximum incorporation of ^{3}H-thymidine in the allogeneic MLR was at the higher responder cell concentrations (25 to 40 x 10^5 per culture) used. However, in all cases, the incorporation in control, autologous cultures at these higher cell concentrations rose dramatically and resulted in a low value for the S.I. This finding was not evident with cells from non-gut associated organs such as spleen or circulating leukocytes.

Table 18 summarizes the results of four different MLR experiments using bone marrow cells as stimulators and indicates the cell concentration at which the optimal response as defined by the S.I. occurred. It can be seen that trends similar to those shown during stimulation with peripheral blood leukocytes occur. However, it is clear that variation exists and one can only define a range of optimal response for the cells of each organ.

In order to compare the response of the cells one organ to another and to determine which organ was superior in its responsiveness in the MLR, the S.I.s were determined for the various lymphoid organs at three different responder cell concentrations in four different experiments (Tables 19, 20 and 21, respectively).

Table 19 shows that, at 5 x 10^5 responder cells per culture, only Peyer's patches and mesenteric lymph node cells give consistently positive responses. Sacculus rotundus responder cells

TABLE 18

VARIATION AMONG DIFFERENT RABBITS WITH RESPECT TO THE CONCENTRATION OF RESPONDER CELLS REQUIRED FOR OPTIMAL ONE-WAY MLR RESPONSE TO MITOMYCIN-C TREATED BONE MARROW CELLS ON DAY 5 OF CULTURE

CELLS OF ORGANS TESTED*	CELL CONCENTRATION X 10^5							
	2.5	5.0	10	15	20	25	30	40
WBC			1**		1			
Spleen			1	3				
Mesenteric LN***		1	2	1			1	
Sacculus rotundus				1	1	1	1	
Appendix						1	3	
Peyer's patches	3					1		

* Bone marrow and thymus do not respond to stimulation with allogeneic mitomycin-C treated cells.

** Each number represents the number of experiments which gave an optimal MLR response (S.I.) at the concentration indicated.

*** Mesenteric lymph node.

TABLE 19

COMPARISON OF MLR RESPONSE (S.I.) OF LYMPHOID ORGANS (5×10^5 CELLS) TO MITOMYCIN-C TREATED BONE MARROW CELLS ON DAY 5 OF CULTURE.

CELLS OF ORGAN TESTED	EXPERIMENTS NO.			
	1	2	3	4
WBC	0.9	1.4	0.7	0.9
Spleen	5.9	2.5	2.7	2.5
Bone marrow	N.D.	0.9	1.1	1.1
Thymus	1.2	2.2	1.4	0.7
Mesenteric LN.	15.0	22.0	5.2	60.0
Sacculus rotundus	0.9	14.0	1.8	0.9
Appendix	1.2	1.3	1.4	0.8
Peyer's patches	30.0	78.0	2.8	47.0

TABLE 20

COMPARISON OF MLR RESPONSE (S.I.) OF LYMPHOID ORGANS (10×10^5 CELLS) TO MITOMYCIN-C TREATED BONE MARROW CELLS ON DAY 5 OF CULTURE.

CELLS OF ORGAN TESTED	EXPERIMENT NO.			
	1	2	3	4
WBC	2.2	1.3	0.9	1.4
Spleen	6.5	7.2	3.1	14.0
Bone marrow	N.D.	0.9	1.0	1.9
Thymus	1.2	1.0	1.3	1.5
Mesenteric LN.	34.0	10.0	7.9	81.0
Sacculus rotundus	3.3	38.0	5.1	2.8
Appendix	1.3	23.0	1.5	1.1
Peyer's patches	21.0	16.0	13.0	38.0

TABLE 21

COMPARISON OF MLR RESPONSE (S.I.) OF LYMPHOID ORGANS (25×10^5 CELLS) TO MITOMYCIN-C TREATED BONE MARROW CELLS ON DAY 5 OF CULTURE.

CELLS OF ORGAN TESTED	EXPERIMENT NO.			
	1	2	3	4
WBC	N.D.	0.9	N.D.	N.D.
Spleen	8.1	5.0	1.2	6.2
Bone marrow	N.D.	0.8	1.0	1.1
Thymus	1.8	1.0	0.8	1.3
Mesenteric LN.	7.6	3.2	2.6	8.0
Sacculus rotundus	15.0	13.0	1.8	4.2
Appendix	4.0	57.0	3.3	4.1
Peyer's patches	3.6	5.6	4.0	13.0

give a positive result in only 1 out of 4 experiments. Spleen cells gave definite but minimal responses. As can be seen in Table 20, at a responder cell concentration of 10 x 10^5 cells per culture, the Peyer's patches cells are already giving a lesser response than at 5 x 10^5 cells per culture (Table 19), while the cells of the other lymphoid organs show increased responses. In Table 20, Exp. 1, mesenteric lymph node cells give the best response, followed by Peyer's patches cells; in Exp. 2 sacculus rotundus cells are best, followed by appendix cells; in Exp. 3 Peyer's patches cells are best, followed by mesenteric lymph node cells; in Exp. 4 mesenteric lymph node cells are best, followed by Peyer's patches cells. It should also be noted that there is a tremendous variation in the magnitude of the response of the cells of any one organ in different experiments and between the cells of the different organs in the same experiment. Table 21 shows similar data at a responder cell concentration of 25 x 10^5 cells per culture. It should be noted that at this cell concentration, the control cultures of the gut-associated organs and mesenteric lymph node are beginning to incorporate tritium to a marked degree. In Table 21, Exp. 1, sacculus rotundus cells are superior to mesenteric lymph node cells; in Exp. 2 appendix cells are superior to sacculus rotundus cells; in Exp. 3 Peyer's patches and appendix cells responded minimally; in Exp. 4 Peyer's patches cells are superior to mesenteric lymph node cells.

TABLE 22

THE RESPONSE (SI) OF SPLEEN, BONE MARROW, THYMUS, AND BONE MARROW PLUS THYMUS CELLS TO MITOMYCIN-C TREATED STIMULATOR CELLS ON DAY 5 OF CULTURE.

RESPONDER CELLS	RESPONDER CELLS PER TUBE	STIMULATOR CELLS (5×10^5 per culture)		
		SPLEEN	WHITE BLOOD CELLS	BONE MARROW
Spleen	1×10^6	11.0	8.5	15.0
Bone marrow	1×10^6	0.8	0.3	1.1
Thymus	1×10^6	0.5	0.6	1.1
Bone marrow plus thymus (1:1)	1×10^6	1.2	0.3	1.2
Bone marrow plus thymus (1:1)	2×10^6	1.1	0.3	0.8

The results of Tables 19, 20, and 21 show that Peyer's patches cells have a greater ability to respond at low responder cell concentrations, mesenteric lymph node cells in the mid-range of cell concentration and sacculus rotundus cells respond optimally at the higher cell concentrations used.

Table 22 shows the response in the MLR of spleen cells, bone marrow cultured with thymus cells (ratio 1:1) to stimulation by mitomycin-C treated allogeneic spleen cells, WBC and bone marrow cells. The spleen cells responded well to all of the three stimulator cells used. Bone marrow cells alone and thymus cells alone did not respond to any of the three stimulator cells. The combinations of bone marrow and thymus cells (ratio 1:1) at the two different concentrations used (1×10^6 and 2×10^6 cells per culture) also did not respond to any of the three stimulators used.

d) Discussion

The primary objective of this study was to investigate the distribution and properties of the cells responding in the MLR in the rabbit. The results are expected to provide a basis for the further investigation of the relationship between the MLR, the in vitro generation of cytotoxic cells, and the transplantation rejection reaction in the rabbit. To determine which of the rabbit lymphoid organs was a good source of rabbit MLR responder cells, the kinetics of the MLR responses of cells of these

organs were studied using two organ sources of stimulator cells. It was felt that this study would not only identify the prime source of the MLR responder cells which could later be used in experiments to generate cytotoxic cells in vitro, but also indicate the T-like cell composition of these organs so that possible functional relationships between these organs could be determined.

i) The lack of response of bone marrow and thymus cells in the MLR.

Tables 12, 14 and 15 show that thymus and bone marrow cells over a wide range of responder cell concentrations do not respond significantly in the five day MLR compared to the cells of other lymphoid organs. This confirms the results of others using rabbit cells in the MLR (Chapman and Dutton, 1965; Ozer, Jr. and Waksman, 1974). Efforts to produce responses by the cells of these organs by increasing the concentration of allogeneic mitomycin-C treated stimulator cells had no effect (Tables 19, 20 and 21). Furthermore, thymus and bone marrow cells also failed to respond on days 3 and 7 of culture.

Viability studies of the thymus and bone marrow responder cells during the MLR using the trypan blue exclusion test were virtually impossible to carry out due to the high number of RBC in the culture (up to 100:1, RBC:WBC), and the high number of mitomycin-C treated stimulating cells which possibly do survive the culture period could not be differentiated from responder cells.

Bone marrow cells cultured alone showed a high degree of viability until day 5 (86%), while thymus cells cultured alone showed poor viability after day 3 (18%). This may not, however, reflect the viability of these cells during the MLR. This indicates that the bone marrow and possibly the thymus cells are still alive at a time when an MLR could take place.

Table 14 and Figure 7 show that there is a high degree of ^{3}H-thymidine incorporation into bone marrow cells cultured alone; the degree of incorporation of ^{3}H-thymidine is much higher on day 3 than on days 5 or 7. Table 15 indicates that there is some incorporation of ^{3}H-thymidine on day 5 into thymus cells cultured with high concentrations of mitomycin-C treated allogeneic stimulator cells. This level of ^{3}H-thymidine incorporation is equivalent to that of WBC or spleen cells cultured with autologous mitomycin-C treated stimulating cells. The same result has been found with thymus cells cultured with high concentrations of mitomycin-C treated allogeneic bone marrow cells. This indicates that under these conditions thymus cells are viable; however no MLR of any consequence occurs. Thymus and bone marrow cells also do not respond to mitomycin-C treated allogeneic cells obtained from any of the other rabbit lymphoid organs (see Section 3 of this chapter).

The lack of response of rabbit thymus cells in the MLR is at variance with the findings using mouse thymocytes (Berman, Puryear and Argyris, 1976), rat thymocytes (Knight and Thorbecke, 1971), and human thymocytes (Schwartz, 1967; Schwartz, 1966).

This may reflect a lack of mature lymphoid cells in the rabbit thymus. Culturing bone marrow cells and autologous thymus cells together with allogeneic mitomycin-C treated stimulating cells also gave no MLR. This lack of synergy between rabbit thymus and bone marrow cells indicates a lack of responder cells, a lack of helper cells, or an excess of suppressor cells in these populations.

ii) The response of the cells of the remaining lymphoid organs in the MLR.

The response in the MLR of the cells of the remaining lymphoid organs may be categorized in two different ways - either on the basis of the magnitude of the response (S.I.) at optimal cell concentration or on the basis of concentration of responder cells necessary to give an optimal response. Peyer's patches cells responded optimally at a low concentration of responder cells (2.5 to 5 x 10^5); mesenteric lymph node cells, WBC and spleen cells responded optimally at the medium concentration of responder cells (15 to 30 x 10^5) while appendix cells did not respond optimally until a concentration range of 24 to 40 x 10^5 responder cells was attained (Tables 13 and 18). Furthermore, in relation to the magnitude of the response, Peyer's patches and mesenteric lymph node cells responded very well exhibiting the highest S.I., spleen cells responded with a moderate S.I. and cells of sacculus

rotundus and appendix responded poorly (Figure 11). WBC cannot be included in this classification as these cells have the unique property, not present in the cells of the other organs, of responding relatively poorly to mitomycin-C treated bone marrow stimulator cells but well to cells of WBC and spleen. On the assumption that the response of the cells of each organ is proportional to the number of responder cells present in each cell population, Peyer's patches cells would have the highest number of responder cells and appendix cells the lowest, on a per cell basis. One would therefore anticipate that, as the optimum responder cell concentration is reached, the magnitude of the response would be the same for each of the cell populations. The results shown in Figure 11, however, do not support this assumption as the magnitude of the responses for the different cell preparations were different, irrespective of the cell concentrations used.

It is clear that an optimum ratio between responder cells and stimulator cells does exist as there is a decrease in the MLR response above a certain concentration of responder cells. This decrease in S.I. occurs at different cell concentrations with the cells of different organs tested and is therefore probably not due to the total number of cells in culture reaching a threshold concentration above which the MLR is inhibited due to nutrient depletion in cultures.

iii) Helper and suppressor cells in the MLR

There are many other explanations as to why the response of the cells of one lymphoid organ in the MLR is better than that of another. There probably are many complex interactions between subpopulations of the cells of each organ, and possibly a difference in the properties of responder cells from one organ to the next. This may explain certain anomolous results such as an occasional good response given by the cells of sacculus rotundus or appendix, which uniformly only respond at high cell concentrations. It is possible that cells that respond well in the MLR such as those of Peyer's patches may contain either more helper cells (Dyminiski and Smith, 1975) or less suppressor cells (Luzzati and Lafleur, 1976; Rich, Chu and Rich, 1977; Hodes and Hathcock, 1976 a,b) than cells of appendix which respond poorly. It is possible that cells of Peyer's patches release many more non-specific mitogenic agents upon stimulation in the MLR than cells of appendix thus resulting in a greater level of DNA synthesis of a non-specific nature (Etheredge, Shons, Hohenthaner and Najarian, 1973). In studies with rats however Wilson (1967), using sex chromosome markers, showed that non-specific recruitment of cells played a minor role in the MLR. It is known that in the mouse MLR system, suppression of the reaction can occur through soluble mediators produced during the MLR by previously in vivo sensitized spleen cells (Rich and Rich, 1975). A similar phenomenon may occur with unsensitized rabbit cells in the MLR. This may occur more readily with

rabbit appendix cells than rabbit Peyer's patches cells in the MLR thus limiting the response of the appendix cells.

iv) Cytotoxic cells in the MLR

Another reason for the differing responses of cells from each of the lymphoid organs could be due to the development of cytotoxic cells in culture. It is possible that appendix cells during the course of the MLR produce many cells cytotoxic to the target cells. This could reduce stimulation of the appendix cells and result in an apparently low response in the MLR. This effect has been found in mice where cells taken from an ongoing MLR will inhibit the response of cells in another MLR directed against the same stimulator cells (Fitch, Engers, Cerottini and Brunner, 1976). The apparent destruction of the stimulator cells in the MLR has, however, not been found to be mediated by previously uncultured cells (Fitch, Engers, Cerottini and Brunner, 1976). It is unlikely, therefore, that this mechanism applies to the primary rabbit MLR studied here.

v) The influence of the duration of the cell cycle on the MLR response

It has been shown by Wilson, Blyth and Nowell (1968) that rat peripheral blood lymphocytes stimulated in the MLR undergo a much quicker cell cycle (13 to 14 hours) than do the same cells stimulated by phytohemagglutinin (>20 hours). It is possible

that rabbit Peyer's patches cells undergo a much quicker cell cycle in the MLR than do rabbit appendix cells. Thus, starting with the same number of responder cells, a much larger response may be expected on day 5 by cells of Peyer's patches than those of appendix. Wilson, Blyth and Nowell (1968) have shown that in the rat MLR the responder cell undergoes 4 to 5 sequential divisions and then stops. If the cells of the various lymphoid organs all undergo the same number of mitoses during the MLR response and one set of cells divides faster than another then one would expect the optimum responses to be on different days. This however is not the case with rabbit responder cells as the maximum response with the cells of all organs occurs on day 5.

vi) The ability of cells to detect stimulating determinants in the MLR

Another possibility which would explain the greater response of Peyer's patches cells than appendix cells in the MLR is that the cells of the Peyer's patches detect a greater number and variety of antigenic determinants on the surface of the stimulating cells than do the cells of the appendix. Accordingly, it might be anticipated that a greater number of Peyer's patches cells would respond to the allogeneic stimulus, as compared to appendix cells.

vii) The classification of rabbit MLR responder cells

From the results of these experiments it would seem that rabbit Peyer's patches cells and mesenteric lymph node cells

contain the most MLR reactive cells. It has been shown that the MLR responder cell in rabbit WBC is sensitive to lysis by anti-thymus cell serum plus complement (Sheppard, Jr., Sell, Poler and Redelman, 1977). This implies that the responder cell is a T cell. This would, in turn, suggest that these organs contain the most T-cells (Raff, 1971; Bach, Bach and Sondel, 1976). However, it is known that classification of cells into B and T cells in the rabbit may be quite different as compared to that in man or mouse. Sell and Sheppard, Jr. (1973) have shown that rabbit peripheral blood leukocytes respond well to T-cell mitogens and yet could be classified as B-cells due to the high concentration of immunoglobulins on their surfaces. It is of interest to note than an example in the mouse of T-cells associated with surface immunoglobulin has been found by Gorcyzski and Rittenberg (1975). They have shown that cells (stimulated two to four days previously in a primary MLR) responding in the secondary MLR (large blast like cells) are not only lysed by anti-theta serum and complement (characteristic of mouse T-cells) but are appreciably depleted by anti-mouse Ig and complement. This indicates that these T-cells also possess immunoglobulins on their surfaces. It is therefore possible that cells involved in the MLR and graft rejection in the rabbit may not be the classical T-cells found in mouse and man.

The results of these experiments show that it is difficult to compare the responses of the cells of the various lymphoid organs in the rabbit MLR without a careful analysis of the dose

response curves. Using only one concentrations of cells for comparisons may give completely erroneous results as to the hierarchy of response.

viii) The autologous MLR response, and the spontaneous incorporation of ^{3}H-thymidine by rabbit lymphoid cells

Another complication in the interpretation of the results is the sharp increase in ^{3}H-thymidine incorporation by cells of the gut-associated organs in the autologous (control) MLR (Tables 14 and 17). This increase has been shown to occur in the absence of autologous mitomycin-C treated stimulator cells (Table 14) and therefore cannot be attributed to autologous stimulation. This response seems to be due to spontaneous blastogenesis of the cells of gut-associated organs at high cell concentrations. Conversely in cultures of responder spleen cells or WBC which also contain autologous, mitomycin-C treated cells from Peyer's patches, appendix or sacculus rotundus apparent autologous stimulation occurs. This response is dependent upon the number of autologous mitomycin-C treated stimulator cells in culture and also occurs in the two-way autologous MLR (see Section 1). This makes determination of the optimum response subject to many interpretations if one only compares the ^{3}H-thymidine incorporation into allogeneic MLR cultures with ^{3}H-thymidine incorporation into autologous cultures.

3. The Distribution of the MLR Stimulator Cells Among the Different Lymphoid Organs

   a) Abstract

   b) Introduction

   c) Results

   d) Discussion

      i) The determination of the optimal source of MLR stimulating cells.

      ii) The marked stimulation of MLR responder cells by cells of the Peyer's patches and mesenteric lymph nodes.

      iii) The relationship of stimulating capacity to cell surface immunoglobulin.

      iv) Blastogenic stimulation of autologous responder cells.

a) Abstract

The distribution in rabbit lymphoid organs of cells capable of stimulating responder cells in the one-way MLR was investigated. Optimal concentrations of responder WBC or spleen cells were cultured with fixed numbers of mitomycin-C treated stimulator cells of the different lymphoid organs for five days. On a per cell basis, the

cells of the Peyer's patches and mesenteric lymph nodes were the most potent stimulator cells. However, the most reliable sources of large numbers of consistently stimulating cells were found to be the spleen and the bone marrow. Mitomycin-C treated cells of the gut associated lymphoid organs - Peyer's patches, sacculus rotundus and appendix, caused apparent autologous stimulation of responder cells. There was no apparent correlation between the MLR stimulating capacity and the number of cells displaying surface membrane immunoglobulin in the stimulator cell population.

b) Introduction

Incubation of rabbit lymphoid cells with allogeneic mitomycin-C treated lymphocytes results in cell proliferation characterized by uptake of ^{3}H-thymidine. This mixed lymphocyte reaction (MLR) in man and mouse is mediated by a responder T-cell (Bach, Bach and Sondel, 1976). The stimulating cell has on its surface lymphocyte determined (LD) determinants which play a direct role in stimulation of the responder cell (Bach and von Rood, 1976). Many investigators have suggested that B-cells are the most potent stimulators in the MLR (Han and Dadely, 1976; Lohrmann, Novikovs and Graw, Jr., 1974) although T-cells and null cells have also been implicated as stimulating cells (Ling and Hardy, 1974; Chess, MacDermott, Sondel and Schlossman, 1974).

The objectives of this investigation were (1) to determine which of the rabbit organs constitutes the optimal source of

stimulating cells in the rabbit MLR which, by definition, would possess the most rabbit LD antigenic determinants and (2) to determine whether a correlation exists in the rabbit between the capacity of a population of cells to stimulate responder cells in the MLR and the number of stimulator cells bearing surface membrane-bound immunoglobulin (a generally accepted indicator of B-cells in mouse and man). It is possible that immunoglobulin-bearing cells are not exclusively B-cells as it has been shown that, in the rabbit, the majority of PHA-responsive cells (a classical T-cell mitogen) have immunoglobulins on their surfaces (Sell and Sheppard, 1973), and in the mouse that blast-like cells produced during a secondary MLR can be lysed not only by anti-mouse immunoglobulin plus complement but also by anti-theta serum and complement (Gorcyzski and Rittenberg, 1975).

c) Results

Table 23 shows the mean recovery of nucleated cells from the various rabbit organs. The greatest yield of nucleated cells was obtained from the thymus and appendix while the lowest yield was from the blood. All the other organs were intermediary in their cell yield.

Table 24 shows the responses of 10×10^5 WBC and spleen cells on day five to stimulation by 5×10^5 mitomycin-C treated cells from the various lymphoid organs. Spleen cells respond well to stimulation by allogeneic WBC, spleen, bone marrow, Peyer's patches and sacculus rotundus cells. Spleen cells consistently

TABLE 23

MEAN RECOVERY OF NUCLEATED CELLS FROM VARIOUS RABBIT ORGANS

ORGAN	EXPERIMENTS	MEAN LYMPHOID CELL NUMBER x 10^8 ± S.E.M.
Appendix (partial)	17	20.0 ± 0.4
Thymus	17	17.0 ± 3.0
Sacculus rotundus	12	6.2 ± 1.2
Bone marrow (2 femurs)	12	5.3 ± 2.1
Mesenteric lymph node	9	4.7 ± 2.1
Spleen	12	2.6 ± 0.4
Peyer's patches	15	2.5 ± 0.5
Blood (70 ml) (Dextran sedimentation)	16	1.5 ± 0.4

TABLE 24

THE RESPONSE (CPM AND SI) IN THE MLR OF RABBIT SPLEEN CELLS AND LEUKOCYTES TO MITOMYCIN-C TREATED ALLOGENEIC STIMULATOR CELLS (5×10^5) OF VARIOUS LYMPHOID ORGANS.

STIMULATOR CELLS	RESPONDER CELLS*					
	SPLEEN			WBC		
	A**+Am***	A+Bm	SI	A + Am	A + Bm	SI
Circulating wbc	1551	8237	5.3	589	5609	9.5
Spleen	4405	25191	5.7	641	14509	23.0
Thymus	1177	1601	1.4	828	732	0.9
Bone marrow	1750	8536	4.9	605	3015	5.0
Mesenteric lymph node	2504	6685	2.7	877	7830	8.9
Appendix	11024	9758	0.9	6178	9543	1.5
Peyer's patches	2303	17510	7.6	1514	42547	28.0
Sacculus rotundus	6449	26386	4.1	2007	22630	11.0

* The responder cell concentration was kept constant throughout at 1×10^6 cells per culture.

** A represents the responder cells from rabbit A.

*** Am and Bm represent the mitomycin-C treated stimulator cells from rabbits A and B, respectively.

respond poorly to mitomycin-C treated thymus and appendix cells and only slightly better to mesenteric lymph node cells. The response of WBC to allogeneic stimulator cells follows essentially the same pattern as that given by spleen cells, the only difference being the good stimulation of WBC by mesenteric lymph node cells. WBC responded well to allogeneic mitomycin-C treated WBC and cells of spleen, mesenteric lymph node, Peyer's patches and sacculus rotundus. WBC responded poorly, if at all, to stimulation with allogeneic mitomycin-C treated cells of thymus and appendix.

In control cultures where spleen responder cells were incubated with autologous mitomycin-C treated stimulating cells from the various lymphoid organs, the ^{3}H-thymidine incorporated by the responder spleen cells varied from 1177 cpm (autologous thymus stimulators) to 11,024 cpm (autologous appendix stimulators). The response of WBC to autologous cells of other organs is similar to that of the spleen responder cells but not as marked. In the case of both spleen cells and WBC responders, ^{3}H-thymidine incorporation was greatest with mitomycin-C treated autologous appendix and sacculus rotundus cells (5×10^{5} cells per culture).

Table 25 depicts the results of an experiment similar to that presented in Table 24 except that the concentration of mitomycin-C treated stimulator cells was increased to 25×10^{5} cells per culture. With this increase of five fold in the number of stimulating cells, the response of responder spleen cells and WBC was enhanced.

TABLE 25

THE RESPONSE (CPM and SI) IN THE MLR OF RABBIT SPLEEN CELLS AND LEUKOCYTES TO MITOMYCIN-C TREATED ALLOGENEIC STIMULATOR CELLS (25×10^5) OF VARIOUS LYMPHOID ORGANS

STIMULATOR CELLS	RESPONDER CELLS*					
	SPLEEN			WBC		
	A**+Am***	A+Bm	SI	A + Am	A + Bm	SI
Circulating wbc	1114	5113	4.6	612	5030	8.2
Spleen	3787	85145	22.0	2609	106975	41.0
Thymus	4071	18363	4.5	1545	8422	5.5
Bone marrow	2858	42172	15.0	1216	25458	21.0
Mesenteric lymph node	753	69617	92.0	276	87445	317.0
Appendix	8179	28850	3.5	3025	9422	3.1
Peyer's patches	5287	104985	20.0	15035	152880	10.0
Sacculus rotundus	5488	70397	13.0	3425	39720	12.0

* The responder cell concentration was kept constant throughout at 1×10^6 cells per culture.

** A represents the responder cells from rabbit A.

*** Am and Bm represent the mitomycin-C treated stimulator cells from rabbits A and B, respectively.

The responder spleen cells were stimulated by all the mitomycin-C treated allogeneic cells, including thymus and appendix. Only mitomycin-C treated WBC seemed to be less stimulatory than at the lower stimulator cell concentration (Table 24). The response of WBC to these cells follows the same pattern as that given by responder spleen cells. Stimulation of WBC by allogeneic mitomycin-C treated Peyer's patches cells led to increased incorporation of ^{3}H-thymidine by responder cells at this higher concentration of stimulator cells (152880 cpm from 42547 cpm; compare Tables 24 and 25), but the S.I. is decreased (to 10 from 28) due to increased cpm in the autologous cultures. It can be seen that there is a slight increase in ^{3}H-thymidine incorporation into autologous control cultures as the number of stimulator cells was increased (compare Tables 24 and 25).

Table 24 shows, using the S.I. as a measure of stimulation, that at low concentrations of stimulator cells, mitomycin-C treated allogeneic cells from Peyer's patches (5×10^5 cells per culture) are the most efficient stimulators of both WBC and spleen cells. However, at the higher concentration of stimulator cells (Table 25), mitomycin-C treated mesenteric lymph node cells (25×10^5 cells per culture) are by far the best allogeneic stimulators for WBC and spleen cells.

Table 26 shows the percentage of cells of the different lymphoid organs capable of binding fluorescein-conjugated sheep anti-rabbit immunoglobulin antibodies. It can be seen from the

TABLE 26

RABBIT CELL SURFACE IMMUNOGLOBULINS

ORGAN*	% FLUORESCENT CELLS** IN EXP. NO.							AVG ± S.D.
	1	2	3	4	5	6	7	
Circulating wbc	30	34	20	40	15	22	22	26.0 ± 8.8
Spleen	32	33	13	22	30	8	15	22.0 ± 10.0
Thymus	1	0	0	0	0	2	1	0.6 ± 0.8
Bone marrow	5	1	1	3	2	3	6	3.0 ± 1.9
Mesenteric lymph node	31	26	18	36	14	8	16	21.0 ± 10.0
Appendix	26	31	10	18	2	12	5	15.0 ± 11.0
Peyer's patches	21	27	19	20	16	10	17	19.0 ± 5.2
Sacculus rotundus	33	19	11	19	17	8	10	17.0 ± 8.5

* Whole cell preparation

** Polyvalent sheep anti-rabbit immunoglobulin serum (anti IgA, IgG, IgM)

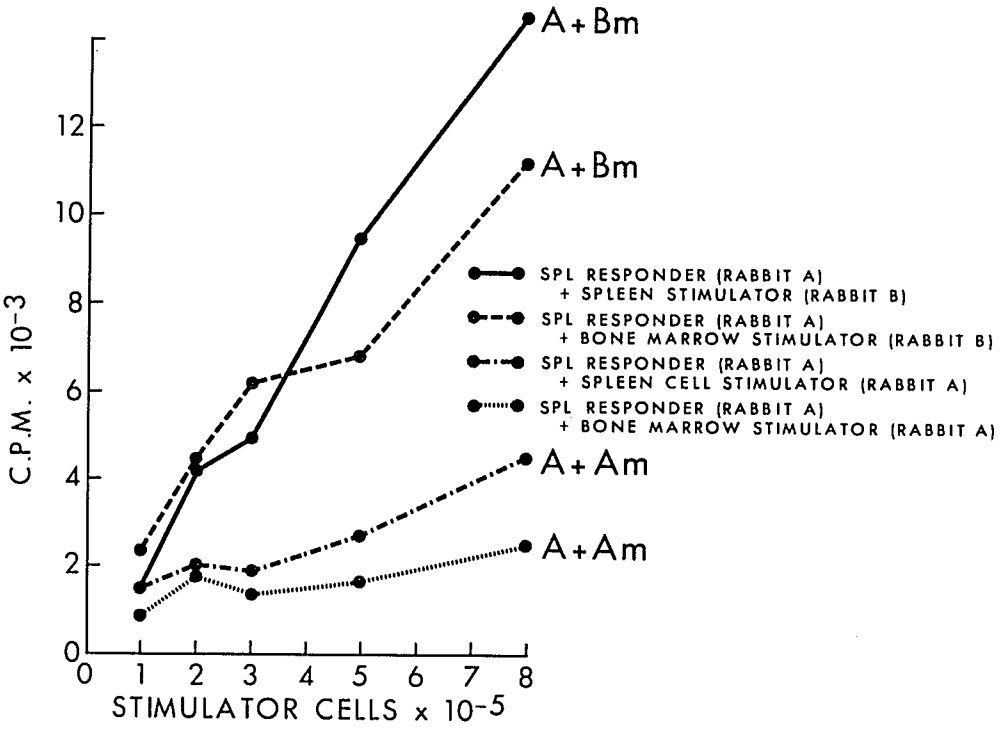


FIGURE 12

THE INCORPORATION IN THE ONE-WAY MLR OF ^{3}H-THYMIDINE INTO RESPONDER SPLEEN (SPL) CELLS STIMULATED WITH VARYING NUMBERS OF ALLOGENEIC MITOMYCIN-C TREATED SPLEEN AND BONE MARROW CELLS. A represents responder cells and Am and Bm represent mitomycin-C treated stimulator cells.

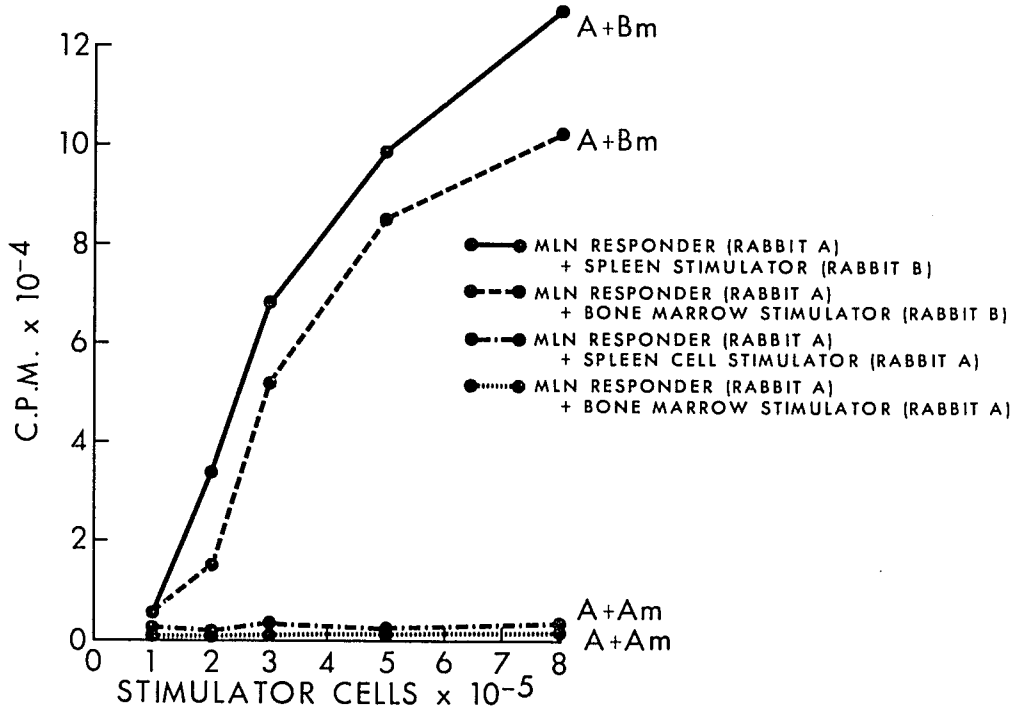


FIGURE 13

THE INCORPORATION IN THE ONE-WAY MLR OF ^{3}H-THYMIDINE INTO RESPONDER MESENTERIC LYMPH NODE (MLN) CELLS STIMULATED WITH VARYING NUMBERS OF ALLOGENEIC MITOMYCIN-C TREATED SPLEEN AND BONE MARROW CELLS.

A represents responder cells and Am and Bm represent mitomycin-C treated stimulator cells.

results of seven experiments that few thymus and bone marrow cells bound the fluorescent conjugate. Approximately 20% of the cells of all the other organs bound the conjugate; however, the results were quite variable.

Figure 12 shows the response of spleen cells to various concentrations of allogeneic mitomycin-C treated spleen or bone marrow cells. It can be seen that, over a concentration range of 1 to 8 x 10^5 stimulator cells per culture, there is an almost linear increase in the amount of ^{3}H-thymidine incorporated.

Figure 13 illustrates the response of mesenteric lymph node cells to various concentrations of allogeneic mitomycin-C treated spleen and bone marrow cells. It can be seen that the amount of ^{3}H-thymidine incorporated into the MLR cultures containing responder mesenteric lymph node cells is ten fold greater than that incorporated into culture containing responder spleen cells (compare Figures 12 and 13). However, the pattern of response of mesenteric lymph node cells to stimulation with allogeneic mitomycin-C treated bone marrow or spleen cells (Figure 13) is similar to that shown by responder spleen cells (Figure 12).

d) Discussion

The primary objectives of this investigation were (1) to determine the organ distribution of the stimulator cells in the rabbit MLR and (2) to determine which of the rabbit lymphoid organs would be the optimal source of stimulator cells for use in the MLR.

i) The determination of the optimal source of MLR stimulating cells.

The stimulator activities of the cells of the different lymphoid organs (circulation, spleen, thymus, bone marrow, sacculus rotundus, Peyer's patches, mesenteric lymph nodes and appendix have been defined and evaluated. Spleen and bone marrow cells were generally used as sources of stimulating cells. They consistently stimulated responder allogeneic cells and did not stimulate autologous cells.

Table 23 shows the mean recovery of lymphoid cells from the eight lymphoid organs studied. It can be seen that the thymus and appendix gave by far the highest cell yield. However, as can be seen in Table 24, cells of these organs were very poor stimulators of WBC and spleen cells in the one-way allogeneic MLR. In the case of thymic stimulator cells, the quantity of ^{3}H-thymidine incorporated into responder cells was quite low in both control (autologous) and allogeneic cultures. With stimulator appendix cells, however, the cpm in allogeneic cultures containing responder spleen cells or WBC were very marked (almost 10,000 cpm) suggesting stimulation by the mitomycin-C treated appendix cells. However, the autologous control cultures also incorporated ^{3}H-thymidine with cpm sometimes exceeding that of the allogeneic MLRs. This made appendix cells unsatisfactory for use as stimulator cells in the allogeneic MLR. Even though

WBC are good stimulator cells, their use generally was precluded as the number of cells required in the majority of experiments exceeded the number recoverable. Table 23 shows that the number of white cells recovered from 70 ml of blood is quite low ($1.5 \pm 0.4 \times 10^8$ SEM, 16 experiments). Cells from the Peyer's patches and sacculus rotundus were available in greater numbers. However in MLR cultures using these cells as stimulators there were increases in cpm in control MLRs as the number of stimulating cells was increased which made the data difficult to interpret (Tables 24,25). In addition, the amounts of ^{3}H-thymidine incorporated into autologous cultures varied considerably from one rabbit to another and from one concentration of stimulator cells to the next making these organs an unreliable source of stimulator cells. The mesenteric lymph nodes contained a large number of cells (Table 23) and showed only minimal autologous stimulation of responder cells. However, one out of every three mesenteric lymph node preparations failed to stimulate, indicating that these cells were also unreliable as stimulators. For the various reasons mentioned above, spleen and bone marrow seemed to be the best sources of stimulatory cells; they consistently stimulated responder allogeneic cells and did not stimulate autologous cells.

ii) <u>The marked stimulation of MLR responder cells by cells of the Peyer's patches and mesenteric lymph nodes</u>.

Table 24 shows that, using the S.I. as a measure of stimulation, the mitomycin-C treated allogeneic cells from Peyer's patches were

the best stimulators at low stimulator cell concentrations, while Table 25 shows that mitomycin-C treated allogeneic mesenteric lymph node cells were the best stimulators at high stimulator cell concentrations. This would indicate that these cells have the greatest number or the most exposed MLR stimulating determinants on their surfaces. It is of interest to note that these cells are also the best responders in the MLR to allogeneic mitomycin-C treated bone marrow and WBC (section 2). Whether these properties of stimulation and response are associated with one cell type or different populations of cells has yet to be determined. This hierarchy of stimulatory ability of the cells from the different lymphoid organs varied from rabbit to rabbit and depended upon the stimulator cell concentration, although either Peyer's patches or mesenteric lymph node cells were usually the best stimulators.

iii) The relationship of stimulatory capacity to cell surface immunoglobulins.

To determine whether cell surface immunoglobulins are characteristic of the cells which stimulate the responder cells in the MLR, cells were incubated with fluorescein-conjugated anti-rabbit immunoglobulin antibodies to detect immunoglobulin bearing cells. One of the characteristics of B-type cells in the mouse is that they have immunoglobulins on their surfaces while T-cells do not (Raff, 1971). In our hands (Table 26) only 3 percent of bone marrow

cells were positive for immunoglobulins (Ig) whereas 22 percent of spleen cells had surface membrane Ig. Wilson, Teodorescu and Dray (1976), using a direct immunocytoadherence technique utilizing anti-rabbit-IgG antibody-coated erythrocytes, showed that rabbit bone marrow cells have significantly less Ig than spleen cells.

Spleen and bone marrow cells, which have highly different preparations of immunoglobulin-bearing cells were then compared for their ability to stimulate responder allogeneic cells.

Although Tables 24 and 25 show that spleen cells are slightly better stimulators than bone marrow cells, this is not always the case. Figures 12 and 13 show that, over a range of 1 to 8 x 10^5 stimulator cells per culture, the efficiency of allogeneic bone marrow and spleen cells in stimulating responder spleen and mesenteric lymph node cells in the one-way MLR is almost equal, suggesting that the presence of surface immunoglobulins do not play any definitive role in the induction of the MLR. The equivalent ability of cells from rabbit bone marrow and spleen to stimulate in the one-way MLR seems therefore to be at variance with the work of others investigating the MLR in mouse and man, who suggest that immunoglobulin-bearing cells (B-cells) are usually the stimulating cells in the MLR (Han and Dadey, 1976; Lohrmann, Novikovs and Graw, Jr., 1974; Harrison, 1973). Our results suggest that, in the rabbit, cells with surface membrane immunoglobulins (B-type cells) are not essential for stimulating responder cells in the MLR or the number of immunoglobulin-bearing cells required for stimulation is very low. The rabbit

MLR stimulating cell, if it is a B-type cell, is possibly not characterized by surface membrane immunoglobulin. Furthermore, it is interesting to note that rabbit T-type cells (characterized by response to mitogens) have abundant surface Ig, unlike those of man and mouse (Sell and Sheppard, Jr., 1973). Mouse T-cells have also been shown to have on their surfaces membrane immunoglobulin (Gorcyzski and Rittenberg, 1975).

iv) Blastogenic stimulation of autologous responder cells.

An unanticipated result was that in autologous cultures of responder spleen cells or WBC and the various mitomycin-C treated lymphoid cells, the cells of gut-associated lymphoid organs produced an apparent autologous stimulation (Tables 24, 25). Since the only cells capable of incorporating ^{3}H-thymidine were the responder spleen cells and WBC, the difference in cpm between responder cells cultured with autologous mitomycin-C treated cells from the same organ and responder spleen cells cultured with autologous mitomycin-C treated cells of other organs must be due to the influence of the stimulating cells. Autologous stimulation has been reported between neonatal thymus and adult spleen or WBC in mice by von Boehmer (1974) and Howe, Goldstein and Battisto (1970), but nothing has been previously reported in rabbits. It is unlikely that the autologous stimulator cells did not receive adequate mitomycin-C treatment, thus allowing incorporation of ^{3}H-thymidine by stimulator cells as no incorporation of ^{3}H-thymidine was evident in cultures when both cell populations were treated

with mitomycin-C. It is possible that appendix cells and cells of other gut-associated organs treated with mitomycin-C release blastogenic factors into the medium (Etheredge, Shons, Hohenthaner and Najarian, 1973) causing apparent autologous stimulation to occur. This is unlikely in our case, however, as good autologous stimulation occurs in two-way MLRs when neither of the cell populations has been treated with mitomycin-C treatment as a cause of incorporation of ^{3}H-thymidine in autologous rabbit cell cultures.

4. The Blastogenic Response of Rabbit Lymphocytes Stimulated with Autologous Cells

a) Abstract

b) Introduction

c) Results

d) Discussion

i) The demonstration of an autologous response

ii) Explanations for the existence of the autologous response in vitro

iii) The significance of the autologous response.

a) Abstract

Rabbit WBC and spleen cells respond with blastogenesis and mitosis when cultured with mitomycin-C treated autologous sacculus rotundus, appendix and Peyer's patches cells, in the rabbit one-way mixed leukocyte reaction (MLR) in a high proportion of experiments. The WBC and spleen cells give a lesser and inconsistent response with mitomycin-C treated cells of the other lymphoid organs. Since this "autologous MLR" response is also observed in the two-way MLR, the blastogenic response in the one-way MLR cannot be attributed to the release of mitogenic

factors as a result of mitomycin-C treatment of the stimulator cells. Several explanations are proposed to account for this autologous MLR.

b) Introduction

The mixed leucocyte reaction (MLR) was first observed by Schrek and Donnelly (1961) and subsequently described in detail by Bain, Vas and Lowenstein (1964) and Hirschorn, Bach, Kolodny, Firschein and Hashem (1963). The reaction consists of blastogenic transformation accompanied by DNA synthesis of the lymphocytes of the two unrelated donors. The reaction is attributed to genetic dissimilarity of the major histocompatibility loci between the donors of the cells (Bach and van Rood, 1976). The MLR using allogeneic cells has been extensively demonstrated in man and mouse (Bach and van Rood, 1976) and in the rabbit (Knight, Walker and Ling, 1971; Ozer, Jr. and Waksman, 1974; Ling and Kay, 1975; Chai and Lerner, 1975; Sheppard, Jr., Sell, Poler and Redelman, 1977).

A major undertaking in this laboratory over the past three years has been the characterization and organ of distribution of responder and stimulator cells in the rabbit MLR. During the course in this study, we have repeatedly observed stimulation of rabbit lymphoid cells by mitomycin-C treated autologous cells. It was decided to carry out a systematic analysis of this

observation in view of the potentially serious ramifications of such a finding with respect to current concepts of the mechanisms of immunological tolerance and autoimmune diseases.

It has been demonstrated in this investigation that rabbit WBC and spleen cells can respond with blastogenesis and mitosis in the presence of autologous gut-associated lymphoid cells in the rabbit MLR.

c) Results

Table 27 shows the blastogenic response of WBC to the mitomycin-C treated cells of autologous lymphoid organs in the one-way MLR. Cultures consisted of 1×10^6 WBC and 2.5×10^6 autologous mitomycin-C treated cells from the different lymphoid organs. It can be seen that the cells of the gut-associated lymphoid organs (appendix, sacculus rotundus, Peyer's patches) induced maximum blastogenesis in autologous WBC. Cells of spleen and mesenteric lymph nodes also stimulated autologous WBC but to a lesser extent.

Similar but not identical results were obtained with responder spleen cells (Table 28). The response of spleen (S.I.) is much lower than that of WBC to autologous gut associated organs. The cpm in the control spleen cell cultures (Table 28) were much higher than in control WBC cultures (Table 27). The cpm found in autologous MLRs of responder WBC and spleen cells to the cells of other autologous organs is similar however.

TABLE 27

THE RESPONSE OF WBC TO THE CELLS OF AUTOLOGOUS LYMPHOID ORGANS IN THE ONE-WAY MLR

Mitomycin-C treated cells incubated with autologous WBC**	Experiment No.* 1	2	3	4
Appendix	4.9	1.7	6.9	10.0
Peyer's Patches	25.0	2.8	20.0	2.6
Sacculus Rotundus	5.6	2.6	6.3	3.4
Spleen	4.4	2.8	3.8	1.1
Bone Marrow	2.0	1.6	2.3	1.0
Thymus	2.5	1.5	5.5	1.4
Mesenteric Lymph Nodes	0.5	2.7	6.9	1.5
Control culture (cpm)***	612	855	560	589

*The figures in all the columns, unless otherwise stated, refer to the S.I.

**Cultures consisted of 1 x 10^6 WBC and 2.5 x 10^6 mitomycin-C treated autologous cells.

***The control culture consisted of mitomycin-C treated WBC plus untreated autologous WBC. The cpm constitute the basis for the calculation of the S.I.s.

TABLE 28

THE RESPONSE OF SPLEEN CELLS TO CELLS OF AUTOLOGOUS LYMPHOID ORGANS IN THE ONE-WAY MLR

Mitomycin-C treated cells incubated with autologous spleen cells**	Experiment No.* 1	2	3
Appendix	2.2	3.4	2.5
Peyer's Patches	2.4	1.2	2.5
Sacculus Rotundus	2.4	2.2	2.5
WBC	0.3	0.6	0.4
Bone Marrow	0.8	0.8	0.4
Thymus	1.1	1.8	0.3
Mesenteric Lymph Nodes	0.2	1.6	0.6
Control culture (cpm)***	3787	1312	4405

*The figures in all the columns, unless otherwise stated, refer to the S.I.

**Cultures consisted of 1×10^6 spleen cells and 2.5×10^6 mitomycin-C treated autologous cells.

***The control culture consisted of mitomycin-C treated spleen cells plus untreated autologous spleen cells. The cpm constitute the basis for the calculation of the S.I.s.

Figure 14 shows the cpm measured in cultures of appendix cells and autologous spleen cells in the two-way MLR. In one set of cultures 5×10^5 spleen cells were cultured with increasing numbers of autologous appendix cells ($2.5, 5, 10, 20, \times 10^5$). It can be seen that spleen cells alone (II) incorporate about 2000 cpm. When appendix cells were added to the cultures (III) there was an increase in cpm which reached a plateau at 5×10^5 added appendix cells. Figure 14 also shows the ^{3}H-thymidine incorporation by appendix cells alone (I). The cultures contain relatively few cpm even at the highest concentration of appendix cells used (2×10^6 per ml). The amount of ^{3}H-thymidine incorporated in cultures consisting of constant numbers of appendix cells (5×10^5) and variable numbers of spleen cells ($2.5, 5, 10, 20 \times 10^5$) and variable numbers of spleen cells ($2.5, 5, 10, 20 \times 10^5$) is also shown in Figure 14 (IV). In these cultures there was increasing incorporation of ^{3}H-thymidine up to the highest number of spleen cells used. The cpm measured were approximately three times the cpm in cultures of constant numbers of spleen cells and increasing numbers of appendix cells. When increasing numbers of spleen cells were cultured alone the maximum incorporation of ^{3}H-thymidine coincided with the maximum number of cells cultured.

Figure 15 indicates the results of an MLR in which constant numbers of responder spleen cells were incubated with increasing numbers of mitomycin-C treated autologous stimulating cells. The stimulating cells were either autologous appendix or autologous spleen. It can be seen that increasing numbers of autologous

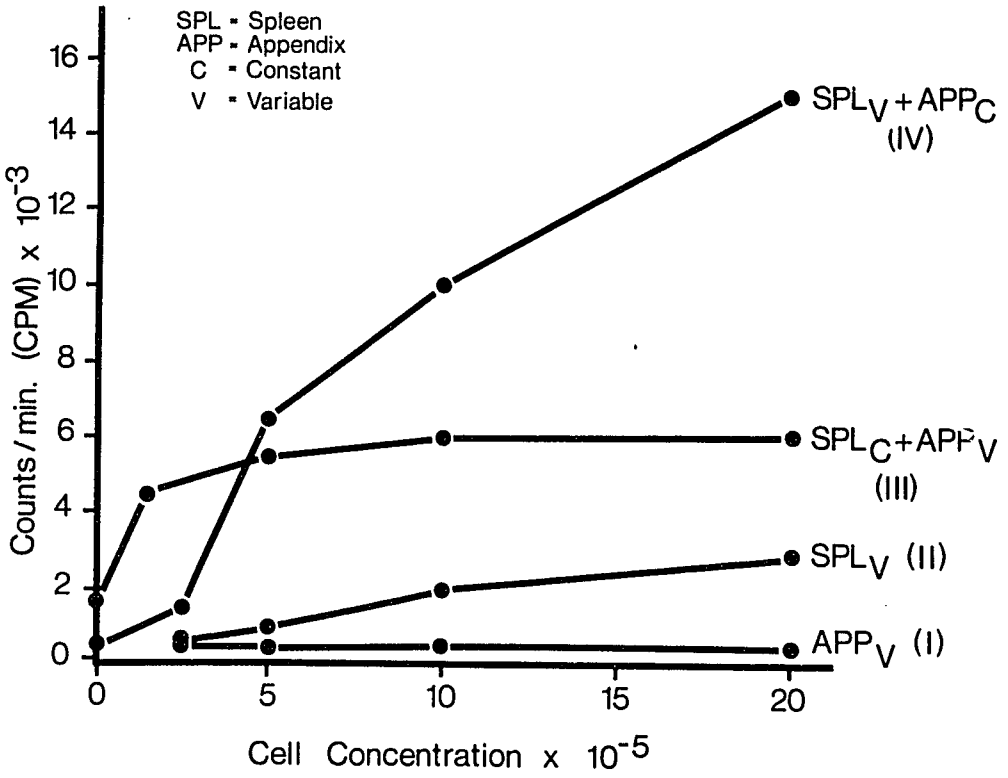


FIGURE 14

THE BLASTOGENIC RESPONSE OF VARYING CONCENTRATIONS OF APPENDIX AND AUTOLOGOUS SPLEEN CELLS IN THE TWO-WAY MLR.

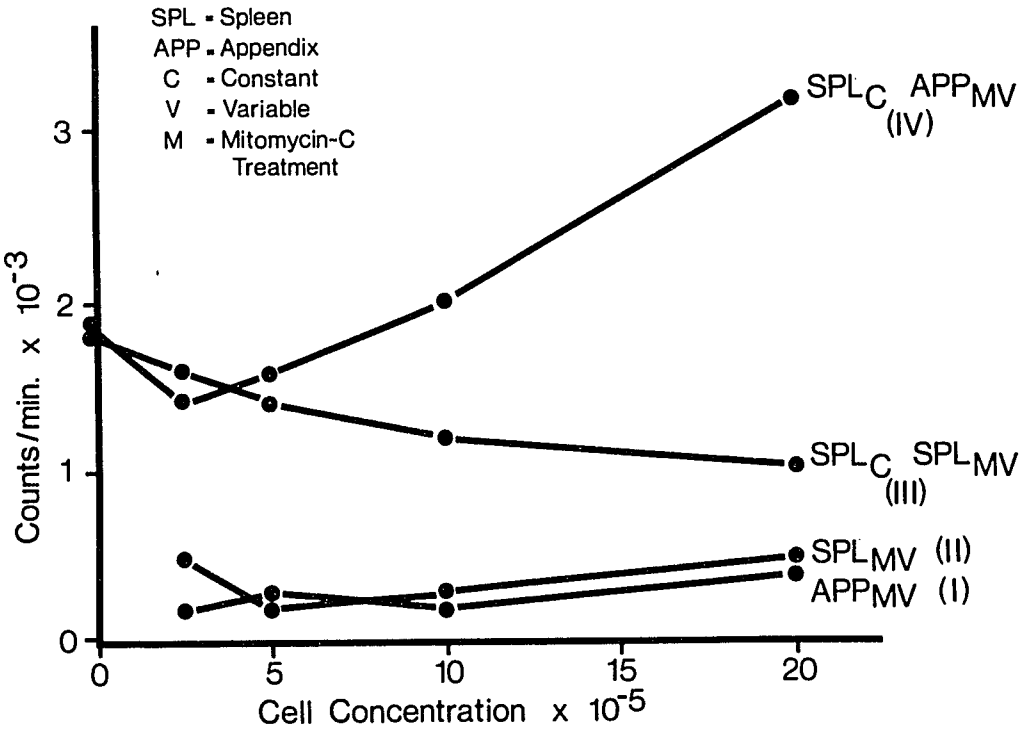


FIGURE 15

THE BLASTOGENIC RESPONSE OF SPLEEN CELLS TO VARYING CONCENTRATIONS OF MITOMYCIN-C TREATED AUTOLOGOUS APPENDIX CELLS IN THE ONE-WAY MLR.

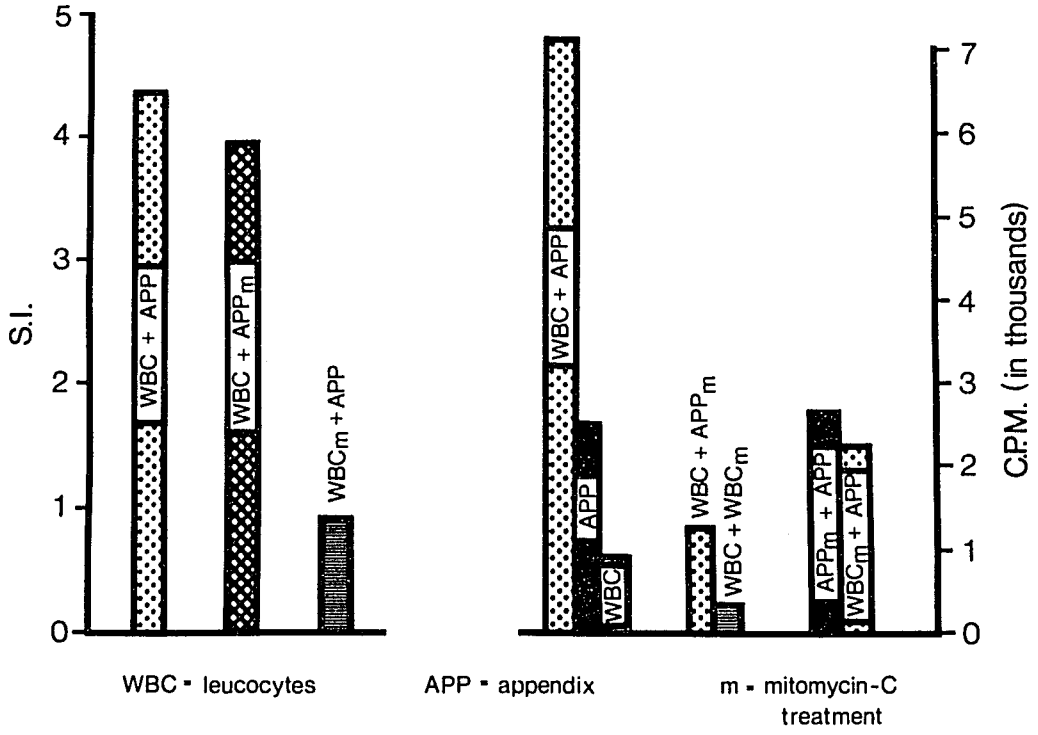


FIGURE 16

THE BLASTOGENIC RESPONSE OF RABBIT CIRCULATING LEUKOCYTES TO STIMULATION WITH MITOMYCIN-C TREATED AUTOLOGOUS APPENDIX CELLS IN THE ONE-WAY MLR.

mitomycin-C treated stimulator spleen cells inhibited progressively the incorporation of ^{3}H-thymidine by the responder spleen cells (III). On the other hand, increasing numbers of autologous mitomycin-C treated appendix stimulator cells caused a definite stimulation of cultures (IV). Figure 15 also shows that increasing numbers of mitomycin-C treated spleen or appendix cells cultured alone showed negligible incorporation of ^{3}H-thymidine at all concentrations used. When appendix cells were used as responders (5×10^{5} per culture) and increasing numbers of mitomycin-C treated autologous spleen or appendix cells were used as stimulators, little incorporation of ^{3}H-thymidine into cultures occurred and consequently no MLR was observed.

The blastogenic response of WBC in the one-way and two-way autologous MLR are presented in Figure 16. It can be seen that a significant degree of blastogenesis, as defined by the S.I., occurred in the two-way autologous MLR, using WBC and autologous appendix cells. WBC were stimulated by mitomycin-C treated autologous appendix cells in the one-way MLR; however, appendix cells were not stimulated by mitomycin-C treated autologous WBC. The cpm of these cultures are also presented in Figure 16.

d) Discussion

Previous results have alluded to an apparent blastogenic response of rabbit WBC and spleen cells to mitomycin-C treated autologous appendix, Peyer's patches, and sacculus rotundus cells (Section 2). The purpose of this investigation was to demonstrate

that the blastogenic response in this "autologous one-way MLR" was due to stimulation by autologous cells and not to blastogenic factors released into the cultures by the mitomycin-C treated stimulator cells.

i) The demonstration of an autologous response.

The response in the autologous two-way MLR is dependent upon the number of cells in culture (Figure 14). This effect could be attributed to (1) an increased number of stimulating antigens in culture, (2) an increased number of responding cells in culture, or (3) a feeder-layer effect (Ling and Kay, 1975) of the increased number of cells in culture. The third possibility seems unlikely as either the spleen or appendix cells which might produce this feeder-layer effect do not incorporate high levels of ^{3}H-thymidine when cultured by themselves at the appropriate cell concentrations. It should also be noted that if a feeder-layer effect is responsible for the autologous MLR then only certain lymphoid populations can produce this effect (Tables 27 and 28). It would seem more likely that antigen stimulation of some sort is responsible for this reaction. To determine whether both appendix and spleen cells were responding or stimulating in the two-way autologous MLR, one-way MLRs were set up with the same cells used in the two-way MLRs (Figure 15). Appendix cells did not respond to autologous mitomycin-C treated spleen cells. The spleen cells, however, did respond to the autologous mitomycin-C treated appendix cells. The results using WBC and appendix cells essentially follow the same pattern shown with autologous cultures

of spleen and appendix. No dose response curves were performed with WBC as the number of cells available precluded this type of experiment.

Spontaneous or baseline blastogenesis of the WBC and spleen was markedly suppressed by the addition of autologous mitomycin-C treated WBC or spleen cells respectively. This was probably due to release from stimulator cells of small amounts of mitomycin-C which would inhibit DNA synthesis in responder cells (Wilson, 1967; Adler, Takasugi, Marsh and Smith, 1970). Since the blastogenic responses recorded in the autologous two-way MLR where no mitomycin-C treatment of the cells was involved, markedly exceeded that observed in the one-way MLR with the identical cells, one cannot attribute the blastogenic response in the autologous MLR to soluble mitogenic factors released from mitomycin-C treated cells in culture as has previously been suggested by Etheridge, Shons, Hohenthoner and Najarian (1973).

Thus, the stimulatory activity of autologous cells must be attributed to as yet undefined intrinsic properties of these cells.

ii) Explanations for the existence of the autologous response in vitro

It has been previously shown that appendix, sacculus rotundus and Peyer's patches cells, which seem to actively stimulate autologous WBC and spleen cells, are also metabolically very active as indicated by a high spontaneous synthesis of DNA in vitro (see Section 2). Cells of the other lymphoid organs which do

not stimulate blastogenesis of autologous cells are metabolically marginally active in vitro. It is therefore possible that antigenic sites are uncovered on the surface of the gut-associated lymphoid cell in vitro as a concomitant or consequence of the active metabolic state of this cell, thus imparting to it the capacity to stimulate autologous lymphocytes.

Several other explanations may be considered in the attempt to attribute a responder-stimulator relationship between the autologous cells in the autologous MLR. Thus, cells in the normal rabbit which stimulate autologous WBC or spleen cells in vitro may, in fact, be normally sequestered or permanently localized within the particular lymphoid organs and do not normally circulate. They would therefore have little or no opportunity to confront autologous responder cells in vivo. Another explanation is that these responses are inhibited in vivo by the presence of suppressor cells or soluble suppressor factors.

iii) The significance of the autologous response.

Over the past decade investigators have reported responses in the autologous MLR utilizing untreated stimulator cells. Howe, Goldstein and Battisto (1970) found that neonatal mouse thymus cells respond to syngeneic adult spleen cells in an MLR. It has also been observed that adult mouse spleen cells can stimulate thymus cells of four day old mice and cortisone resistant thymus cells of syngeneic adult mice (v. Boehmer and Byrd, 1972; v. Boehmer, 1974). Llancer and Uyeki (1969) have also reported that

rat thymus cells react with autologous spleen cells in an autologous two-way MLR.

Jerne (1970) has suggested that recognition of self and consequent tolerance to self antigens by elimination of specific clones occurs only in the thymus. Our experiments indicate that self recognition does occur outside the thymus in the rabbit. Other evidence for similar autologous reactivity of peripheral organs has recently been found by Takasugi, Kiuchi and Opelz (1977). They have recently found that human unseparated leukocytes, B plus null cells or T-enriched cells separated by rosetting will respond to autologous null cells or null cells plus B cells in the MLR. A similar autologous reaction between human responder T cells and stimulator B cells has been shown by Vande Stouwe, Kunkel, Halper and Weksler (1977). The above observations contradict the theory that cells reacting against self exist only for short periods of time within the thymus until that particular clone of reactive cells is eliminated.

These results of a blastogenic response in the autologous MLR using normal untreated cells should not be confused with results of investigations which have reported blastogenic responses in the autologous one-way MLR using stimulating cells which have been modified by the introduction of chemical groups (Shearer, Lozner, Rehn and Schmitt-Verhulst, 1975), or have been treated with mitogens, such as phytohemagglutinin (Ling and Hardy, 1974; Loew, 1971). Lymphoid tumor cells have also been shown to be capable of inducing a blastogenic response in the autologous

MLR (Green and Sell, 1970; Bernstein, Wright and Cohen, 1976; Boyer and Fahey, 1976). These responses can be attributed to (i) uncovering of normally inaccessible surface structures on the stimulating cells, or (ii) the modification of normally accessible surface structures, or (iii) the synthesis of new cell surface constituents. The effect is to impart a degree of "foreigness" to these cells in relation to autologous responder lymphocytes. One must consider that conditions in vitro do not permit the maintenance of a normal homeostatic balance nor regulation of cellular activity which would normally be effected by suppressor cells and/or soluble mediators in vivo. This imbalance may permit autologous stimulation to occur in the artificial environment of the test tube.

5. The Effect of in vivo Radiation on the Blastogenic Response of Lymphoid Cells Stimulated with Allogeneic Cells or Plant Mitogens

a) Abstract

b) Introduction

c) Results

d) Discussion

i) The effect of in vivo irradiation on MLR activity.

ii) Explanations for the loss of MLR activity in irradiated rabbits.

iii) The organ of origin of the MLR responder cell.

iv) The effect of in vivo irradiation on the mitogen response.

a) Abstract

The radio-sensitivity of the MLR responder cells of irradiated rabbits was investigated in the five day one-way MLR using optimal numbers of WBC, spleen, thymus, mesenteric lymph node, appendix, Peyer's patches and sacculus rotundus cells. The lymphoid cells of rabbits subjected to 50R, 100R or 200R retained their capacity to respond in the MLR. However, the lymphoid cells of rabbits subjected to 400R or 600R whole body irradiation failed to respond in the one-way MLR in the immediate post-irradiation period. In

analyzing the time of recovery of MLR responder cells after 600R whole body irradiation, it was found that the responder activity of the cells of all the lymphoid organs did not fully recover until at least four weeks after irradiation. These results cannot be interpreted to suggest that any one lymphoid organ constitutes a primary source of mature MLR responder cells in the rabbit, but does indicate that all the rabbit lymphoid organs contain precursor or a small number of mature MLR responder cells which are able to reconstitute the MLR response of these organs after 600R irradiation.

The WBC of the irradiated rabbits also exhibited greatly reduced blastogenic responses to the plant mitogens concanavalin-A, phytohemagglutinin and pokeweed. However, the responses were not uniformly suppressed. The cells responding to pokeweed mitogen appeared to be most sensitive to in vivo irradiation and the cells responsive to concanavalin-A the least sensitive.

b) Introduction

It has been shown that subpopulations of lymphoid cells differ in their sensitivity to irradiation when assayed for certain functional properties such as immunocompetence in antibody production (Abdou, Rose and Richter, 1969; Anderson and Warner, 1975), or responsiveness to mitogens (Stobo and Paul, 1973).

The radiosensitivity of rabbit lymphoid cells capable of responding in the mixed leukocyte culture reaction (MLR) has not been thoroughly or systematically investigated. Chapman and Dutton (1965)

have shown that spleen cells or lymph node cells of rabbits subjected to 400R irradiation no longer give a detectable response in the two-way MLR. Ozer, Jr. and Waksman (1974) have shown that appendix, spleen and mesenteric lymph node cells of bone-marrow deprived rabbits (animals exposed to 900R whole body irradiation and reconstituted with 5×10^9 allogeneic thymocytes) responded to the mitogens concanavalin-A (Con-A), phytohemagglutinin (PHA), and pokeweed mitogen (PWM). On the other hand appendix, mesenteric lymph node and spleen cells of thymus-deprived rabbits (extirpation of thymus, popliteal lymph nodes and spleen followed by three doses of 450R whole body irradiation with femoral bone marrow shielding) did not show significant responses to Con-A, PHA or PWM. However, these cells showed a significant response to anti-immunoglobulin sera (Calkins, Ozer, Jr. and Waksman, 1974). It is instructive to note that neither spleen cells nor appendix cells nor circulating lymphocytes of any of the irradiated and reconstituted rabbits referred to above were capable of a blastogenic response in the one-way MLR (Ozer, Jr. and Waksman, 1974).

The purpose of this investigation was to systematically analyze the in vivo radio-sensitivity of the MLR responder cells of rabbits subjected to varying doses of whole body irradiation. The cells of the different lymphoid organs were compared for their responsiveness in the MLR at intervals of time post-irradiation. It was found that, with the appropriate dose of irradiation, all

MLR responsiveness was eliminated in the immediate post-irradiation period and the responses of the cells of all the lymphoid organs recovered in approximately the same period of time.

c) Results

The average blastogenic responses in the one-way MLR of WBC, spleen and appendix cells from a number of rabbits are presented in Table 29. The concentrations of cells used have previously been shown to permit an optimal response. Rabbits were irradiated with 50R to 800R and the WBC, spleen and appendix cells were tested the next day for their responses in the one-way MLR (Table 30). It can be seen that the cells of rabbits 1 to 6 subjected to less than 200R responded normally in the one-way MLR. Cells of rabbits exposed to 200R gave barely significant responses. Cells of rabbits exposed to 400R, 600R and 800R all failed to respond in the one-way MLR.

The WBC of rabbits exposed to 200R and 400R were tested for their MLR and mitogen-induced blastogenic responses (Table 31). The MLR responses of cells of the rabbits irradiated with 200R were virtually abolished and their responses to PWM were also markedly reduced. The responses to Con-A and PHA were also reduced significantly. The cells of the rabbits irradiated with 400R did not respond in the one-way MLR nor following stimulation with PWM. The responses to PHA and Con-A, although significant, were much reduced compared to those obtained with cells of the unirradiated rabbits.

TABLE 29

THE RESPONSE OF WBC, SPLEEN CELLS AND APPENDIX CELLS TO MITOMYCIN-C TREATED ALLOGENEIC WBC IN THE ONE-WAY MLR.

Organ source of responder cells	Lymphoid cell conc ($x10^5$)	Average SI ± S.E.M.*	Number of experiments performed
WBC	5	23 ± 14	8
	10	31 ± 21	7
Spleen	10	80 ± 33	5
	15	31 ± 12	4
Appendix	15	13 ± 9	4
	25	14 ± 9	4

*S.E.M. represents standard error of the mean.

TABLE 30

THE IN VITRO MLR RESPONSIVENESS (S.I.) OF LYMPHOID CELLS OF THE RABBIT WITHIN ONE DAY FOLLOWING WHOLE BODY ^{60}Co-IRRADIATION.

Responder rabbit	Stimulator rabbit (Mitomycin-C treated cells)	No. of rads. applied	Responder cells tested (x 10^5)					
			WBC		Spleen		Appendix	
			5	10	10	15	15	25
1	7	0	0.6*	0.7	ND**	ND	ND	ND
2	7	0	23.0	ND	148.0	ND	351.0	ND
3	7	50	30.0	25.0	347.0	212.0	391.0	227.0
4	7	50	101.0	54.0	77.0	116.0	243.0	182.0
5	7	100	3.3	ND	153.0	ND	164.0	ND
6	7	100	50.0	32.0	49.0	32.0	220.0	301.0
8	14	0	121.0	158.0	20.0		3.1	10.0
9	14	200	2.8	3.9	2.6	1.5	ND	ND
10	14	200	1.4	1.0	1.1	1.1	4.0	11.0
11	14	400	1.6	1.3	0.6	0.9	1.0	2.2
12	14	400	1.0	2.3	0.7	0.9	1.8	1.4
15	21	0	9.3	11.0	ND	ND	ND	ND
16	21	0	3.5	3.9	ND	ND	ND	ND
17	21	600	< 1	< 1	< 1	< 1	< 1	< 1
18	21	600	< 1	< 1	< 1	< 1	< 1	< 1
19	21	800	< 1	< 1	< 1	< 1	< 1	< 1
20	21	800	< 1	< 1	< 1	< 1	< 1	< 1

* The figures represent the S.I.s

** ND = Not done.

TABLE 31

THE EFFECT OF WHOLE BODY IRRADIATION ON THE MLR AND MITOGEN-INDUCED BLASTOGENIC RESPONSES OF RABBIT WBC

Responder Rabbit A	No. of Rads Applied (cpm)	Control (No stimulus)	Stimulus: Mitogens			Stimulus: Allogeneic Cells (MLR)	
			Con-A	PHA	PWM	A + Am**	A + Bm*
1	0	529***	842	466	105	537	173.0
2	0	3461	124	54	39	ND****	ND
3	200	331	558	215	21	723	3.9
4	200	388	120	34	3.3	858	1.0
5	400	243	44	7.4	0.8	652	0.9
6	400	276	45	8.1	1.8	483	1.4

*The results are expressed as Stimulation Index (S.I.) (see Methods and Materials).

**A represents the responder rabbit (1 to 6) and Am and Bm represent mitomycin-C treated cells used as stimulator cells in the MLR. Bm are mitomycin-C treated cells of rabbit No. 2 which were used as allogeneic stimulator cells for the entire experiment.

***These numbers represent cpm of triplicate samples.

****ND = Not Done.

The lymphoid cells of rabbits subjected to 600R whole body irradiation were tested for their responses in the MLR for up to 47 days post-irradiation (Table 32). No MLR activity was detected in the cells of any of the lymphoid organs studied one to two days after 600R irradiation. The cpm were very low in all allogeneic mixtures of irradiated responder cells and mitomycin-C treated stimulator cells. Five to six days after irradiation, the mesenteric lymph node (MLN) cells exhibited relatively high blastogenic activity in comparison to the activity in the cells of the other organs. However, in another set of identical experiments MLN cells of irradiated rabbits did not show this high blastogenic activity five days after whole body irradiation. The cells of all the irradiated lymphoid organs showed inconsistent recovery of MLR responsiveness; and generally the cpm of these cultures were also markedly lower than those observed in cultures of cells from non-irradiated rabbits. The cells of all the lymphoid organs did not approach normal responsiveness in the MLR until 43 to 47 days after irradiation (Table 32). At this time the cells of spleen and WBC of irradiated rabbits showed a higher S.I. than that of normal rabbits, possibly due to overcompensation by these organs.

It is evident that throughout the time period studied, no MLR responder activity could be detected in the cells of the bone marrow or thymus which are normally unresponsive. Although thymus cells do not generally respond in the MLR (see Section 2), it should be noted that thymus cells of two out of the eleven normal rabbits tested responded with highly significant S.I.s (rabbits 1N and 1ON; Table 32).

FOLLOWING WHOLE BODY ^{60}Co. IRRADIATION (600 R).

Responder rabbit (irradiated(R) or normal (N) rabbit)	Stimulator rabbit (Mitomycin-C treated cells)	Days after Irradiation	Responder cells tested (1 x 10^6)							
			Spleen	WBC	Bone Marrow	Thymus	MLN*	Sacculus rotundus	Peyer's patches	Appendix
1N	2	1	84.0*	55.0	2.4	14.0	20.0	133.0	21.0	95.0
3R	2	1	2.8	0.8	1.7	2.9	3.1	3.8	3.3	1.6
4N	5	2	20.0	92.0	1.9	3.3	31.0	27.0	8.7	15.0
6R	5	2	0.5	0.4	1.8	1.4	2.6	1.0	1.3	1.6
7N	8	5	49.0	169.0	0.8	0.6	11.0	32.0	4.5	13.0
9R	8	5	2.0	4.6	0.8	2.9	26.0	6.6	2.6	9.9
10N	11	6	62.0	117.0	3.8	14.0	27.0	59.0	2.2	10.0
12R	11	6	17.0	15.0	6.9	5.5	36.0	11.0	5.3	3.7
13N	14	14	27.0	134.0	1.0	1.2	27.0	3.7	6.7	1.1
15R	14	14	2.5	77.0	3.2	1.4	2.4	1.3	0.7	1.0
19N	20	22	76.0	45.0	0.7	1.3	103.0	16.0	108.0	67.0
21R	20	22	13.0	6.5	0.5	1.4	4.3	2.2	1.1	1.3
22N	23	23	67.0	73.0	3.3	3.3	7.0	7.8	7.3	4.9
24R	23	23	7.6	16.0	1.8	2.3	20.0	3.0	4.6	3.3
25N	26	29	2.2	8.6	2.9	0.4	15.0	6.9	8.8	2.9
27R	26	29	1.7	1.7	2.5	0.5	9.1	2.8	1.3	1.0
28N	29	30	15.0	71.0	5.5	0.8	22.0	13.0	1.5	-
30R	29	30	2.8	1.3	4.0	0.5	0.6	0.2	4.7	-
31N	32	43	15.0	35.0	3.8	1.1	17.0	71.0	37.0	5.3
33R	32	43	34.0	54.0	3.5	0.8	5.1	12.0	11.0	2.2
34N	35	47	6.4	14.0	13.0	2.6	197.0	91.0	53.0	45.0
36R	35	47	40.0	122.0	3.9	7.6	23.0	52.0	89.0	38.0

* MLN represents mesenteric lymph node cells.

** The figures represent the S.I.s.

d) Discussion

The primary objectives of this study were to determine (1) the radiosensitivity of the MLR responder cells of the various lymphoid organs and (2) the time required for the recovery of MLR responder cells in the various lymphoid organs following irradiation. These results suggest that all the lymphoid organs studied, with the exception of bone marrow and thymus contain a reservoir or act as a site of maturation of MLR responder cells. No evidence of migration of mature MLR responder cells from one organ to another was observed.

i) The effect of in vivo irradiation on MLR activity.

Initial experiments indicated that only irradiation with 600R or 800R could totally abolish MLR responsiveness of the rabbit lymphoid cells. Consequently 600R, a dose of irradiation less likely to result in the death of the rabbit than 800R, was chosen for subsequent experiments designed to determine the rate of recovery of the MLR responder cells in the lymphoid organs. In some cases cells of the mesenteric lymph nodes showed an apparent recovery of normal activity in the one-way MLR by day 5 after irradiation, as measured by the S.I. It should also be noted that at later times post-irradiation the recovery of the activity of the cells of the MLN was inconsistent and therefore the indication that the cells of the MLN had recovered by day 5 are not decisive. The MLR cultures consisting of MLR responder cells from irradiated rabbits did not exhibit cpm in the normal range until 43 to 47 days after irradiation. There was no indication that the cells of one organ recovered MLR activity faster than those of another. These

data suggest that the proportion of MLR responding cells within each organ rose from day 7 to day 47.

ii) Explanations for the loss of MLR activity in irradiated rabbits.

The results indicate that either the MLR responder cells are more radiosensitive than non-responder cells in the lymphoid organs and are eliminated from these organs preferentially leaving only non-responder cells or that responder cells that have been irradiated in vivo remain viable in the rabbit lymphoid organs (i.e. are not eliminated) but cannot respond by blastogenesis during the in vitro MLR, and that the recovery of responder activity is due to replacement in vivo of damaged MLR responder cells by new functional responder cells.

It is also possible that the lymphoid organs harbour populations of MLR helper cells and that the reduction of MLR activity following 600R irradiation is due to depletion of helper cells and not MLR responder cells. Although our data neither support nor reject the existence of MLR helper cells in the rabbit, results of other investigators infer the presence of such a cell. Dyminski and Smith (1975,1977) have demonstrated the presence of a B-like helper cell in mouse mixed lymphocyte cultures. In man, the MLR is enhanced by adherent, phagocytic cells (Blomgren, 1977). There is also evidence of helper cell activity in the graft vs. host reaction (GvH) which is considered to be immunologically the in vivo counterpart of the MLR (Cantor and Asofsky, 1970; Tigelaar and Asofsky, 1973). Furthermore, there is also evidence of a helper cell in the mitogen-induced response which is relevant

in terms of this discussion as the responder cell in this case is also a T-cell. Lipsky, Ellner and Rosenthal (1976) and Rosenstreich (1976) have found that activation of guinea pig lymphocytes by PHA requires macrophages which function as helper cells in the T-cell response to PHA. Helper cells have been implicated in the blastogenic response of rabbit lymphocytes to Con-A and PHA (Chou, Cinader and Dubiski, 1977; Bona, Cinader and Dubiski, 1977) which, like the MLR response, is regarded as a typical T-cell response (Ozer,Jr. and Waksman, 1974; Sheppard, Jr., Sell, Poler and Redelman, 1977; Bona, Cinader and Dubiski, 1977). These helper cells in the rabbit are not T-cells but are cells with appendix-specific antigens (Chou, Cinader and Dubiski, 1977). Although Chou, Cinader and Dubiski (1977) and Bona, Cinader and Dubiski (1977) consider these cells to be analogous to the B-cells in man and the mouse. In the mouse, B-cells are more susceptible to in vivo irradiation than are T-cells (Anderson, Olson, Autry, Howarth, Troup and Bartels, 1977). Thus, if the situation in the rabbit is similar to that in the mouse, irradiation may deplete lymphoid organs in the rabbit of helper cells for the MLR.

iii) <u>The organ of origin of the MLR responder cell</u>

Extensive investigations of the MLR in a number of animal species suggest that the organ of origin of the MLR-responsive cells in the normal untreated adult animal is the thymus. It has been repeatedly demonstrated that the MLR responder cells of the mouse (Bach, Bach and Sondel, 1976), man (Chess, MacDermott, Sondel and Schlossman, 1974; Owen and Fanger, 1974) and rabbit

(Bona, Cinader and Dubiski, 1977; Sheppard, Jr., Sell, Poler and Redelman, 1977) possess thymus-associated antigens. By implication, this finding indicates that the MLR responder cells are generated in the thymus or are the progeny of thymus-derived cells in the peripheral lymphoid organs. Subpopulations of adult mouse (Blomgren and Svedmyr, 1971), neonatal mouse (Howe, Goldstein and Battisto, 1970), and human (Han, Minowada, Subramanian and Sinks, 1976) thymus cells have been shown to be responsive in the MLR. The human fetal thymus also contains MLR responder cells (Granberg, Mamunen and Toivanen, 1976; Asantila, Vahala and Toivanen, 1974). In spite of the above findings in a number of animal species, no evidence for a role of the thymus in the generation or sustaining of MLR responder cells was obtained in either normal or irradiated rabbits.

iv) The effect of in vivo irradiation on the mitogen response

Lymphocytes of rabbits exposed to 200R whole body irradiation showed a disproportionate loss in responsiveness to PWM, as compared to PHA and Con-A. 200R reduced the MLR and PWM responses to the level of control cultures but did not eliminate the responses to PHA and Con-A. This suggests that the cells responsible for the mitogen response are less susceptible to irradiation damage than are the cells responding in the MLR.

Our results consequently suggest the existence of distinct populations of lymphocytes responding to the different plant mitogens. These data are supported by other investigations in the mouse (Stobo and Paul, 1973) and in the rat (Milthorp and Forsdyke, 1973). Stobo and Paul (1973) showed that mouse PHA-responder cells

are more susceptible to in vivo irradiation than are Con-A responder cells.

6. The Response in the One-Way MLR of WBC from Allograft Sensitized Rabbits to Stimulation with WBC from the Allograft Donors

a) Abstract

b) Introduction

c) Results

d) Discussion

i) The accelerated MLR response after allograft sensitization.

ii) The premature decrease of the accelerated MLR response.

iii) The absence of cytotoxic cells in allograft sensitized animals.

a) Abstract

The relationship between allograft rejection and the response in the one-way mixed leukocyte reaction (MLR) of the WBC of allograft recipients was investigated. The responses of the WBC were assessed prior to skin allografting and subsequent to rejection of the allograft. The optimal MLR response of the WBC from the allografted rabbit stimulated with cells from the allograft donor occurred on days 3 to 4 of culture and by day 6 the MLR response was down to control levels. This accelerated response by the

allograft-sensitized cells was specific to stimulator cells from the allograft donor as stimulator cells from a third party rabbit induced a normal MLR response by these cells, that is, an optimal response on days 5 to 7 of culture.

No evidence of cytotoxic cells was obtained in cultures of cells from allograft-sensitized rabbits. The implications of these results are discussed.

b) Introduction

The mixed leukocyte reaction (MLR) was first described in man by Schrek and Donnelly (1961) and the reaction was subsequently linked to histocompatibility differences between leukocyte donors (Bain, Vas and Lowenstein, 1964; Hirschorn, Bach, Kolodny, Firschein and Hashem, 1963). The histocompatibility antigens detected in the MLR were referred to as lymphocyte-determined (LD) antigens. However, it quickly became apparent that other histocompatibility antigens exist on the lymphocyte which are detected by their interaction with specific antisera (tissue typing). These are referred to as serologically determined (SD) antigens. After development of the one-way MLR technique (Bach and Voynow, 1966) LD antigens were mapped in the major histocompatibility loci closely linked to the SD antigens detected by tissue typing.

More recently, in vitro experiments have shown that cytotoxic cells are produced during the MLR which specifically kill cells with SD-antigens identical to those present on the MLR stimulator cells. It has been recently suggested that the proliferating

cells (MLR responder cells) which react to different LD antigens in the MLR and which are not the precursors of cytotoxic cells, "help" in the maturation of the cytotoxic cells. The cytotoxic cells are specifically directed against SD antigens (Bach, Bach and Sondel, 1976; Hayry and Andersson, 1973). An identical model has been suggested for in vivo graft rejection (Bach, Kuperman, Sollinger, Zarling, Alter and Bach, 1977) and the suggestion has been made that the targets of cytotoxic cells may not be identical to SD antigens and should be redefined as CD (cytotoxically defined) antigens. It is generally assumed in these models that the cytotoxic cells responsible for graft rejection are T-cells as allografts are not rejected in the absence of T-lymphocytes (Cerottini and Brunner, 1974). However, recent experiments have indicated that the majority of cytotoxic cells at the site of the rejected allograft in mice are non-T in nature (Roberts and Hayry, 1977).

It has also been shown in vitro that, along with the generation of cytotoxic cells in the MLR, memory MLR responder cells are produced in the cultures several days after the initial MLR has subsided. These cells exhibit a "memory" as their MLR response is accelerated only if stimulated by LD-antigens identical to those on the initial stimulating cells (Zier and Bach, 1975; Hayry and Andersson, 1975; MacDonald, Engers, Cerottini and Brunner, 1974).

Wilson and Nowell (1971) have shown that the MLR blastogenic responses of cells of allografted rats when stimulated with the cells of the graft donor are accelerated for up to three weeks

following allograft rejection. Similar results were observed by Wilson, Silvers and Nowell (1967) using rats and Adler, Takiguchi, Marsh and Smith (1970) using mice. Harrison, Wei and Ahie (1971) have shown that the MLR response between cells of allograft recipient and allograft donor rabbits is greatly enhanced. However, Chapman and Dutton (1965) and Sheppard, Jr., Sell, Poler and Redelman (1977) did not confirm these results.

The purpose of this investigation was to study the temporal aspects of the one-way MLR blastogenic response of the WBC of rabbits before and after allograft rejection in order to ascertain whether the rejection of an allograft is accompanied by changes in the MLR responsiveness of WBC of allografted rabbits.

c) Results

Figure 17 illustrates the protocol for the investigation of the effect of allograft rejection on the one-way MLR response in the rabbit.

Figure 18 shows the results of an MLR with the cells of the same rabbits before and after allografting. Curve A shows the results of a one-way MLR using responder leukocytes from rabbit A (prospective allograft recipient) and mitomycin-C treated stimulator leukocytes from rabbit B (prospective allograft donor) eight days before skin allografting. It can be seen that no detectable MLR has taken place by 2 to 3 days of culture, a slight response has occurred by days 4 to 5, and the MLR response has become evident by day 6. This is the normal time course of the rabbit MLR response.

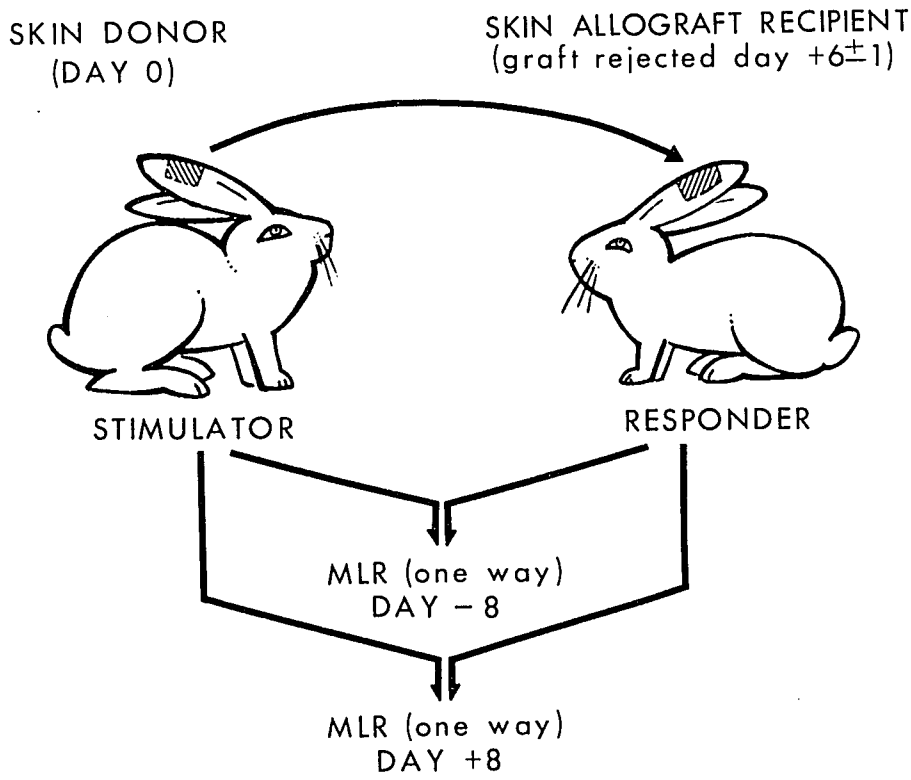


FIGURE 17

PROTOCOL FOR THE DEMONSTRATION OF THE EFFECT OF ALLOGRAFT REJECTION ON THE KINETICS OF THE ONE-WAY MLR.

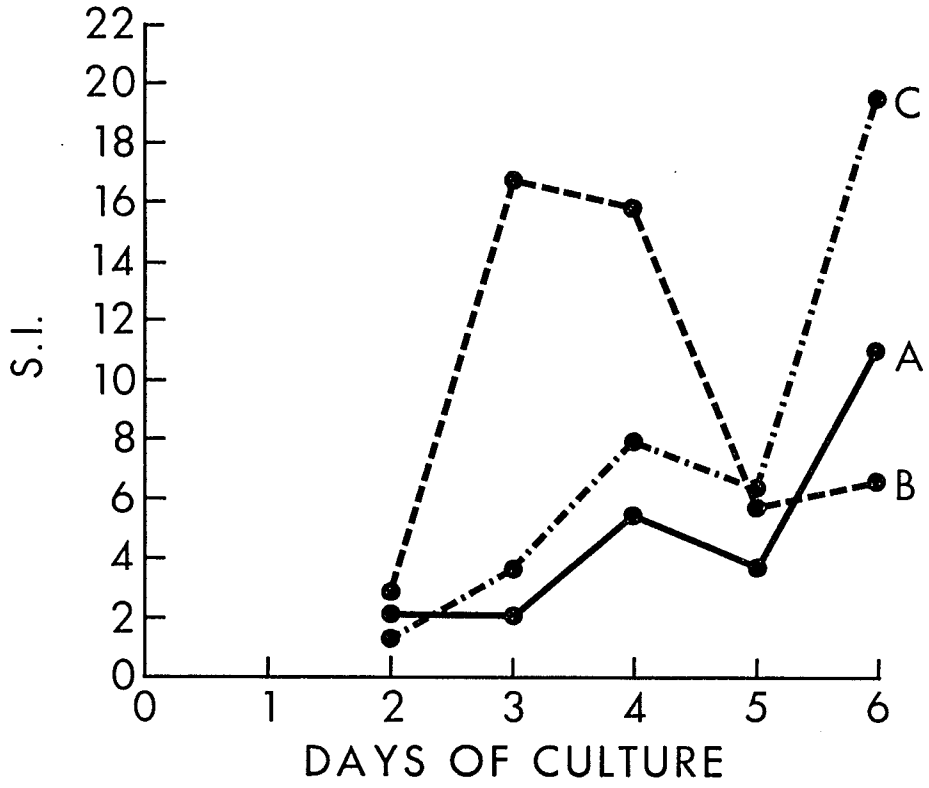


FIGURE 18 THE ACCELERATED ONE-WAY MLR RESPONSE OF CIRCULATING RABBIT WBC FOLLOWING SKIN ALLOGRAFTING.

Curve A = Rabbit A responder cells (pre-grafting) vs. rabbit B stimulator cells (Mitomycin-C treated).

Curve B = Rabbit A responder cells (day 8 post-allografting) vs. rabbit B (skin graft donor) stimulator cells (Mitomycin-C treated).

Curve C = Rabbit A responder cells (day 8 post allografting) vs. rabbit C (innocent 3rd party) stimulator cells (Mitomycin-C treated).

Rabbit A was then given a skin graft from Rabbit B (Figure 17). The graft took and was sloughed off within 5 to 6 days. Autografts remained healthy. Eight days after the transplant and 2 to 3 days after complete rejection of the allograft, another MLR was carried out using responder cells of rabbit A (recipient of graft from rabbit B) and stimulator WBC of rabbit B (curve B in Figure 18). The response in the MLR of WBC from the allografted rabbit A was accelerated with the peak response recorded on days 3 to 4 of culture. By days 5 to 6 the response has decreased dramatically. At the same time another MLR was carried out between the WBC of responder rabbit A and stimulator WBC of an unrelated rabbit C to which rabbit A had not been sensitized (curve C in Figure 18). The results were similar to those obtained previously with responder WBC of rabbit A prior to allografting and stimulator rabbit B, with the main MLR response evident on day 6.

The cpm in the cultures of the experiment illustrated in Figure 18 are presented in Table 33. Maximum incorporation of ^{3}H-thymidine using pre-allograft responder cells of rabbit A and stimulator cells of rabbit B occurred on day 6. Furthermore, the MLR between the responder WBC of allografted rabbit A and the stimulator WBC of rabbit C shows a similar pattern with the cpm increasing until day 6. Using responder WBC from the allografted rabbit A and stimulator WBC from the allograft donor rabbit B, maximum ^{3}H-thymidine incorporation occurred on day 4 followed by a decrease on days 5 and 6 at which point the cpm are similar to those on day 2. The maximum S.I. was recorded on day 3.

TABLE 33

THE ACCELERATED RESPONSE (CPM) IN THE ONE-WAY MLR OF CIRCULATING WBC FOLLOWING SKIN ALLOGRAFTING*.

Rabbits		WBC of rabbit A cultured	Days after initiation of MLR in vitro				
Responder	Stimulator**		2	3	4	5	6
A	A	Day 8 Before Allografting	296	480	299	428	428
A	B		585	971	1644	1555	4917
A	A	Day 8 following allografting (day 2 post-rejection)	888	520	1039	1234	495
A	B		2276	8495	16268	7037	3315
A	C		1083	1904	8361	7870	9560

* The data given in this table consists of the incorporation of ^{3}H-thymidine as counts per minute, of the experiments illustrated in Figure 2.

** Stimulator cells were treated with 50 ug/ml of mitomycin-C.

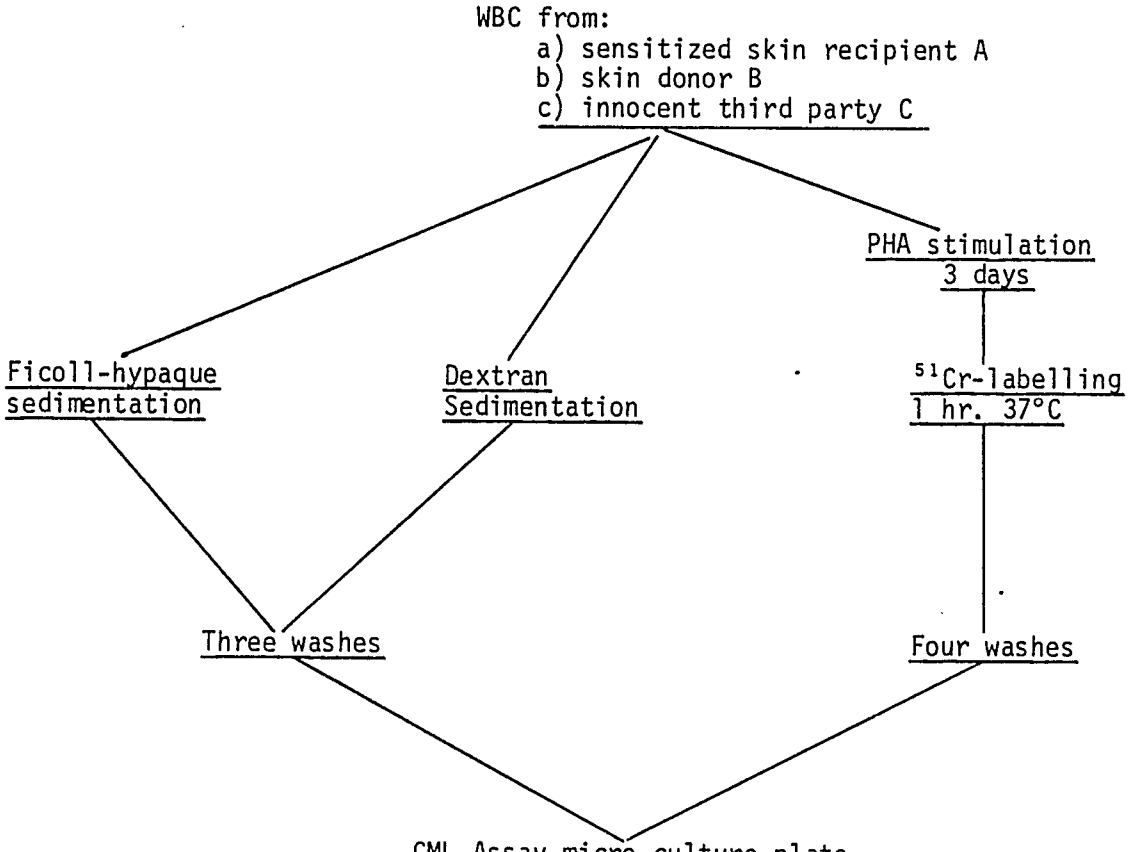
TABLE 34

THE RESPONSE (S.I.) IN THE MLR OF CIRCULATING WBC BEFORE AND AFTER TRANSPLANTATION OF SKIN FROM STIMULATOR RABBIT TO RESPONDER RABBIT.

Rabbits		MLR initiated at day following allografting**	Maximum SI	Day of maximum MLR response in the in vitro culture
Responder	Stimulator*			
A	B	- 8	11	6
A	B	+ 8	17	3
C	D	- 8	1.4	6
C	D	+ 8	6.6	4
E	F	- 8	35	6
E	F	+ 8	51	4

* Stimulator cells were treated with 50 ug/ml of mitomycin-C

** The skin allograft was carried out on day 0 and was rejected in six days. The MLR was carried out 8 days prior to allografting and two days after rejection.



COMBINATIONS USED

effector A plus target A
effector A plus target B
effector A plus target C
effector B plus target B
effector C plus target C

FIGURE 19

PROTOCOL FOR THE DEMONSTRATION OF CELL MEDIATED LYMPHOLYSIS ASSAY IN THE RABBIT.

Table 34 shows the optimal MLR response (S.I.) of WBC before and after allografting in three different experiments. It can be seen that in each case the maximum S.I. before allografting occurred on day 6 and the maximum S.I. after allografting occurred on days 3 to 4. It should also be noted that the maximum response (S.I.) after allografting was always higher than before allografting.

Figure 19 illustrates the protocol used to investigate cell mediated lympholysis in the rabbit. The target cells were obtained from the allograft donor, stimulated to undergo blastogenesis by incubation with PHA for 3 days and then labelled with ^{51}Cr. Effector cells were prepared using dextran sedimentation or ficoll-hypaque gradients. The ratio of effector to target cells was varied from 100:1 to 6.25:1 with the target cell concentration kept constant at 2.5×10^4 cells. No cell mediated lympholysis was detected in these experiments.

d) Discussion

A number of recent studies have disclosed that cytotoxic cells, in addition to proliferating cells, are generated in culture during the primary allogeneic MLR with mouse or human lymphoid cells. Furthermore, both the proliferative and cytotoxic responses are enhanced following restimulation of the cultured cells with stimulating cells from the original donor(s) (Hayry and Andersson, 1975; MacDonald, Engers, Cerottini and Brunner, 1974; Zier and Bach, 1975; Gorczynski and Rittenberg, 1975). It is generally assumed, more on the basis of circumstantial and coincidental findings than on objective unequivocal evidence, that

the in vitro MLR response mirrors the in vivo response of host cells to allogeneic transplantation antigens on allografted cells. The primary objective of this study was to determine whether immunization of the rabbit to allogeneic transplantation antigens, by skin allografting followed by graft rejection, modifies the specific MLR between the responder lymphocytes of the allografted rabbit and stimulator lymphocytes of the graft donor. Our results suggest that this is in fact the case.

i) The accelerated MLR response after allograft sensitization

Eight days after allograft implantation and two days after its rejection, the MLR blastogenic response of the host's cells to the cells of the allograft donor was markedly accelerated compared to the pre-allograft MLR response. The optimal MLR blastogenic response occurred on days 3 and 4 of culture, instead of days 5 and 6 observed with cells of normal rabbits (Figure 18). On the other hand, the response in the MLR of the WBC of the allografted rabbit to stimulator cells of a rabbit unrelated to the skin graft donor was not accelerated. These findings indicate that the accelerated MLR blastogenic response is a reflection of prior sensitization of the responder cells to determinants that are also found on the lymphoid cells of the allograft donor. In view of the fact that the S.I. at the optimal response with the post-grafted responder cells was not significantly different from that obtained with the responder cells of the same rabbit

prior to allografting, it is not very likely that the accelerated responses of cells of allografted rabbits can be attributed to increases in the proportions of specific responder cells. Rather, the accelerated response appears to be a property of "presensitized" or "memory" cells, which are more rapidly stimulated to respond in the MLR. These results are consistent with those obtained by other investigators in the allogeneic rat MLR using responder cells of rats either presensitized with allogeneic spleen cells (Wilson and Nowell, 1971) or allogeneic skin grafts (Wilson, Silvers and Nowell, 1967).

ii) The premature decrease of the accelerated MLR response

An interesting feature of the accelerated MLR response presented here was its dramatic decrease by day 5, which is a characteristic feature of the secondary MLR response (Hayry and Andersson, 1975; MacDonald, Engers, Cerottini and Brunner, 1974; Zier and Bach, 1975), and of the MLR response of cells obtained from animals presensitized in vivo to the appropriate transplantation antigens present on the stimulator cells (Wilson, Silvers and Nowell, 1967; Wilson and Nowell, 1971). It has been suggested that this decrease in the secondary MLR response by day 5 may be attributed to the elimination of stimulating cells by specific cytotoxic cells (Fitch, Engers, Cerottini and Brunner, 1976), or to increased activity of suppressor cells (Rich, Chu and Rich, 1977).

Our results are not in agreement with those of Chapman and Dutton (1965) and Sheppard, Jr., Sell, Poler and Redelman (1977) who did not observe an enhanced MLR blastogenic response by the cells of post-allografted rabbits to stimulator cells of the graft donor. These results may, however, be explained by the fact that the investigators evaluated the MLR blastogenic response only on or after day 5 of culture, by which time the accelerated response would have subsided and the response of the cells would be reduced to the level of "unsensitized" cells.

iii) The absence of cytotoxic cells in allograft sensitized animals.

In our hands no cytotoxic cells were found among the WBC of the rabbits before or after allograft rejection although the MLR responses given by the WBC at these times were very different. The target cells were obtained from the allograft donor, stimulated to undergo blastogenesis by incubation with PHA for 3 days and then labelled with ^{51}Cr (Lightbody, Bernoco, Miggiano and Ceppelini, 1971). There are a number of explanations which can account for our failure to detect cytotoxic cells. It is possible that, in the rabbit, direct cell-mediated immunity involving non-cytotoxic sensitized cells may be the major mechanism used in allograft rejection. If rejection involves a humoral component such as antibody plus complement or antibody-dependent cellular cytotoxicity, it would not have been detected in our assays which lacked serum from the allografted rabbit and complement. It is

possible that cytotoxic cells do mediate the rejection of the allograft but are sequestered in the graft and stored in a lymphoid organ other than the blood (Andersson and Hayry, 1975). The WBC in this case may contain very few cytotoxic cells and no reactivity would be detected. It is also possible that the conditions of our assay used to detect cytotoxic cells in the rabbit are inadequate.

CHAPTER V

GENERAL DISCUSSION

The long-term objectives of this investigation were to define, in a systematic fashion, the organ sources of the MLR responder and stimulator cells, to study their in vivo migration characteristics following stimulation with allogeneic transplantation antigens and to evaluate the roles which the humoral and cell-mediated immune responses play in the allograft rejection.

The outbred adult rabbit constitutes an excellent animal in which to investigate immunologic phenomena and characterize and evaluate the cellular and non-cellular mechanisms recruited in the initiation and maintenance of the response. A large number of different lymphoid organs and tissues can be investigated from the same rabbit. Furthermore, the physiological anatomy of the rabbit is more closely related to that of the human than is that of other experimental animals used, especially mice, rats and guinea pigs. In all of these latter animal species, the spleen and lymph node are hematopoietic organs, quite unlike the situation in the human and the rabbit where only the bone marrow in the adult is hematopoietic. Furthermore, the mice, rats and guinea pigs utilized are often inbred, in contradistinction to the rabbit (and the human). Results obtained with animals of one strain are not often observed in animals of a different strain and therefore, more often than not, the investigator is tempted

to select that strain of animal which has been previously shown to respond in the desired manner. It is for these reasons that the outbred rabbit was selected for this present investigation.

The responder and stimulator activities of the cells of the different rabbit lymphoid organs (circulation, spleen, thymus, bone marrow, sacculus rotundus, Peyer's patches, lymph nodes and appendix) have been defined and evaluated. The results have revealed a number of previously unreported relationships between responder and stimulator cells in the rabbit MLR. They also show that contrary to the generally held view, the rabbit WBC respond well in the MLR and that an autologous MLR can be induced under certain circumstances.

The composition of the culture medium plays a major role in facilitating the blastogenic response of the rabbit MLR. Experiments were carried out using different basic culture media and different types and concentrations of serum (fetal calf serum, normal rabbit serum) used to supplement these basic culture media. It was observed that media NCTC-109 and CMRL-1066 supported the blastogenic response only minimally, irrespective of the type or concentration of the supplementing serum. Medium 199 consistently supported the blastogenic response using normal rabbit serum in the appropriate concentration, but the degree of blastogenesis observed was uniformly less than that recorded with the same responder cells cultured simultaneously in similarly supplemented medium RPMI-1640. The medium which supported the optimal MLR

response was therefore RPMI-1640 supplemented with homologous rabbit serum at a concentration of 2.5 percent. The blastogenic response generally diminished with increasing concentration of serum in the medium and was often totally suppressed at serum concentrations of 25 to 30 percent. This is an important point and explains why some investigators have, in the past, reported failure to induce an MLR blastogenic response by rabbit WBC (see below).

The time course of the response in the MLR was another aspect that was investigated. The response time was similar for the cells of all lymphoid organs and was maximum on day 5 of culture. The temporal aspects of the response therefore do not show any differences between the participating cells of the different organs. In general, responder cells cultured alone incorporated more ^{3}H-thymidine than responder cells cultured in the presence of autologous mitomycin-C treated cells. This finding suggested some inhibitory effect of the mitomycin-C treated cells on the culture, a suggestion previously made by Wilson (1967).

The cells of the majority of the lymphoid organs responded in the MLR. Peyer's patches cells responded optimally at a low number of responder cells (2.5 to 5 x 10^5 cells per culture); mesenteric lymph node cells, WBC and spleen cells responded optimally at the intermediate number of responder cells (15 to 30 x 10^5 cells per culture); while appendix cells did not respond optimally unless 25 to 40 x 10^5 responder cells were used per culture. Furthermore, with respect to the magnitude of the response as measured by

the S.I., Peyer's patches and mesenteric lymph node cells responded the best; spleen cells responded moderately and cells of the sacculus rotundus and appendix responded poorly. On the assumption that the response of the cells of each organ is proportional to the percentage of responder cells present in each cell population, Peyer's patches cells would have the highest number of responder cells on a per cell basis and appendix cells the lowest. One would therefore anticipate that, as the optimum responder cell concentration is reached, the magnitude of the response (S.I.) would be the same for each of the cell populations. The results do not, however, support this assumption as the responses for the different cell preparations were different, irrespective of the cell concentrations used.

Only thymus and bone marrow cells did not respond in the MLR irrespective of whether they were cultured, individually or together for up to 7 days over a wide range of cell concentrations, with varying numbers of mitomycin-C treated allogeneic stimulating cells. This finding confirms the experiences of others using rabbit thymus and bone marrow cells in the MLR (Chapman and Dutton, 1965; Ozer Jr., and Waksman, 1974; Knight, Walker and Ling, 1971).

The lack of a significant response by rabbit thymus cells in the MLR is at variance with the positive findings using mouse thymocytes (Berman, Puryear and Argyris, 1976), rat thymocytes (Knight and Thorbeck, 1971) and human thymocytes (Schwarz, 1967; Schwarz, 1966). This may reflect a lack of mature MLR responder lymphoid cells in the rabbit thymus.

The rabbit WBC seem unique among the WBC of mammalian species in that they have been reported to respond poorly or not at all in the MLR (Knight, Walker and Ling, 1971; Ling and Kay, 1975; Ozer Jr. and Waksman, 1974; Sheppard Jr., Sell, Poler and Redelman, 1977) while the cells of organs such as the spleen respond normally. In our hands, rabbit WBC responded in the MLR in contrast to the findings reported by other investigators. WBC of the majority of the rabbits tested gave an S.I. greater than 5 (63% of 79 rabbits; average S.I. = 40 $\pm$ 6 S.E.M.). A positive response (S.I. >2) was observed with the WBC of the majority of the rabbits (82% of the 79 rabbits tested). A small number of the rabbits (18% of the 79 rabbits tested) had WBC which did not respond in the MLR. The lack of response by the WBC of these rabbits could be attributed to genetic similarities between stimulator and responder cells, as the rabbits were randomly selected and not tissue-typed prior to use. One should anticipate that genetic similarities will occur even between the outbred rabbits since they are obtained from a single breeder. All breeders unintentionally begin to inbreed their rabbits by mating only selected members of their stock.

The identity of the MLR responder cell in the rabbit in terms of it being a T, B or null cell, has been the subject of a number of investigations. The MLR responder cell has been shown to be lysed by anti-thymus serum in the presence of complement (Sheppard Jr., Sell, Poler and Redelman, 1977), implying

that the responder cell is a T cell. However, it is known that the classification of cells into B and T cells in the rabbit may be quite different as compared to that in man or the mouse. Sell and Sheppard, Jr. (1973) have shown that the rabbit lymphocyte which responds to T-cell mitogens can also be classified as a B-cell on the basis of a high concentration of surface immunoglobulins. It is therefore possible that the cells involved in the rabbit MLR and the graft rejection reaction may be cells other than those defined as T-cells in the mouse and man by the accepted criteria pertaining to these two animal species.

An interesting and consistent observation was that bone marrow cells were generally markedly inferior to WBC in their capacity to stimulate WBC blastogenesis in the MLR. This finding is in contradistinction to the equal responses given by the responder cells of the various lymphoid organs stimulated with either WBC or bone marrow cells. Any explanation for this observation would be strictly conjectural at this time.

A reproducible finding was the superior blastogenic response of cells of one organ, i.e. Peyer's patches, compared to that of the cells of another organ, i.e., appendix. Any explanation for this finding should take into account, (i) the augmenting or inhibiting effects in the MLR of helper or suppressor cells in these different responder cell populations, (ii) the possible generation of cells cytotoxic toward the stimulator cells in the

MLR, thus limiting their stimulating activity, (iii) the possible different sensitivity to stimulation by allogeneic cells of the different responder organs, and (iv) the differing percentage of responder cells in the cell populations tested.

It is possible that cells which respond best in the MLR, such as those of the Peyer's patches, may contain either more helper cells (Dyminski and Smith, 1975; Dyminski and Smith, 1977) or less suppressor cells (Luzzati and Lafleur, 1976; Rich, Chu and Rich, 1977; Hodes and Hathcock, 1976) than cells of an organ such as the appendix, which respond poorly. A second explanation for the differing responses of cells of the lymphoid organs could be due to the generation of cytotoxic cells in culture. Appendix cells during the course of the MLR could produce cells cytotoxic to the stimulating cells. This would reduce stimulation of the appendix cells and result in a low response in the MLR. An effect of this type has indeed been found in experiments with mice where cells taken from an ongoing MLR are found to inhibit the response of syngeneic responder cells in a second MLR using the same allogeneic stimulator cells (Fitch, Engers, Cerottini and Brunner, 1976). However, the destruction of the stimulator cells in the MLR has not been found to be mediated by previously uncultured cells (Fitch, Engers, Cerottini and Brunner, 1976) and therefore this mechanism cannot be considered to apply in the primary rabbit MLR system utilized in this study. A third possibility to explain the greater response of Peyer's patches cells than appendix cells in

the MLR is that the cells of the Peyer's patches detect a greater number and variety of antigens on the surfaces of the stimulating cells, via receptors on their surface, than do the cells of the appendix. Accordingly, we might anticipate that a greater number of Peyer's patches cells would respond to the allogeneic stimulus, as compared to appendix cells.

A recurrent noticeable deletion in the numerous investigations previously carried out to analyze the mechanism of the MLR in the rabbit has been the failure to assess the stimulating capacity of cells of the different lymphoid organs. Although WBC, spleen or mesenteric lymph node have been used as stimulator cells in various studies (Chapman and Dutton, 1965; Ozer Jr. and Waksman, 1974; Sheppard Jr., Sell, Poler and Redelman, 1977), cells of other lymphoid organs have generally been neglected in this regard. This matter was systematically analyzed in this study. Cells from the different lymphoid organs were compared with respect to their MLR stimulating capacity to determine their distribution, the optimal yield of these cells per organ and their capacity to stimulate the MLR in terms of the consistency of stimulation and the degree of stimulation.

Spleen and bone marrow cells consistently stimulated responder allogeneic cells and did not stimulate autologous cells. Though WBC are good stimulator cells, their use generally was precluded as the number of cells required in the majority of experiments exceeded the number recoverable from the blood of the donor rabbit.

The number of white cells recovered from 70 ml of blood is quite low ($1.5 \pm 0.4 \times 10^8$ cells; 16 experiments). Thymus and appendix gave by far the highest cell yield; however, these cells were comparatively poor stimulators in the one-way MLR. In the case of thymic stimulator cells, the quantity of ^{3}H-thymidine incorporated into responder WBC or spleen cells was quite low in both control and allogeneic cultures, indicating a definite lack of stimulation at the different concentrations of stimulator cells used. With stimulator mitomycin-C treated appendix, sacculus rotundus and Peyer's patches cells, the incorporation of ^{3}H-thymidine by the allogeneic responder spleen cells or WBC was very marked. However, ^{3}H-thymidine incorporation into the allogeneic cultures did not always exceed to any significant degree that observed in cultures of spleen cells and WBC stimulated with autologous mitomycin-C treated Peyer's patches, sacculus rotundus and appendix cells. This made these cells unsatisfactory as stimulator cells in the allogeneic MLR as the incorporation of ^{3}H-thymidine in the control cultures was so high as to mask the specific uptake of ^{3}H-thymidine. However, a second explanation for these findings is that appendix cells are capable of inducing a true MLR in autologous WBC and spleen cells (see below).

The mesenteric lymph nodes contained a larger number of cells which only minimally stimulated autologous responder cells. However, one out of every three mesenteric lymph node preparations

failed to stimulate in the MLR, indicating that these cells were also unreliable as stimulators.

Using the S.I. as a measure of stimulation, the mitomycin-C treated Peyer's patches cells were the best stimulators at a low stimulator cell concentration. This indicates that these cells have the greatest number or the most exposed MLR stimulating determinants on their surface. It is also of interest to note that Peyer's patches cells are the best responders in the MLR. Whether these properties of stimulation and response are associated with one cell type or different populations of cells has yet to be determined.

To determine whether cell-surface immunoglobulins are characteristic of the cells which stimulate responder cells in the MLR cells were incubated with fluorescein-conjugated anti-rabbit immunoglobulin antibodies to detect immunoglobulin-bearing cells. Spleen and bone marrow cells, which have highly different proportions of immunoglobulin-bearing cells were compared for their ability to stimulate responder allogeneic cells. The bone marrow and spleen cell were equally capable of stimulating the MLR over a wide range of cell concentrations, suggesting that surface immunoglobulins do not play any definitive role in the induction of the MLR.

An unexpected finding was the uniformly observed sharp increase in ^{3}H-thymidine incorporated by cells of the gut-associated organs at high cell concentrations in the autologous (control) MLR.

This increase in ^{3}H-thymidine incorporation was also observed in the total absence of autologous mitomycin-C treated stimulator cells and therefore can only be attributed to spontaneous blastogenesis of these cells.

Another unanticipated observation was that cultures of responder spleen cells or WBC in the presence of mitomycin-C treated stimulator cells obtained from the gut associated organs responded in a manner which, for all intents and purposes, resembled a conventional MLR. This response was dependent upon the number of autologous mitomycin-C treated "stimulator" cells in culture and also occurred in the two-way autologous MLR in which mitomycin-C treatment of the "stimulator" cells was omitted. This suggests that mitomycin-C induced production of mitogenic agents by the "stimulating" cells played no part in the induction of this autologous response.

The in vivo radiosensitivity of rabbit MLR responder cells was investigated in the one-way MLR. The lymphoid cells of rabbits subjected to up to 200R retained all or part of their ability to respond to allogeneic cells. Doses of 400R or 600R whole body irradiation, however, eliminated responder cell activity in the immediate post-irradiation period. In analyzing the recovery of MLR activity of the cells of the rabbits subjected to 600R no preferential recovery could be detected in any particular lymphoid organ. This suggests that no organ acted as a source of mature MLR responder cells which could consequently be seeded via

the circulation to the other lymphoid organs. Repopulation of the lymphoid organs with MLR responsive cells probably occurred by replication of a few undamaged mature or precursor MLR responder cells indigenous to the particular organ or by seeding of precursor MLR responder cells from another lymphoid organ.

It is likely that any seeding of rabbit precursor MLR cells is associated with the thymus as it has been demonstrated that the MLR responder cells of the rabbit (Bona, Cinader and Dubiski, 1977; Sheppard, Jr., Sell, Poler and Redelman, 1977) as well as those of mouse (Bach, Bach and Sondel, 1976) and man (Chess, MacDermott, Sondel and Schlossman, 1974; Owen and Fanger, 1974) possess thymus associated antigens. By implication, this indicates that MLR responder cells are derived from the thymus.

It is of interest to note that during the time of recovery of the rabbits from irradiation, no MLR responder cell activity was detected in the thymus or bone marrow, indicating that in the rabbit even during extreme loss of functional MLR responder cells in the peripheral organs no mature MLR responder cells are generated in these central lymphoid organs.

The mitogenic responses of lymphoid cells from irradiated rabbits was also studied. Stobo and Paul (1973) have shown that increasing the dose of whole body irradiation dramatically reduces the mitogenic response of mouse spleen cells to PHA to a greater degree than to Con-A. This indicates that mouse spleen cells responding to Con-A are more radioresistant than those responding to PHA. Similar results were found in the lymphoid cells of rabbits subjected to increasing doses of whole body irradiation.

It has been previously mentioned that a blastogenic response of rabbit WBC and spleen cells could be stimulated with mitomycin-C treated autologous appendix, Peyer's patches and sacculus rotundus cells in an apparent autologous MLR. It has been demonstrated that the blastogenic response in this "autologous one-way MLR" is in fact due to stimulation by autologous cells and not to non-specific blastogenic factors released into the cultures by the mitomycin-C treated stimulator cells.

Mitomycin-C treated cells of the gut-associated lymphoid organs uniformly stimulated blastogenesis of autologous WBC and spleen cells whereas mitomycin-C treated thymus and bone marrow cells generally had no stimulatory activity. Spontaneous or baseline blastogenesis of these same WBC was markedly suppressed by the addition of autologous mitomycin-C treated WBC. Furthermore, the blastogenic responses recorded in the autologous two-way MLR using untreated stimulator cells markedly exceeded those observed in the one-way MLR with the identical cells. One cannot therefore attribute the blastogenic response in the autologous one-way MLR to soluble mitogenic factors released from cultured mitomycin-C treated cells as has been suggested by Etheredge, Shons, Hohenthoner and Najarian (1973).

It has been shown that appendix, sacculus rotundus and Peyer's patches cells, which stimulate autologous WBC and spleen cells to undergo blastogenesis and mitosis, are also metabolically more

active than other lymphoid cells as indicated by a high spontaneous synthesis of DNA in vitro. Cells of the other lymphoid organs which do not stimulate blastogenesis of autologous cells or do so to a much lesser degree are metabolically marginally active in vitro. It is therefore possible that antigenic sites are uncovered on the surface of the gut-associated lymphoid cells in vitro as a concomitant or consequence of the active metabolic state of these cells, thus imparting to them a degree of foreigness sufficient to stimulate blastogenesis of autologous lymphocytes.

Over the past decade, a number of investigators have reported responses in the autologous MLR utilizing untreated stimulator cells. The stimulation of human unseparated or T-enriched lymphocyte populations by autologous non-T cells was first described by Opelz, Kiuchi, Takasugi and Terasaki (1975). These findings have been extended by several other investigators using human cells who found that (i) B cells and null cells, as well as T cells, respond in the autologous MLR (Takasugi, Kiuchi and Opelz, 1977), (ii) null cells as well as B cells stimulate in the autologous MLR (Takasugi, Kiuchi and Opelz, 1977), (iii) immunological memory and specificity can be observed during this reaction (Weksler and Kozak, 1977), (iv) cytotoxic T cells can be generated during the autologous MLR (Vande Stowe, Kunkel, Halper and Weksler, 1977), and (v) the B cell antigens responsible for stimulation of T cells during the autologous MLR may not be the same as those B cell antigens responsible for stimulation in the allogeneic MLR (Smith, 1978).

Investigations of the autologous MLR have also been carried out in other species. Howe, Goldstein and Battisto (1970) found that neonatal mouse thymus cells respond to syngeneic adult spleen cells. It has also been observed that adult mouse spleen cells can stimulate thymus cells of four day old allogeneic mice and cortisone resistant thymus cells of syngeneic adult mice (von Boehmer and Byrd, 1972; von Boehmer, 1974). Llancer and Uyeki (1969) have also reported that rat thymus cells react with autologous spleen cells in an autologous two-way MLR. More recently Werkerle (1977) has demonstrated immunological memory and specificity in the MLR between responder rat lymphocytes and autologous stimulator lymph node and testicular cells.

These results of a blastogenic response in the autologous MLR using normal untreated cells should not be confused with reported blastogenic responses using stimulating cells which have been modified by the introduction of chemical groups (Shearer, Lozner, Rehn and Schmitt-Verhulst, 1975), or have been treated with mitogens such as phytohaemagglutinin (Ling, Hardy and Steele, 1974; Lowe, 1971). Lymphoid tumour cells have also been shown to be capable of inducing a blastogenic response in the autologous MLR (Green and Sell, 1970; Bernstein, Wright and Cohen, 1976; Boyer and Fahey, 1976). These responses can be attributed to (i) uncovering of normally-inaccessible surface structures on the stimulator cells, (ii) the modification of normally-accessible surface structures, or (iii) the synthesis of new cell surface

constituents. The effect is to impart a degree of "foreignness" to these cells in relation to autologous responder lymphocytes.

Several other explanations may be considered in the attempt to attribute a responder-stimulator relationship to the cell in the autologous MLR. Thus, cells in the normal rabbit which stimulate autologous WBC or spleen cells in vitro may, in fact, be normally sequestered or permanently localized within the particular lymphoid organs and do not normally circulate. They would therefore have little or no opportunity to confront autologous responder cells in vitro. Another possible explanation is that these responses are inhibited in vivo by the presence of suppressor cells or soluble suppressor factors. However, these latter have not as yet been detected. Thus, the stimulatory activity of autologous cells must be attributed to as yet undefined intrinsic properties of these cells.

One must also consider that in vitro conditions do not necessarily mimic in vivo conditions as they do not permit the maintenance of a homeostatic balance of immunologic activity presumably maintained by the continual interaction of regulatory suppressor and stimulating cells and/or soluble mediators in vivo. The imbalance attained in vitro may therefore permit stimulation to occur in the artificial environment of the test tube.

A number of recent studies have disclosed that cytotoxic cells, in addition to proliferating cells, are generated in culture during the primary allogeneic MLR with mouse or human lymphoid cells.

Furthermore, both the proliferative and cytotoxic responses are enhanced following restimulation of the cultured cells with stimulating cells from the original donor(s) (Hayry and Andersson, 1975; MacDonald, Engers, Cerottini and Brunner, 1974; Zier and Bach, 1975). It is generally assumed, more on the basis of circumstantial and coincidental findings than on objective unequivocal evidence, that the in vitro MLR response mirrors the in vivo response of host cells to allogeneic transplantation antigens on allografted cells. It was of interest therefore to determine whether immunization of the rabbit to allogeneic transplantation antigens, by skin allografting followed by graft rejection, modified the specific MLR between the responder lymphocytes of the allografted rabbit and stimulator lymphocytes of the graft donor. The results suggest that this is in fact the case.

Eight days after allograft implantation and two days after its rejection, the MLR blastogenic response of the host's cells to the cells of the allograft donor was markedly accelerated compared to the pre-allograft MLR response. The optimal MLR blastogenic response occurred on days 3 and 4 of culture, instead of days 5 and 6 observed with cells of normal rabbits. On the other hand, the response in the MLR of the WBC of the allografted rabbit to stimulator cells of a rabbit unrelated to the skin graft donor was not accelerated. These findings indicate that the accelerated MLR blastogenic response by lymphocytes of allografted rabbits stimulated with cells of the graft donor is a reflection of prior sensitization of the responder cells to determinants

found on the cells of the allograft donor. In view of the fact that the S.I. at the optimal response with the post-grafted responder cells was not significantly different from that obtained with the responder cells of the same rabbit prior to allografting, it is not very likely that the accelerated responses of cells of allografted rabbits can be attributed to increases in the proportions of specific responder cells. Rather, the accelerated response appears to be a property of "presensitized" or "memory" cells, which are more rapidly stimulated to respond in the MLR. These results are consistent with those obtained by other investigators of the allogeneic rat MLR using responder cells of rats either presensitized with allogeneic spleen cells (Wilson and Nowell, 1971) or allogeneic skin grafts (Wilson, Silvers and Nowell, 1967).

An interesting feature of the accelerated MLR response presented here was its dramatic decrease by day 5, which is a characteristic feature of the secondary MLR response (Hayry and Andersson, 1975; MacDonald, Engers, Cerottini and Brunner, 1974; Zier and Bach, 1975), and of the MLR response of cells obtained from animals presensitized in vivo to the appropriate transplantation antigens present on the stimulator cells (Wilson, Silvers and Nowell, 1967; Wilson and Nowell, 1971). It has been suggested that this decrease in the secondary MLR response by day 5 may be attributed to the elimination of stimulating cells by specific cytotoxic cells (Fitch, Engers, Cerottini and Brunner, 1976), or to increased activity of suppressor cells (Rich, Chu and Rich, 1977).

The results presented in this thesis are not in agreement with those of Chapman and Dutton (1965) and Sheppard, Jr., Sell, Poler and Redelman (1977) who did not observe an enhanced MLR blastogenic response by the cells of post-allografted rabbits to stimulator cells of the graft donor. Their results may, however, be explained by the fact that the investigators evaluated the MLR blastogenic response only on or after day 5 of culture, by which time the accelerated response would have subsided and the response of the cells would be reduced to the level of "unsensitized" cells.

No cytotoxic cells were found among the WBC of the rabbits before or after allograft rejection although the MLR responses given by the WBC at these times were very different. The target cells were obtained from the allograft donor, stimulated to undergo blastogenesis by incubation with PHA for 3 days and then labelled with ^{51}Cr (Lightbody, Bernoco, Miggiano and Ceppelini, 1971). There are a number of explanations which can account for our failure to detect cytotoxic cells. It is possible that, in the rabbit, direct cell-mediated immunity involving non-cytotoxic sensitized cells may be the major mechanism used in allograft rejection. If rejection involves a humoral component such as antibody plus complement or antibody-dependent cellular cytotoxicity, it would not have been detected in the assays for cytotoxicity which lacked serum from the allografted rabbit and complement. It is possible that cytotoxic cells do mediate the rejection of the allograft but are sequestered in the graft and stored in a

lymphoid organ other than the blood (Andersson and Hayry, 1975). The WBC in this case may contain very few cytotoxic cells and no reactivity would be detected. It is also possible that the conditions of our assay used to detect cytotoxic cells in the rabbit are inadequate.

In summary, it has been shown that the MLR blastogenic response of WBC of allografted rabbits stimulated with cells of the allograft donor is markedly accelerated with the MLR response of these same cells stimulated with cells of a third party rabbit. This accelerated response is specific with respect to stimulator cells obtained from the allograft donor. These results suggest that in the rabbit, as in mouse and man, the MLR plays an important role in the allograft rejection reaction.

It is clear from these results that many of the objectives of this research project have been met - the characterization of the MLR responses by cells from the different lymphoid organs, the determination of which organs contain stimulating cells, the observation of the recognition of self by the cells of peripheral lymphoid organs, the production of an accelerated MLR response by sensitizing lymphoid cells in vivo using an allogeneic skin graft. These results have increased the knowledge of the rabbit MLR to a great extent so that in some areas such as the investigation of the reactivity of the cells of the different lymphoid organs, the observations made are far in excess of those made in man and mouse.

The observed relationship between allograft rejection and increased reactivity in the rabbit MLR although not demonstrably linked to cytotoxic cells, nevertheless does elevate rabbit lymphoid cell interactions closer to the level of sophistication demonstrated in mouse and man. One major aspect that has yet to be investigated in the rabbit is the subclassification of lymphocytes into groups that can be distinguished by function and surface membrane structures such as the T, B, null cell classifications found in mice and humans. This only awaits the correct antiserum. Another area in which rabbit transplantation immunology is deficient is the mapping of genes responsible for the various immune functions. The work presented in this thesis may facilitate the realization of these future projects.

CHAPTER VI

CONTRIBUTION TO KNOWLEDGE

1. The candidate has determined the culture conditions required for the generation of consistently high blastogenic responses utilizing rabbit WBC as a responder cell in the two-way and one-way MLR.

2. The candidate has determined that the optimal time of culture required for the optimal blastogenic response in the rabbit MLR is five days. This applies to the cells of all the organs except bone marrow and thymus cells which did not respond in the MLR.

3. The candidate has determined that the optimal concentration of responder cells used to generate the MLR response is characteristic of each of the different lymphoid organs of the rabbit.

4. The candidate has determined that the magnitude of the S.I. in the MLR response, irrespective of the cell concentration used, is a characteristic of the cells of each of the different lymphoid organs of the rabbit.

5. The candidate has determined that sacculus rotundus cells respond in the rabbit MLR, a fact not before mentioned in the literature.

6. The candidate has determined that the cells of the appendix, Peyer's patches, sacculus rotundus and bone marrow will stimulate allogeneic responder cells in the rabbit MLR.

7. The candidate has determined that the cells of the appendix, Peyer's patches and sacculus rotundus when cultured alone at high cell concentrations, consistently undergo a high degree of spontaneous blastogenesis.

8. The candidate has determined that the ability of cells to stimulate in the rabbit MLR is not correlated with the percentage of stimulator cells bearing cell surface immunoglobulin. This is contrary to the findings in other species such as the mouse and the human where cells bearing the most surface immunoglobulin (B cells) are considered the primary source of MLR stimulating cells.

9. The candidate has determined that the cells of the appendix, Peyer's patches and sacculus rotundus are able to stimulate autologous WBC and spleen cells to undergo a blastogenic response. This indicates that in the rabbit recognition of self occurs with cells of the peripheral lymphoid organs.

10. The candidate has determined that the WBC of the rabbit have a memory for the recognition of transplantation antigens. This memory can be detected in an MLR between cells of a skin graft donor and responder cells from the recipient of that skin graft.

CHAPTER VII

BIBLIOGRAPHY

Abdou, N.I., Rose, B., and Richter, M., (1969). J. Exptl. Med. 130, 867.

Abdou, N.I., Sagawa, A., Pascual, E., Hebert, J., and Sadeghee, S., (1976). Clin. Immunol. Immunopathol. 6, 192.

Adler, W.H., Takiguchi, T., Marsh, B., and Smith, R.T., (1970). J. Immunol. 195, 984.

Allen, S.L., (1955). Genetics 40, 627.

Alling, D.W., and Kahan, B.D., (1969). Transplantation 8, 1.

Alm, G.V., and Peterson, R.D.A., (1970). Fed. Proc. 29, 430.

Alter, B.J., and Bach, F.H., (1970). Cell. Immunol. 1, 207.

Alter, B.J., Grillot-Courvalin, C., Zier, K.S., and Bach, M.L., (1976). In Leukocyte Membrane Determinants Regulating Immune Reactivity, V.P. Eijsvoogel, D. Roos, and W.P. Zeijlemaker, Eds. Academic Press, New York. p. 519.

Amos, D.B. and Bach, F.H., (1968). J. Exptl. Med. 128, 623.

Amos, D.B., Gorer, P.A. and Mikalska, Z.B., (1955). Proc. roy. Soc. B. 144, 369.

Anderson, R.E., Olson, G.B., Autry, J.R., Howarth, J.L., Troup, G.M. and Bartels, P.M., (1977). J. Immunol. 118, 1191.

Anderson, R.E., and Warner, N.L., (1975). J. Immunol. 115, 161.

Andersson, L.C., and Hayry, P., (1973). Eur. J. Immunol. 3, 595.

Andersson, L.C. and Hayry, P., (1974). Scand. J. Immunol. 3, 461.

Andersson, L.C., Wordling, S., and Hayry, P., (1973). J. Exptl. Med. 138, 324.

Appelman, A.W.M., and Balner, H., (1972). Transplant. Proc. 4, 17.

Asantila, T., and Toivanen, P., (1976). J. Immunol. 117, 555.

Asantila, T., Vahala, J., and Toivanen, P., (1974). Immunogenetics 3, 272.

Bach, F.H., (1976). Clinical Immunol. 3, 273.

Bach, M.L., Alter, B., Zosche, D., and Bach F.H., (1971). Transplant. Proc. 3, 844.

Bach, F.H., and Amos, D.B., (1967). Science 156, 1506.

Bach, F.H., and Amos, D.B., (1967). Science 156, 1506.

Bach, F.H., Bach, M.L., Kuperman, O.J., Sollinger, H.W., and Sondel, P.M., (1976). Cold Springs Harb. Symp. Quant. Biol. 41, 429.

Bach, F.H., Bach, M.L., and Sondel, P.M., (1976). Nature 259, 273.

Bach, F.H., Bach, M.L., Sondel, P.M., and Sundharadas, G., (1972). Transplant. Revs. 12, 30.

Bach, M.L., Bach, F.H., Widmer, M., Oranen, H., and Wolberg, W.H., (1971), Transplantation 12, 283.

Bach, F.H., and Hirschom, K., (1964). Science 143, 813.

Bach, F.H., Kuperman, O.J., Sollinger, H.W., Zarling, J.M., Sonder, P.M., Alter, B.J. and Bach, M.L., (1977). Transplant. Proc. 9, 859.

Bach, F.H., and van Rood, J.J., (1976a). New Engl. J. Med. 295, 806.

Bach, F.H., and van Rood, J.J., (1976b). New Engl. J. Med. 295, 872.

Bach, F.H., and van Rood, J.J., (1976c). New Engl. J. Med. 295, 927.

Bach, F.H., and Voynow, N.K., (1966). Science 153, 545.

Bach, F.H., Zeir, K.S., and Sondel, P.M., (1973). Transplant. Proc. 5, 1717.

Bain, B., and Lowenstein, L., (1964). Science 145, 1315.

Bain, B., and Pshyk, K., (1972). Transplant. Proc. 6, 163.

Bain, B., Vas, M.R., and Lowenstein, L., (1964). Blood 23, 108.

Benacerraf, B., and Dorf, M.E., (1974). In Progress in Immunology II, Vol. 2, L. Brent and J. Holborow, Eds. North Holland Publishing Company, Amsterdam. p. 181.

Berman, M., Puryear K. and Argyris, B.F., (1976). Cellular Immunol. 23, 126.

Bernstein, I.D., Wright, P.W., and Cohen, E., (1976). J. Immunol. 116, 1367.

Bernoco, D., Cullen, S., Schudeller, G., Trinchieri, G., and Ceppellini, R., (1973). In Histocompatibility Testing 1972, J. Dausset, and J. Colombani, Eds. Munksgaard, Copenhagen. p. 527.

Betel, I.K., van den Berg, J., Martijnse, J., and van den Berg, R., (1974). In Lymphocyte Recognition and Effector Mechanisms, K. Lindahl-Kiessling and D. Osoba, Eds. Academic Press, New York. p. 639.

Beverly, P.C.L., Feldman, M., Dunkley, M., and McKenzie, I., (1977). Transplant. Proc. 9,703.

Bianco, C., Patrick, R., and Nussenzweig, V., (1970). J. Exptl. Med. 132, 702.

Blomgren, H., (1977). Scan. J. Immunol. 6, 857.

Blomgren, H., and Svedmyr, E., (1971). Cellular Immunol. 2, 285.

Boehmer, H.v. (1974). In Lymphocyte Recognition and Effector Mechanisms, K. Lindahl-Kiessling and D. Osoba, Eds. Academic Press, New York. p. 289.

Boehmer, H.v., and Byrd, W.J., (1972). Nature New Biology 235, 50.

Bona, C., Cinader, B., Dubiski, S., (1977). Ann. Immunol. (Inst. Past.) 128, 572.

Bonnard, G.D., Chappuis, M., Glauser, A., Mempel, W., Baumann, P., Grosse-Wilde, H., and Albert, E.D., (1973). Transplant. Proc. 5, 1679.

Boyer, P.J.J., and Fahey, J.L., (1976). J. Immunol. 116, 202.

Bradley, B.A., (1974). In Lynphocyte Recognition and Effector Mechanisms, K. Lindahl-Kiessling and D. Osoba, Eds. Academic Press, New York. p. 85.

Burnet, F.M., (1968). Nature 218, 426.

Calkins, C.E., Ozer, Jr., H., and Waksman, B.H., (1974). Fed. Proc. 33, 593.

Callewaert, D.M., Kaplan, J., Peterson, Jr., W.D., and Lightbody, J.J., (1975). Cell. Immunol. 19, 276.

Cantor, H., and Asofsky, R., (1970). J. Exptl. Med. 235, 131.

Cantor, H. and Boyse, E.A., (1975a). J. Exptl. Med. 141, 1376.

Cantor, H., and Boyse, E.A., (1975b). J. Exptl. Med. 141, 1390.

Cantor, H., and Boyse, E.A., (1976). Cold Springs Harb. Symp. Quant. Biol. 41, 23.

Cantor, H., and Jandinski, J., (1974). J. Exptl. Med. 140, 1712.

Carr, M.C., Stites, D.P., and Fudenberg, H.H., (1973). Nature New Biology 241, 279.

Cavaillon, J.M., Bona, C., Cazenave, P.A., and Cinader, B., (1977). J. Immunol. Methods 14, 355.

Ceppellini, R., Bonnard, G.D., Coppo, F., Miggiano, V.C., Pospisil, M., Curtoni, E.S., and Pellegrino, M., (1971). Transplant. Proc. 3, 58.

Ceppellini, R., Franceschini, P., Maggiano, V.C., and Trident, G., (1965). In Histocompatibility Testing 1965, H. Balner, F.J. Cleton, and J.G. Eernisse, Eds. Munksgaard, Copenhagen. p. 225.

Cerottini, J.C., and Brunner, K.T., (1974). Advan. Immunol. 18, 67.

Cerottini, J.C., Engers, H.D., MacDonald, H.R., and Brunner, K.T., (1974). J. Exptl. Med. 140, 703.

Cerottini, J.C., Nordin, A.A., and Brunner, K.T., (1970). Nature 227, 71.

Chapman, N.D., and Dutton, R.W., (1965). J. Exptl. Med. 121, 85.

Chai, C.K., and Lerner, C.P., (1975). Immunogenetics 2, 363.

Chen, C., and Hirsch, J.G., (1972a). Science 176, 60

Chen, C., and Hirsch, J.G., (1972b). J. Exptl. Med. 136, 604.

Chess, L., MacDermott, R.P., Sondel, P.M., and Schlossman, S.F. (1974). In Progress in Immunology II, Vol. 2, L. Brent and J. Holborow, Eds. North Holland Publishing Company, Amsterdam. p. 125.

Chou, C.T., and Cinader, B., Dubiski, S., (1977). Cell. Immunol. 28, 334.

Click, R.E., Benck, L. and Alter, B.J., (1972). Cell. Immunol. 3, 156.

Cochrum, K.C., Perkins, H.A., Payne, R.O., Kountz, S.L. and Belzer, F.O., (1973). Transplant. Proc. 5, 391.

Colas de la Noue, H., Koperstych, S., and Richter, M., (1972). Immunology, 23, 655.

Colas de la Noue, H., and Richter, M., (1974a). Immunology 27, 413.

Colas de la Noue, H., and Richter, M., (1974b). Immunology 27, 421.

Cohen, C., and Tissot, R.G., (1974). Transplantation 18, 150.

Cooper, A.J., (1969). In Proceedings of the Third Leukocyte Culture Conference, W.O. Rieke, Ed. Appleton Century Crofts, New York. p. 137.

Corley, R.B., Dawson, J.R., and Amos, D.B., (1976). Eur. J. Immunol. 6, 235.

Croy, B.A., and Osoba, D., (1974). J. Immunol. 113, 1626.

Cunningham, A.J., (1974). Curr. Topics Mol. Immunol. 3, 1.

Cunningham, A.J., (1975). Cell. Immunol. 19, 368.

Currie, G.A., (1967). Nature 215, 164.

Daguillard, F., and Richter, M., (1969). J. Exptl. Med. 130, 1187.

Demant, P., (1973). Transplant. Rev. 15, 162.

Dupont, B., Hansen, J.A., Yunis, E.J., (1976). Advan. Immunol. 23, 107.

Dutton, R.W., (1965). J. Exptl. Med. 122, 759.

Dyminski, J.W., and Argyris, B.F., (1971). Transplantation 11, 514.

Dyminski, J.W., and Smith, R.T., (1975). J. Exptl. Med. 141, 360.

Dyminski, J.W., and Smith, R.T., (1977). J. Exptl. Med. 146, 412.

Eijsvoogel, V.P., du Bois, M.J.G.J., Meinesz, A., Bierhorst-Eijlander, A., Zeijlemaker, W.P., Schellekens, P.Th.A., (1973). Transplant. Proc. 5, 1675.

Eijsvoogel, V.P., Schellekens, P.Th. A., du Bois, M.J.G.J., and Zeijlemaker, W.P., (1976). Transplant. Rev. 29, 125.

El-Arini, M.O., and Osoba, D., (1973). J. Immunol. 110, 1476.

Elkins, W., Karathas, P., and Bach, F.H., (1973). Transplant. Proc. 5, 1759.

Etheredge, E.E., Shons, A.R., Hohenthaner, K.L., Najarian, J.S., (1973). Transplantation 15, 331.

Evans, R.L., Breard, J.M., Lazarus, H., Schlossman, S.F., and Chess, L., (1977). J. Exptl. Med. 145, 221.

Fanger, M.W., Hart, D.A., Wells, J.V., and Nisonoff, A., (1970). J. Immunol. 105, 1043.

Fathman, C.G., Collavo, D., Davies, S., and Nabholz, M., (1977). J. Immunol. 118, 1232.

Feldmann, M., (1973). Transplant. Proc. 5, 1803.

Fernald, G.W., and Metzgar, R.S., (1971). J. Immunol. 107, 456.

Festenstein, H., (1966). Ann. N.Y. Acad. Sci. 129, 567.

Festenstein, H., (1973). Transplant. Rev. 15, 62.

Festenstein, H., Abbasi, K., Sachs, J.H., and Oliver, R.T.D., (1971). Transplant. Proc. 4, 219.

Festenstein, H., Davies, A.J.S., Leuchars, E., Wallis, V.J., and Doehoff, M.J., (1969). In Lymphatic Tissue and Germinal Centers in the Immune Response, L. Fiore-Donate and M.G. Hanna, Jr., Eds. Plenum Publishing Company, New York. p. 121.

Festenstein, H., and Demant, P., (1974). In Progress in Immunology II, Vol. 2, L. Brent and J. Holborow, Eds. North Holland Publishing Company, Amsterdam. p. 45.

Fitch, F.W., Engers, H.D., Cerottini, J.C., and Brunner, K.T., (1976). J. Immunol. 116, 716.

Folch, H. and Waksman, B.H., (1974). J. Immunol. 113, 140.

Ford, W.L., and Atkins, R.C., (1972). In Microenvironmental Aspects of Immunity, B.D. Jankovic and K. Isokavic, Eds. Plenum Publishing Company, New York. p. 255.

Fradelizi, D., and Dausset, J., (1975). Eur. J. Immunol. 5, 295.

Fujiwara, S., Armstrong, R.M., and Cinader, B., (1974). Immunological Communications 3, 275.

Gershon, R.K., Eardley, D.D., Naidorf, K.F., and Ptak, W., (1976). Cold Springs Harb. Symp. Quant. Biol. 41, 85

Gershon, R.K., Eardley, D.D., and Ptak, W., (1976). Nature 216, 262.

Goldstein, P., Blomgren, H., Svedmyr, E.A.J., and Wigzell, H., (1973). Transplant. Proc. 5, 1441.

Goldstein, P., Wigzell, H., Blomgren, H., and Svedmyr, E.A.J., (1972). J. Exptl. Med. 135, 890.

Gorezynski, R.M., and Rittenberg, M.B., (1975). Cell. Immunol. 16, 171.

Gowans, J.L., (1977). Transplant. Proc. 9, 685.

Granberg, C., Manninen, K., and Toivanen, P., (1976). Clinical Immunol. Immunopathol. 6, 256.

Graziano, K.D., and Mardiney, Jr., M.R., (1976). Transplantation 21, 317.

Greaves, M.F., and Janossy, G., (1972). Transplant. Revs. 11.

Green, S.S., and Sell, K.W., (1970). Science 170, 989.

Hadden, J.W., Hadden, E.M., Meetz, G., Good, R.A., Haddox, M.K., and Goldberg, N.D., (1973). Fed. Proc. 32, 1022.

Hallgren, H.M., and Yunis, E.J., (1977). J. Immunol. 118, 2004.

Han, T., (1972). Immunology 23, 355.

Han, T., (1976). Clin. Exp. Immunol. 25, 338.

Han, T., and Dadey, B., (1976). Immunology 31, 643.

Han, T., Minowada, J., Subramanian, S., and Sinks, L.F., (1976). Immunology 30, 361.

Hardy, D.A., and Ling, N.R., (1969). Nature 221, 545.

Hardy, D.A., Wallin, J.M., and Ling, N.R., (1970). In Proceedings of the Fifth Leukocyte Culture Conference, J.E. Harris, Ed. Academic Press, New York. p. 278.

Harris, J.W., MacDonald, H.R., Engers, H.D., Fitch, F.W., and Cerottini, J.C., (1976). J. Immunol. 116, 1071.

Harrison, H.N., Wei, F., and Ahie, M., (1971). Transplantation 11, 341.

Harrison, M.R., (1973). J. Immunol. 111, 1270.

Hayry, P., and Andersson, L.C., (1973). Transplant. Proc. 5, 1697.

Hayry, P., and Andersson, L.C., (1975). J. Exptl. Med. 141, 508.

Hayry, P., and Andersson, L.C., (1976). Scan. J. Immunol. 5, 391.

Hayry, P., Andersson, L.C., and Nordling, S., (1973). Transplant. Proc. 5, 87.

Hayry, P., Andersson, L.C., and Roberts, P.J., (1976). In Leukocyte Membrane Determinants Regulating Immune Reactivity, V.P. Eijsvoogel, D. Roos, and W.P. Zeijlemaker, Eds. Academic Press, New York. p. 525.

Hayry, P., and Defendi, V., (1970). Science 168, 133.

Hayry, P., and Roberts, P.J., (1977). Transplant. Proc. 9, 691

Heber-Katz, E., and Click, R.E., (1972). Cell. Immunol. 5, 410.

Hersh, E.M., and Harris, J.E., (1968). J. Immunol. 100, 1184.

Hirano, T., and Nordin, A., (1976a). J. Immunol. 116, 1115.

Hirano, T., and Nordin, A., (1976b). J. Immunol. 117, 2226.

Hirschberg, H., and Thorsby, E., (1977). Scand. J. Immunol. 6, 809.

Hirschorn, K., Bach, F., Kolodny, R.L., Firschein, L.L., and Hachem, N., (1963). Science 142, 1185.

Hirschorn, K., Firschein, L.L., and Bach, F.H., (1965). Nat. Acad. Sci. Nat. Res. Council Publ. 1229, 131

Hodes, R.J., and Hatcock, K.S., (1976a). J. Immunol. 116, 167.

Hodes, R.J., and Hathcock, K.S., (1976b). In Leukocyte Membrane Determinants Regulating Immune Reactivity, V.P. Eijsvoogel, D. Roos, and W.P. Zeijlemaker, Eds. Academic Press, New York. p.

Howard, J.C., Hont, S.V., and Gowans, J.L., (1972). J. Exptl. Med. 135, 200.

Howe, M.L., and Cohen, L., (1975). J. Immunol. 115, 1233.

Howe, M.L., Goldstein, A.L., and Battisto, J.R., (1970). Proc. Natl. Acad. Sci. (U.S.) 67, 613.

Jeme, N.K., (1971). Eur. J. Immunol. 1, 1.

Johnston, J.M., and Wilson, D.B., (1970). Cell. Immunol. 1, 430.

Kamin, R.M., Henry, C., and Fudenberg, H.H., (1974). J. Immunol. 113, 1151.

Kasakura, S., (1977). Immunology 33, 23.

Kasakura, S., and Lowenstein, L., (1965a). In Histocompatibility Testing 1965, H. Balner, F.J. Cleton, J.G. Eernisse, Eds. Munksgaard, Copenhagen. p. 211.

Kasakura, S., and Lowenstein, L., (1965b). Nature 208, 794.

Kasakura, S., and Lowenstein, L., (1968). J. Immunol. 101, 12.

Kennedy, J.C., and Elepaha-Mensah, J.A., (1973). J. Immunol. 111, 1939.

Kisiclow, P., Hirst, J.A., Shiku, H., Beverley, P.C.L., Hoffman, M.K., Boyse, E.A., and Oettgen, H.F., (1975). Nature 253, 219.

Kisken, W.A., and Swenson, W.A., (1969). Nature 225, 76

Klein, J., Klein, D., and Shreffler, D.C., (1970). Transplantation 10, 309.

Knight, S.C., Hardy, D.A., and Ling, N.R., (1970). Immunology 19, 343.

Knight, S.C., Newey, B., and Ling, N.R., (1973). Cytobios 7, 35.

Knight, S.C., and Thorbecke, G.J., (1971). Cell. Immunol. 2, 91.

Knight, S., Walker, D., and Ling, N.R., (1971). Cytobios 3, 65.

Lafferty, K.J., Cooley, M.A., Woolnough, J., and Walker, K.Z., (1975). Science 188, 259.

Lafferty, K., Ryan, M., and Misko, I., (1974). J. Immunol. Methods 4, 263.

Lawton, A.R., Self, K.S., Royal, S.A., and Cooper, M.D., (1972). Clin. Immunol. Immunopathol. 1, 84.

Lightbody, J., Bernoco, D., Miggiano, V.C., and Ceppellini, R., (1971). G. Batt. Virol. 64, 243

Lightbody, J.J., Brown, M. and Kong, Y.C.M., (1976). Fed. Proc. 35, 354.

Lightbody, J.J., and Kong, Y.C.M., (1976). J. Immunol. 117, 1336.

Lilliehook, B., Jacobsson, H., and Blomgren, H., (1976). Scan. J. Immunol. 5, 417.

Lindahl, K.F., and Bach, F.H., (1976). J. Exptl. Med. 144, 305.

Lindh, J., (1973). Scan. J. Immunol. 2, 417.

Ling, N.R., Hardy, D.A., and Steele, C.M., (1974). In Lymphocyte Recognition and Effector Mechanisms, K. Lindahl-Kiessling and D. Osoba, Eds. Academic Press, New York. p. 235.

Ling, N.R., and Kay, J.E., (1975). Lymphocyte Stimulation. North-Holland Publishing Company, Amsterdam. p. 123.

Linthicum, D.S., and Sell, S., (1977). Immunology 32, 701

Lipsky, P.E., Ellner, J.J., and Rosenthal, A.S., (1976). In Leukocyte Membrane Determinants Regulating Immune Reactivity, V.P. Eijsvoogel, D. Roos, and W.P. Zeijlemaker, Eds. Academic Press, New York. p. 317.

Livnat, S., Klein, J. and Bach, F.H., (1973). Nature New Biology 243, 42.

Llacer, V., and Uyeki, E.M., (1969). Intern. Arch. Allergy 35, 88.

Lohrmann, H.P., Novikovs, L., Graw, Jr., R.G., (1974). Nature 250, 144.

Lowe, J., (1971). Cytobios 3, 137.

Luzzati, A.L., and Lafleur, L., (1976). Eur. J. Immunol. 6, 125.

MacDermott, R.P., Chess, L., and Schlossman, S.F., (1975). Clin. Immunol. Immunopathol. 4, 415.

MacDonald, H.R., Engers, H.D., Cerottini, J.C., and Brunner, K.T., (1974). J. Exptl. Med. 140, 718.

Mage, M.G., and McHugh, L.L., (1973). J. Immunol. 111, 652.

Main, R.K., Cole, L.J., Jones, M.J., and Haire, H.M., (1967). J. Immunol. 98, 417.

Mardiney, M.R., Bock, G.N., and Chess, L., (1972). Transplantation 14, 274.

Mawas, C., Sasportes, M., Christen, Y., Bernard, A., Dausset, J., Alter, B.J., and Bach, M.L., (1973). Transplant. Proc. 5, 1683.

Mempel, W., Grosse-Wilde, H., Baumann, P., Netzel, B., Steinbauer-Rosenthal, I., Scholz, S., Bertrams, J., and Albert, E.D., (1973). Transplant. Proc. 5, 1529.

Metcalf, D., and Moore, M.A.S., (1971). Haemopoetic Cells. North-Holland Publishing Company, Amsterdam.

Miggiano, V.C., Bemoco, D., Lightbody, J., Trinchieri, G., and Ceppellini, R., (1972). Transplant. Proc. 9, 231.

Miller, R.G., and Phillips, R.A., (1976). J. Immunol. 117, 1913.

Milthorp, P., and Forsdyke, D.R., (1973). Biochem. J. 132, 803.

Milton, J.D., Mowbray, J.F., Ruskiewicz, M., and Carpenter, C.B., Transplantation 15, 579.

Mosier, D., and Canter, H., (1971). Eur. J. Immunol. 1, 459.

Nadler, L.M., and Hodes, R.J., (1977). J. Immunol. 118, 1886.

Nisbet, N.W., Simonsen, M., and Zaleski, I.M., (1969). J. Exptl. Med. 129, 459.

Nordling, S., Andersson, L.C., and Hayry, P., (1972). Eur. J. Immunol. 2, 405.

Olding, L.B., and Oldstone, M.B.A., (1974). Nature 249, 161.

Oppenheim, J.J., Whang, J., and Frei, E., (1965). J. Exptl. Med. 121, 651.

Owen, J.T.T., (1972). In Ontogeny of Aquired Immunity, a Ciba Foundation Symposium. Associated Science Publishers, Amsterdam. p. 35.

Owen, J.T.T., Cooper, M.D., and Raff, M.C., (1974). Nature 249, 361.

Owen, F.L., and Fanger, M.W., (1974). J. Immunol. 113, 1128.

Ozer, Jr., H., and Waksman, B.H., (1974). J. Immunol. 113, 1780.

Peavy, D.L., and Pierce, C.W., (1974). J. Exptl. Med. 140, 356.

Peck, A.B., Alter, B.J., and Lindahl, K.F., (1976). Transplant. Rev. 29, 189.

Piguet, P.F., and Vassalli, P., (1972). J. Exptl. Med. 136, 962.

Pizzaro, O., Hoecker, G., Rubinstein, P., and Ramos, A., (1961). Proc. Nat. Acad. Sci. (U.S.) 47, 1900.

Plate, J.D., (1973). Transplant. Proc. 5, 1351.

Plate, J.M., Ward, F.E., and Amos, D.B., (1970). In Histocompatibility Testing 1970, P.I. Terasaki, Ed. Baltimore, Williams and Wilkins. p. 531.

Potter, M.R., and Moore, M., (1977). Immunology 32, 359.

Raff, M.C., (1970). Immunology 19, 637.

Raff, M.C., (1971). Transplant. Rev. 6, 52.

Raff, M.C., and Wortiss, H.H., (1970). Immunology 18, 931.

Ragab, A.H., and Cowan, D.H., (1973). Cell. Immunol. 7, 336.

Redelman, D., Scott, C.B., Sheppard, H., and Sell, S., (1976). J. Exptl. Med. 143, 919.

Rich, R.R., Chu, L., and Rich, S.S., (1977). J. Immunol. 118, 1252.

Rich, R.R., and Rich, S.S., (1975). J. Exptl. Med. 142, 1391.

Rich, S.S., and Rich, R.R., (1976). In Leukocyte Membrane Determinants Regulating Immune Reactivity, V.P. Eijsvoogel, D. Roos, and W.P. Zeijlemaker, Eds. Academic Press, New York. p. 285.

Richter, M., Colas de la Noue, H.C., and Hamdy, H., (1975). Immunology 29, 103.

Rieke, W.O., (1966). Science 152, 535.

Roberts, P.J., and Hayry, P., (1977). Cell. Immunol. 30, 236.

Rode, H.N., and Gordon, J., (1969). J. Immunol. 102, 786.

Rode, H.N., and Gordon, J., (1974). Cell. Immunol. 13, 87.

Rosentreich, D.L., (1976). In Leukocyte Membrane Determinants Regulating Immune Reactivity, V.P. Eijsvoogel, D. Roos, and W.P. Zeijlemaker, Eds. Academic Press, New York. p. 363.

Rudolph, R.H., Hered, B., Epstein, R.B., and Thomas, E.D., (1969). Transplantation 8, 141.

Rychlikova, M., and Ivanyi, P., (1969). Folia Biol. (Praha) 15, 126.

Schellekens, P.T.A., and Eijsvoogel, V.P., (1970). Clin. Exp. Immunol. 7, 229.

Schendel, D.J., Alter, B.J., and Bach, F.H., (1973). Transplant. Proc. 5, 1651.

Schrader, J.W., Henning, R., Milner, R.J., and Edelman, G.M., (1976). Cold Springs Harb. Symp. Quant. Biol. 41, 547.

Schrek, R., and Donnelly, W.J., (1961). Blood 18, 561.

Schwartz, M.R., (1966). Immunology 10, 281.

Schwartz, M.R., (1967). Proc. Soc. Exptl. Biol. Med. 127, 879.

Sell, S., and Sheppard, Jr., H.W., (1973). Science 182, 586.

Segall, M., Bach, F.H., Bach, M.L., Hussey, J.L., and Uehling, D.T., (1975). Transplant. Proc. 7 (Suppl. 1) 41.

Shearer, G.M., Lozner, E.C., Rehn, T.G., and Schmitt-Verhulst, A.M., (1975). J. Exptl. Med. 141, 930.

Sheehy, M.J., Sondel, P.M., Bach, M.L., Wank, R., and Bach, F.H., (1975). Science 188, 1308.

Shek, P.N., Chou, C.T., Dubiski, S., and Cinader, B., (1975). Cell. Immunol. 18, 331.

Sheppard, Jr., H.W., Sell, S., Poler, S.M., and Redelman, D.D., (1977). J. Immunol. Methods 16, 185.

Shreffler, D.C., Amos, D.B., and Mark, R., (1966). Transplantation 4, 300.

Shreffler, D.C., and David, C.S., (1972). Tissue Antigens 2, 232.

Silvers, W.K., Wilson, D.B., and Palm, J.E., (1967). Science 155, 703.

Singal, D.P., (1977). Transplant. Proc. 9, 709.

Singal, D.P., Mickey, M.R., and Terasaki, P.I., (1969). Transplantation 7, 246.

Smith, J.B., (1978). Cell. Immunol. 36, 203.

Solheim, B.G., Ek, J., Thune, P.O., Baklien, K., Bratlie, A., Rankin, B., Thoresen, A.B., and Thorsby, E., (1976). Tissue Antigens 7, 57.

Solliday, S., and Bach, F.H., (1974). Science 170, 1406.

Sollinger, H.W., and Bach, F.H., (1976). Nature 259, 487.

Solomon, J.B., (1971). Foetal and Neonatal Immunology. North-Holland Publishing Company, Amsterdam.

Sondel, P.M., Chess, L., MacDemott, R.P., and Schlossman, S.F., (1975). J. Immunol. 114, 982.

Sondel, P.M., Jacobson, M.W., and Bach, F.H., (1977). Eur. J. Immunol. 7, 38.

Sondel, P.M., O'Brien, C., Porter, L., Schlossman, S.F., and Chessin, L., (1976). J. Immunol. 117, 2197.

Sorensen, S.F., (1971). Acta Pathol. Microbiol. Scand. Sect. B. 79, 483.

Sorensen, S.F., (1972). Acta Pathol. Microbiol. Scand. Sect. B. Suppl. 230.

Soulillou, J.P., Carpenter, C.B., Lundin, A.P., and Strom, T.B., (1975). J. Immunol. 115, 1566.

Sprent, J., and Miller, J.F.A.P., (1971). Nature New Biology 234, 195.

Sprent, J., and Miller, J.F.A.P., (1972). Eur. J. Immunol. 2, 384.

Stimpfling, J.H., and Reichart, A.E., (1970). Transplant. Proc. 2, 39.

Stimpfling, J.H., Reichart, A.E., and Hudson, P., (1971). Immunogenetics of the H-2 system. Kargel, Basel.

Stimpling, J.H., and Richardson, A., (1965). Genetics 51, 831.

Stites, D.P., Caldwell, J., Carr, M.C., and Fudenberg, H.H., (1975). Clin. Immunol. Immunopathol. 4, 519.

Stobo, J.D., and Paul, W.E., (1973). J. Immunol. 110, 362.

Strom, T.B., Tilney, N.L., Paradysz, J.M., Bancewics, J., and Carpenter, C.B., (1977). J. Immunol. 118, 2020.

Takasugi, M., Kiuchi, M., and Opelz, G., (1977). Transplant. Proc. 9, 789.

Terasaki, P.I., Purk, M.S., Opelz, G., Saito, S., and Mickey, M.R., (1977). Transplant. Proc. 9, 469.

Thorsby, E., du Bois, R., Bondevik, H., Dupont, B., Eijsvoogel, V., Hansen, J.A., Jersild, C., Jorgensen, F., Kissmeyer-Nielsen, F., Lamm, L.U., Schellekens, P.T.A., Svejgaard, A., and Thomsen, M., (1974). Tissue Antigens 4, 507.

Thorsby, E., and Piazza, A., (1975). In Histocompatibility Testing 1975, F. Kissmeyer-Neilsen, Ed. Munksgaard, Copenhagen, p. 414.

Tigelaar, R.E., and Asofsky, R., (1973). J. Exptl. Med. 137, 239.

Tilney, N.L., Strom, T.B., Booth, D.B., Finnegan, A., Lundin, P., and Carpenter, C.B., (1977). Transplant. Proc. 9, 713.

Ting, C.C., and Bonnard, G.D., (1976). J. Immunol. 116, 1419.

Tissot, R.G., and Cohen, C., (1974). Transplantation 18, 142.

Tyan, M.L., and Ness, D.B., (1972). Transplantation 13, 198

Vande Stouwe, R.A., Kunkel, H.G., Halper, J.P., and Weksler, M.E., (1977). J. Exptl. Med. 146, 1809.

van Oers, M.H.J., and Zeijlemaker, W.P., (1977). Cell. Immunol. 31, 205.

van Rood, J.J., Eernisse, J.G., and van Leeuwen, A., (1958). Nature 181, 1735.

van Rood, J.J., van Leeuwen, A., and Eernisse, J.G., (1959). Vox Sang 4, 427.

Virolainen, M., Hayri, P., and Defendi, V., (1969). Transplantation 8, 179

Vitale, B., Jaksic, B., Matosic, M., Silorcic, V., and Tomazic, V., (1976). Transplantation 21, 502.

Wagner, H., (1972). J. Immunol. 109, 630.

Wagner, H., Gotze, D., and Rollinghoff, M., (1976). In Leukocyte Membrane Determinants Regulating Immune Reactivity, V.P. Eijsvoogel, D. Roos, and W.P. Zeijlemaker, Eds. Academic Press, New York. p. 599.

Wagner, H., Harris, A.W., and Feldman, M., (1972). Cell. Immunol. 4, 39.

Wagner, H., Rollinghoff, M., and Nossal, G.J.V., (1973). Transplant. Rev. 17, 3.

Wagner, H., Rollinghoff, M., and Shortman, K., (1974). In Progress in Immunology II, Vol. 3, L. Brent and J. Holborow, Eds. North Holland Publishing Company, Amsterdam. p. 111.

Waldman, T.A., Broder, S., Krakaver, R., Durm, M., Goldman, C., Meade, B., and Strober, W., (1977). In Regulatory Mechanisms in Lymphocyte Activation, D.O. Lucas, Ed. Academic Press, New York. p. 269.

Weber, W.T., (1970). Clin. Exptl. Immunol. 6, 919.

Weksler, M.E., and Kozak, R., (1977). J. Exptl. Med. 146, 1833.

Werkerle, H., (1977). Nature 267, 357.

Widmer, M.B., and Bach, F.H., (1972). J. Exptl. Med. 135, 1204.

Widmer, M.B., Peck, A.B., and Bach, F.H., (1973). Transplant. Proc. 5, 1501.

Wilson, B.S., Teodorescu, M., and Dray, S., (1976). J. Immunol. 116, 1306.

Wilson, D.B., (1967). J. Exptl. Med. 126, 625.

Wilson, D.B., Blyth, J.L., and Nowell, P.C., (1968). J. Exptl. Med. 128, 1157.

Wilson, D.B., and Elkins, W.L., (1969). In Proceedings of the Third Leukocyte Culture Conference, W.O. Rieke, Ed. Appleton Century Crofts, New York. p. 391.

Wilson, D.B., Howard, J.C., Nowell, P., (1972). Transplant. Revs. 12, 3.

Wilson, D.B., and Nowell, P.C., (1971). J. Exptl. Med. 133, 442.

Wilson, D.B., Silvers, W.K., and Nowell, P.C., (1967). J. Exptl. Med. 126, 655.

Wioland, M., Sabulovic, D., and Burg, C., (1972). Nature New Biology 237, 275.

Yunis, E.J., and Amos, D.B., (1971). Proc. Natl. Acad. Sci. (U.S.) 68, 3031.

Zarling, J.M., Raich, P.C., McKeough, M., and Bach, F.H., (1976). Nature 262, 691.

Zier, K.S., and Bach, F.H., (1975). Scan. J. Immunol. 4, 607.

Zier, K.S., and Bach, F.H., (1976). In Leukocyte Membrane Determinants Regulating Immune Reactivity, V.P. Eijsvoogel, D. Roos, and W.P. Zeijlemaker, Eds. Academic Press, New York. p. 517.

Zinkernagel, R.M., and Doherty, P.C., (1975). J. Exptl. Med. 141, 1427.

Zinkernagel, R.M., and Doherty, P.C., (1976). Cold Springs Harb. Symp. Quant. Biol. 41, 505.

Zoschke, D.C., and Bach, F.H., (1971). J. Immunol. Methods 1, 55.

CHAPTER VIII

VITA

Name:	Peter Milthorp
Place and Year of Birth:	Hitchin, Herts., England, 1947
Education:	McGill University, 1964-1968 B.Sc. 1968
	Queen's University, 1968-1970 M.Sc. 1970
	School of Graduate Studies, University of Ottawa, 1974-1978
Experience:	Research Technician, University of Ottawa, 1971-1974
Societies:	Canadian Society for Immunology
	Canadian Society for Cell Biology
	Canadian Society of Biochemistry

LIST OF PUBLICATIONS

1. Milthorp, P., and Forsdyke, D.R. (1970) Inhibition of Lymphocyte Activation at High Ratios of Concanavalin A to Serum Depends on Complement. Nature, Lond. 227, 1351.

2. Milthorp, P., and Forsdyke, D.R. (1970) Activation by Concanavalin A and Phytohemagglutinin of Rat Lymphocytes in Homologous or Heterologous Serum: Dependence of Inhibition at High Concanavalin A / Serum Ratios on Complement. In "Proceedings of the Fifth Leukocyte Culture Conference" (Jules Harris, ed.) Academic Press, Inc., New York 5, 143.

3. Milthorp, P. (1971) A Study of the Activation of Rat Lymphocytes by Concanavalin A. M.Sc. Thesis, Queen's University, Kingston.

4. Forsdyke, D.R., and Milthorp, P. (1972) Isotope-Dilution Analysis of Cell Stimulation or Inhibition by Biological Media. J. Immunol. Methods 1, 207.

5. Milthorp, P., and Forsdyke, D.R. (1973) Serum Factors Affecting the Incorporation of ^{3}H-Uridine by Lymphocytes Stimulated by Concanavalin-A: Studies of the Role of Complement. Biochem. J. 132, 803.

6. Milthorp, P., and Forsdyke, D.R. (1973) A Comparison of the Activation of Thymus and Lymph-Node Cells by Concanavalin A and Phytohemagglutinin: Effects of Complement. J. Immunol. Methods 2, 269.

7. Quastel, M.R., Milthorp, P., Kaplan, J.G., Vogelfanger, I.J. (1974) Further Studies on M-ATPase in Lymphocytes and Plaque-Forming Cells: Possible Species and Functional Differences Between Lymphocyte Subclasses. In "Proceedings of the Eighth Leukocyte Culture Conference" (K. Lindahl-Kiessling, D. Osoba, eds.) Academic Press, Inc., New York 8, 493.

8. Milthorp, P., Quastel, M.R., Kaplan, J.G., and Vogelfanger, I.J. (1974) Effect of the Inhibition of Cation Transport by Ouabain on Plaque Formation by Splenic Lymphoid Cells: Time Course and Dose Response. Cell. Immunol. 14, 128.

9. Vogelfanger, I.J., Milthorp, P., Behelak, Y., Barron, P.T., Steele, D., and Richter, M. (1975) Naturally Occurring Immunosuppressive Agents: 1. The Presence in Normal Pig Liver of a Factor Possessing Immunosuppressive Properties with Respect to Pig Lymphoid Cells in Vitro. Transplantation 20, 130.

10. Vogelfanger, I.J., Milthorp, P., Behelak, Y., Barron, P.T., Steele, D., and Richter, M. (1976) Naturally Occurring Immunosuppressive Agents: II. The Ability of Cell-Free Liver Extracts to Modify the Rejection of Allogeneic Skin Grafts in the Rabbit. Immunol. Commun. 5, 125.

11. Milthorp, P., and Richter, M. (1978) The Cells Involved in Cell Mediated and Transplantation Immunity in the Rabbit. X. The Organ Sources of the Stimulator and Responder Cells in the Mixed Leukocyte Reaction. Immunology (In Press).

12. Milthorp, P., Belanger, R., and Richter, M. (1978). The Cells Involved in Cell Mediated and Transplantation Immunity in the Rabbit. XI. The Accelerated Response in the One-Way MLR of Rabbit WBC from Skin Graft Recipients. Immunology (In Press).

13. Milthorp, P., and Richter, M. (1978). The Blastogenic Response of Rabbit Lymphocytes Stimulated with Autologous Cells. Immunology (In Press).

14. Milthorp, P., and Richter, M. (1978). The Optimal Conditions for the One-Way Mixed Leukocyte Culture in the Rabbit. J. Immunol. Methods (Submitted for Publication)

LIST OF ABSTRACTS

1. Milthorp, P., and Forsdyke, D.R. (1970) Activation by Concanavalin A or Phytohemagglutinin of Rat Lymphocytes in Homologous and Heterologous Serum: Dependence of Inhibition at High Con A/Serum Ratios on Complement. Proc. Can. Fed. Biol. Soc. 13, 58.

2. Milthorp, P., and Forsdyke, D.R. (1972) Activation and Inactivation by Concanavalin A of Rat Lymph-Node or Thymus Cells in Heated or Unheated Calf Serum. Proc. Can. Fed. Biol. Soc. 15, 147.

3. Milthorp, P., Quastel, M.R., Kaplan, J.G., and Vogelfanger, I.J. (1973) Effect of Inhibition of Cation Transport by Ouabain on Plaque Forming Cells. Proc. Can. Fed. Biol. Soc. 16, 52.

4. Forsdyke, D.R., and Milthorp, P. (1978) Early-onset Inhibition of Lymphocytes in Heterologous Serum by High Concentrations of Concanavalin-A. The Role of Complement. Proc. Can. Fed. Biol. Soc. 21, 151.