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THE CELLS INVOLVED IN THE BLASTOGENIC RESPONSE TO STIMULATION BY ALLOGENEIC CELLS IN THE MIXED LEUCOCYTE CULTURE REACTION OF THE RABBIT

BY

NICOLE LYSCOM

This thesis is submitted to the school of graduate studies of the University of Ottawa as partial fulfilment of the requirements for the degree of Master of Science in Pathology (Immunology).

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ABSTRACT

The roles of thymus-derived cells, bone marrow-derived cells, appendix-derived cells and phagocytic cells in the response of rabbit cells in the mixed leucocyte reaction (MLR) have been evaluated. This was carried out using spleen cells depleted of each of these cell subpopulations. The specific subpopulations of lymphoid cells were eliminated by lysis using specific antisera in the presence of complement. Phagocytic cells (macrophages and polymorphonuclear cells) were removed with a strong magnet after incubation with carbonyl iron particles. The results indicate that the MLR-responding cells are thymus-derived and that neither bone marrow-derived cells nor appendix-derived cells are essential for the blastogenic response to stimulation by allogeneic cells. A minimum number of phagocytic cells is required for a significant response. However, these cells exhibit a non-specific accessory role and can be supplied by either the responder or the stimulator cell population.

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ABBREVIATIONS

ATS	Anti-thymocyte antiserum
B	bursal-derived or bursal-equivalent-derived (lymphocytes)
CI	cytotoxic index
E-rosette	erythrocyte-rosette
GvH	graft versus host (reaction)
HARAAS	horse anti-rabbit appendix antiserum
HARBMAS	horse anti-rabbit bone marrow antiserum
HARTAS	horse anti-rabbit thymus antiserum
HBSS	Hank's balanced salt solution
HvG	host versus graft (reaction)
LD	lymphocyte derived (determinants)
MHC	major histocompatibility complex
MLR	mixed leucocyte (culture) reaction
RBC	red blood cell
SAPP	sacculus rotundus, appendix and Peyer's patches
SD	serologically defined (antigens)
SI	stimulation index
T	thymus-derived (lymphocytes)
WBC	white blood cell

CHAPTER 1

INTRODUCTION

When leucocytes from two unrelated individuals are cultured together, some of the cells undergo blast transformation resulting in cellular proliferation. This response, referred to as the mixed leucocyte (culture) reaction (MLR), was first observed by Schrek and Donnelly (1961) and later described in detail by Bain, Vas and Lowenstein (1963; 1964) and Hirschhorn, Bach, Kolodny, Firschein and Hashem (1963). Treatment of one of the leucocyte populations with mitomycin-C prevents that population from proliferating and enables the measurement of the reaction in one direction only. This is referred to as the one-way MLR (Bach and Voynow, 1966). It is generally accepted that the blastogenic response in the MLR is the *in vitro* counterpart of the *in vivo* recognition of foreign antigens on allografted cells which is followed by rejection of the transplanted tissues.

The responder cell has been identified as a thymus-derived (T) cell in the human (Potter and Moore, 1977), the mouse (Mosier and Cantor, 1971; Berman Puryear and Argyris, 1976) the rat (Wilson, Silvers and Nowell, 1967) and the chicken (Alm, 1971). For a significant response to ensue in the human MLR, a minimal proportion of the cells must be macrophages which can be supplied by either the responder or the stimulator cell population (Jones, 1966; Gordon, 1968; Twomey, Sharkey, Brown, Laughter and Jordon, 1970; Rode and

Gordon, 1970; Rode and Gordon 1974; Berlinger, Lopez and Good, 1976). They may be untreated or mitomycin-C treated.

Rabbit cells were first investigated for their response in the MLR by Chapman and Dutton (1965). The cells of each of the lymphoid organs of the rabbit have been thoroughly investigated recently in terms of the <u>in vivo</u> localization of responding cells (Milthorp and Richter, 1979) but no detailed study of the ontogenic identity of the responding cells has been carried out.

It has been demonstrated, by the use of specific anti-lymphocyte antisera and complement, that the thymus (Colas de la Noue, Koperstych and Richter, 1972) bone marrow (Colas de la Noue and Richter, 1974a) and appendix (Colas de la Noue and Richter, 1974b) of the rabbit each possesses an antigenically-unique subpopulation of lymphocytes. It is possible to eliminate each of these three lymphocyte subpopulations from a heterogeneous cell suspension by lysis with the appropriate antiserum in the presence of complement. In this thesis the development of methods is described for the removal of each of these three subpopulations of lymphoid cells, and also for the removal of phagocytic cells (i.e. macrophages and polymorphonuclear cells) from a heterogeneous cell suspension. The primary objective was to use spleen cell suspensions depleted of each of the above mentioned leucocyte sub-populations to determine the roles, if any, of thymus-derived cells, bone marrow-derived cells, appendix-derived cells and phagocytic cells in the blastogenic response of the rabbit to stimulation by allogeneic cells.

CHAPTER II

REVIEW OF THE LITERATURE

1. CHARACTERISTICS OF THE MIXED LEUCOCYTE CULTURE REACTION

1a) Introduction

When leucocytes from two unrelated individuals are cultured together, some of the cells undergo blast transformation, resulting in cell proliferation. This *in vitro* reaction, which is dependent on antigenic differences coded in the major histocompatibility complex (MHC), is known as the mixed leucocyte (culture) reaction (MLR).

The MLR was first described in 1961 by Schrek and Donnelly. They accidentally cultured cells from two patients together and noticed the appearance of large dividing cells on the fifth day. This was further explored by Bain, Vas and Lowenstein (1963; 1964) and Hirschhorn, Bach, Kolodny, Firschein and Hashem (1963) who co-cultured leucocytes from pairs of healthy individuals and observed the same response. No MLR is exhibited by the cells of identical twins which indicates that the reaction reflects a genetic disparity between the donors of the two populations of reacting cells (Bach and Hirschhorn, 1964; Bain, Vas, and Lowenstein, 1964). Since identical twins do not reject grafts transferred from one another it was suggested that the *in vitro* MLR is the counterpart of the *in vivo* allograft rejection reaction (Bain, Vas and Lowenstein, 1964).

It appears that circulating MLR responding cells are universal amongst vertebrates. They have been detected in many animals including humans (Schrek and Donnelly, 1961) mice (Dutton, 1965) rats (Wilson, 1967) chickens (Weber, 1970) and rabbits (Chapman and Dutton, 1965).

Early measurement of the MLR involved examination of the cells under a microscope. Using stained preparations, the percentages of blast cells and cells in mitosis can be determined (Bach and Hirschhorn, 1964). Alternatively, the extent of proliferation can be quantified by the uptake by the dividing cells of radioactive thymidine added to the cells several hours before termination of the culture. The dividing cells can then be revealed by autoradiography (Bain, Vas and Lowenstein, 1964). In this tecnhique, cell smears made after termination of the culture are treated with a photographic emulsion and then developed. The cells with radioactive thymidine exhibit microscopically-detectable black dots.

The technique currently used by most investigators is the measurement of the incorporation of radioactive thymidine into the DNA of the proliferating cells by scintillation counting. The labelled cultures are exposed to trichloroacetic acid which precipitates the DNA-bound radioactive thymidine. The latter is then dissolved by the addition of sodium hydroxide. This solution is mixed with scintillation fluid which enables the radioactivity to be measured in terms of counts per minute. This technique has been thoroughly reviewed

by Thorsby, du Bois, Bondevik, Dupont, Eijsvoogel, Hansen, Hersild, Jorgensen, Kissmeyer-Nielsen, Lamm, Schellekens, Svejgaard and Thomsen, (1974).

The MLR between untreated leucocytes of two unrelated individuals is a two-way reaction because the cells of both of the participating populations respond. As described in II.3, the *in vitro* MLR is used to predict allograft rejection; that is, the response of the cells of the allograft recipient to the cells of the allograft donor. The response of the cells of the allograft donor to the cells of the allograft recipient is not of interest unless the allograft contains immunologically competent cells. Using the two-way MLR it is impossible to ascertain the individual contribution to the response of each of the two participating cell populations. For this reason methods were developed for the measurement of the reaction in one direction only, that is, a one-way MLR. This is done by inhibiting the proliferation of one of the cell populations without affecting its stimulatory capacity. The first method, which was introduced by Kasakura and Lowenstein (1965), involves X-irradiation of the stimulator cells. A dose of 4,000 rads was found to be sufficient to inhibit the proliferative response and to have no effect on the stimulatory capacity of the cells (Kasakura and Lowenstein, 1968). The advantage of this technique is that no proliferation inhibitory factor can be carried into the

culture by the treated stimulator cells. However, the cells treated this way may become damaged and their long term viability, and hence their stimulatory capacity, may become affected. Another method was introduced by Bach and Voynow (1966). This involves treatment of the stimulator cells with the drug mitomycin-C which prevents cellular proliferation. This method is simple to use but care must be taken to ensure that all the drug is washed out of the stimulating cell suspension before the latter is added to the culture as otherwise it would also inhibit the proliferation of the responding cells. This method is currently used by most investigators.

1b) <u>The generation of cytotoxic cells in the MLR</u>

The proliferative response which occurs in the MLR is usually accompanied by the generation of specific cytotoxic cells which are able to lyse target stimulating cells in the absence of antibody and complement (Cerottini and Brunner, 1974). The *in vitro* generation of cytotoxic cells was first described by Hirschhorn, Firschein and Bach (1965) who observed that leucocytes cultured with fibroblasts develop a cytotoxic potential for these cells. The production of cytotoxic cells in the MLR has been observed in the mouse by Hayry and Defendi (1970) and in the human by Hardy, Wallin and Ling (1970). In their experiments, cell death was measured by the release of radioactive chromium from labelled target cells. The generation of cytotoxic cells has not yet been demonstrated in the MLR of the rabbit.

The cytotoxic cells are not the same as those which proliferate in the MLR since removal of the former does not affect the proliferative capacity of the remaining cells. This was demonstrated by Bach, Segall, Zier, Sondel, Alter and Bach (1973) who reported that cytotoxic cells, but not proliferatory cells, adhered to monolayers of the specific stimulator cells. Cantor and Boyse (1975) have observed that the mouse lymphocytes which exhibit cytotoxicity have different antigenic determinants from the proliferating lymphocytes.

Bach, Segall, Zier, Sondel, Alter and Bach (1973) have demonstrated that the proliferating cells and the cytotoxic cells respond to different alloantigens on the surface of the stimulating cell. (These antigenic determinants will be discussed in section 3a of this chapter). They have suggested that the proliferating cells are helper cells and that the potential cytotoxic lymphocytes require stimuli, both from the particular cellular alloantigens and from the proliferating cells, in order to become effective.

1c) Memory in the MLR

The cells which respond in the MLR exhibit memory with respect to the alloantigens used for stimulation. The proliferative and cytotoxic responses of the MLR reach their peaks between the fifth and seventh days of culture. Six to seven days later the blast cells revert to small non-dividing lymphocytes and cytotoxic activity against the cellular alloantigens used for stimulation disappears (Andersson and Hayry, 1973; Dupont, Hansen and Yunis, 1976).

Upon subsequent exposure to cells bearing the alloantigens used for stimulation, the responder cells display a secondary, modified MLR which differs from the primary MLR in kinetics and magnitude (Hayry and Andersson, 1973; Fradelizi and Dausset, 1975). Maximum activity is reached between the second and fourth days and the overall response is greater. The secondary MLR will only occur in response to cells bearing the same cellular alloantigens as those used in the primary MLR.

After the MLR, the blast cells revert to small lymphocytes and these cells follow a lymphocyte-blast-lymphocyte cycle upon repeated stimulation by specific allogeneic cells. Secondary proliferative responses can be maintained indefinitely by means of repeated stimulation, but cytotoxic activity disappears after five to seven cycles (Hayry, 1976).

The investigations of Hayry (1976) suggest that the cells which respond in the secondary MLR are the same as those which respond in the primary reaction. In order to determine this, blast cells were separated from small lymphocytes at the peak of the primary response by velocity sedimentation. The separated fractions were tested for their activities after secondary stimulation and it was found that all of the responder cell activity lay in the blast cell fraction.

1d) Clonal selection in the MLR

On the basis of experiments by Asantila, Vahala and Toivanen (1974), it appears that the proliferating cells in the MLR represent specific clones of cells. These investigators cultured human foetal thymus and spleen cells with allogeneic cells in the presence of 5-bromodeoxyuridine, a drug which becomes incorporated into the DNA of the dividing cells rendering them sensitive to ultra-violet light so that they die upon exposure to it. It was observed that the cells remaining after ultra-violet light treatment were unable to respond to cells bearing the alloantigens used for the primary stimulation. However, their response to stimulation by unrelated third party allogeneic cells was unimpaired.

It appears that the cytotoxic effector cell is also clonally derived as the ability of cytotoxic cells to lyse particular allogeneic cells can be abbrogated following adsorption to monolayers of the stimulating cells without affecting the ability of the remaining cells to lyse other allogeneic cells (Bach, Segall, Zier, Sondel, Alter and Bach, 1973).

2. THE CELLS INVOLVED IN THE MIXED LEUCOCYTE CULTURE REACTION

2a) Characteristics of the different types of leucocytes

In order to discuss the cells involved in the MLR it is necessary to review some of the characteristics and properties of the different mature nucleated circulating

cells found in the blood and lymphoid organs of vertebrates. These cells, referred to collectively as leucocytes, consist primarily of polymorphonuclear cells, monocytes and lymphocytes and they can be identified on stained slide preparations. Mature monocytes, also referred to as circulating macrophages, and polymorphonuclear cells are phagocytic. Lymphocytes, which have immunological functions, can be subdivided into at least two different subpopulations, thymus-derived (T) cells and bursal-derived (in avian species only) or bursal-equivalent-derived (B) cells.

Early evidence for the existence of two functional types of lymphocytes was provided by investigations of the effects on the immune response of the removal of the thymus and the bursa of Fabricius. Miller, Marshall and White (1962) and Good and Gabrielsen (1964) observed that neonatal thymectomy of mice resulted in a failure of the animals to produce antibodies and to reject skin allografts. Glick, Chang and Jaap (1954) observed that bursectomized chickens were unable to produce antibodies. However, bursectomy does not affect the animals' ability to reject skin allografts (Mueller, Wolfe, and Meyer, 1960; Warner, Szenberg and Burnet, 1962).

The experiments of Claman, Chaperon and Triplett (1966) demonstrated that both thymus cells and bone marrow cells are required for the production of antibodies in the mouse. They rendered mice totally immunoincompetent by

subjecting them to a sublethal dose of whole body X-irradiation and divided them into three groups. The first group was injected with syngeneic thymus cells, the second with syngeneic bone marrow cells, and the third with a mixture of syngeneic thymus and bone marrow cells. When challenged with antigen, only the third group, i.e. mice which had been injected with both thymus cells and bone marrow cells, were able to produce antibodies. Later experiments by Miller and Mitchell (1968) revealed that the cells of the thymus do not produce antibodies but are required as helper cells for antibody production by B cells. In their experiments, the repopulation of neonatally thymectomized mice with semi-allogeneic thymus cells enabled the animals to produce antibodies. However, subsequent killing of the grafted thymus cells with a specific anti-serum and complement did not prevent the continued production of antibodies by the remaining cells.

From these and many other experiments, the concept of distinct subpopulations of immunocompetent lymphocytes has evolved, namely B lymphocytes and T lymphocytes. B lymphocytes are the antibody producing cells and T lymphocytes are primarily involved in cell-mediated immunity, such as allograft rejection, but also function as helper cells for antibody production. However, this classification of lymphocyte subpopulations is an over-simplification as it has been shown that these cells can be further subdivided.

Non-avian species do not possess a bursa of Fabricius and there is still some controversy as to its equivalent in mammalian species. It is considered to be the bone marrow in humans (Golub, 1977). In some mammalian species the bursal equivalent may reside in one or more of the anatomically distinct lymphoid organs or amongst the diffuse aggregates of lymphoid cells found within the gastro-intestinal tract.

Cooper, Perey, Gabrielsen, Sutherland, McKneally and Good (1968) removed the gut-associated lymphoid tissues (the sacculus rotundus, appendix and Peyer's patches (SAPP)) of the rabbit in the immediate postnatal period. This significantly impaired the production of antibodies by the animals when they attained immunological maturity. On the other hand, Haasz and Richter (1972) demonstrated that the removal of these organs from adult rabbits did not significantly effect antibody production up to one year following surgery. Haasz and Richter (1972) postulated that the antibody-forming cells are originally found in the SAPP organs and that they are released into the circulation following birth. These cells infiltrate the other lymphoid organs where they exist as SAPP-derived cells, no longer under the influence of the SAPP organs. This hypothesis is further substantiated by the experiments of Behelak and Richter (1972). They immunized rabbits with

sheep red blood cells and examined several lymphoid organs for the presence of antibody-forming cells at different periods of time after immunization. Immediately after immunization, none of the lymphoid organs tested contained antibody-forming cells or had the potential to generate these cells in culture. However, one to two days after immunization, the Peyer's patches were able to generate specific antibody-forming cells in culture. Antibody-forming cells were not detectable in the spleen until at least four days after immunization. At this time none of the other lymphoid organs tested, including the Peyer's patches, either contained antibody-forming cells or had the ability to generate them in culture. These results suggest that the Peyer's patches may constitute the primary sites for the generation and/or maturation of antibody-forming precursor cells.

Colas de la Noue, Koperstych and Richter (1972) and Colas de la Noue and Richter (1974a; 1974b) used specific anti-lymphocyte antisera in the presence of complement to demonstrate that the lymphoid cells of the rabbit consist of at least three antigenically-defined subpopulations, namely thymus-derived cells, bone marrow-derived cells and appendix-derived cells, which constitute the major population of cells in the thymus, bone marrow and appendix, respectively. These cells are also found, in varying proportions, in the spleen, lymph nodes and circulation (Richter, Colas de la Noue and Hamdy, 1975).

The roles of the above-mentioned subpopulations of leucocytes in the MLR have been investigated by many methods, particularly ones using separated populations of cells. The most commonly used techniques for the separation of these cells are described in the appendix to this chapter.

2b). Stimulating cells in the MLR

The MLR was first discovered using leucocytes from two genetically unrelated individuals (Schrek and Donnelly, 1961). Not long after this is was established that the stimulation for this reaction is provided by mononuclear cells. Polymorphonuclear cells are unable to stimulate in the MLR (Rode and Gordon, 1970; Bach, Bach, Widmer, Oranen and Wolfberg, 1971; Mardiney, Bock and Chess, 1972).

A number of investigators have presented evidence that macrophages are extremely stimulatory in the MLR. Greineder and Rosenthal (1975) and Oehler and Herbermann (1977) demonstrated that the depletion of glass-adherent cells and phagocytic cells (macrophages) from the MLR-stimulator cells of the guinea pig and rat, respectively, resulted in a much reduced MLR. The ability to stimulate an MLR was restored to these cells by the addition of peritoneal exudate cells which are rich in macrophages. These investigators concluded that macrophages are the pre-dominant stimulating cells in the MLRs of these animals. Macrophages are also highly stimulatory in the MLRs of the mouse (Schirrmacher, Pena-Martinez and Festenstein,

1975) and the human (Rode and Gordon, 1974). Using human cells, Rode and Gordon (1974) observed that pure populations of macrophages are ten times as effective as an equal number of lymphocytes in eliciting an MLR response.

Contradictory results have been published concerning the ability of human T and B lymphocytes to stimulate in the MLR and it appears that the results obtained may depend on the methods used for cell preparation. Potter and Moore (1977) observed that T cell-enriched preparations, which were depleted of B cells using the nylon wool adherence technique, provided poor stimulation in the MLR. This may have been due to the removal of nylon fibre-adherent macrophages which are highly stimulatory (Rode and Gordon, 1974).

The same investigators (Potter and Moore, 1977) observed that T cells were better stimulators than B cells when the cells were separated by means of the E-rosette sedimentation technique. However, neither of these separated cell populations stimulated the MLR as well as an equal number of unseparated cells. These results suggest that the E-rosette sedimentation technique separates two distinct populations of cells which are both required for stimulation. On the other hand, Kuntz, Innes and Weksler (1974) and Blomgren (1977), who used cells separated by E-rosette sedimentation, reported that B lymphocytes were more stimulatory than T lymphocytes.

Sondel, Chess and Schlossman (1975) separated human lymphocytes using immunoglobulin absorbent columns and observed that T cells, B cells and unseparated cells were equally stimulatory in the MLR. Using this technique they achieved a high recovery rate of T lymphocytes. They suggested that the preparations of T lymphocytes shown by other investigators to be poor stimulators probably lacked a subpopulation of stimulatory T lymphocytes. This hypothesis could explain the results of Kuntz, Innes and Weksler (1974) and Blomgren (1977) because the E-rosette separation technique used by them gives a poor recovery of cells.

Results from experiments concerning the mouse MLR are also contradictory. B lymphocytes were observed to be more stimulatory than T lymphocytes by Plate and McKenzie (1973), Fathman, Handwerger and Sachs (1974), Simpson (1975) and Berman, Puryear and Argyris (1976). On the other hand, the results of Harrison (1973) and Cheers and Sprent (1973) indicate that mouse B and T lymphocytes are equally stimulatory.

Milthorp and Richter (1979) observed that the bone marrow cells of the rabbit are excellent stimulators of the MLR and that rabbit thymocytes are not stimulatory. The thymus cells of the human are also poor stimulators of the MLR (Han, Minowada, Subramanian and Sinks, 1976). Berman, Puryear and Argyris (1976) demonstrated that the thymus cells of the mouse are able to stimulate allogeneic

spleen cells. However, nylon column filtration of these mouse thymus cells removed the cells responsible for MLR stimulation and it was concluded that the stimulatory cells are either B cells or a nylon-adherent subpopulation of T cells. These investigators appear to have overlooked the possibility that macrophages (which are also nylon-adherent) could have been responsible for the MLR stimulation by the thymus cells of the mouse.

The cells which stimulate in the MLR have lymphocyte-derived (LD) determinants on their surfaces. These antigenic determinants will be discussed in II.3a below. Some cells which possess these antigenic determinants are unable to elicit an MLR, for example, fibroblasts (Shellekens and Eijsvoogel, 1970). It has been suggested that the MLR depends not only on the amount of LD antigenic determinants on the cell surfaces of the stimulating cells but also on their distribution. It is possible, for example, that the cells which are the most immunogenic have their antigenic determinants clustered together (Ling and Kay, 1975).

2c) <u>Responding cells in the MLR</u>

The cells which proliferate in the MLR of all species studied to date are T lymphocytes. On the other hand B lymphocytes of most species do not appear to be involved. The MLR responses of cells of children suffering from a congenital absence of the thymus (Ling and Kay, 1975), of thymectomized rats (Wilson, Silvers and Nowell, 1967),

and of thymectomized chickens (Alm, 1971) are severely diminished, but the MLR response of cells of bursectomized chickens (Alm, 1971) appears to be unimpaired.

Further evidence in favour of T cells being the MLR-responding cells is provided by experiments involving the use of responder cells depleted of or enriched for a sub-population of cells. The techniques involved are discussed in the appendix to this chapter. In the human, B cell enrichment brought about by the removal of E-rosette-forming cells causes a decrease in the MLR response (Lohrmann, Novikovs and Graw, 1974; Potter and Moore, 1977; Blomgren, 1977). The cells which adhere to immunoglobulin absorbent columns (B cells) do not respond (Chess, MacDermott and Schlossman, 1974). Conversely, human cells enriched for T cells as a result of the removal of non-E-rosette-forming cells (Lohrmann, Novikovs and Graw, 1974; Potter and Moore, 1977; Blomgren, 1977), cells adhering to nylon wool (Chess, MacDermott, Sondel and Schlossman, 1974; Potter and Moore, 1977), and cells adhering to columns coated with anti-immunoglobulin antibodies (Chess, MacDermott and Schlossman, 1974), exhibit an increased MLR response. On the other hand evidence for the involvement of B cells in the human MLR has been provided by Phillips and Weisrose (1974), who observed that a proportion of the lymphoblasts produced in the MLR are immunoglobulin-bearing cells.

Similar results have been reported in the mouse. Mouse cells depleted of cells which are susceptible to lysis by anti-thymus antiserum and complement lose their ability to respond to allogeneic stimulation (Mosier and Cantor, 1971; Gorczynski and Rittenberg, 1974; Berman, Puryear and Argyris, 1976). T cell-enriched mouse responder cells, which have been depleted of cells adhering to nylon wool, exhibit an increased MLR response (Berman, Puryear and Argyris, 1976). On the other hand, it appears that some mouse B cells may indeed respond in the MLR. Wagner (1972) observed that spleen cells of athymic mice were able to respond in the MLR and attributed this to B cells. Similar evidence was provided by von Boehmer (1974) but he stressed that B cell proliferation could only occur in the presence of T cells. In his experiments the latter were prevented from proliferating by treatment with mitomycin-C. Piquet and Vassalli (1972) also reported B cell proliferation in the mouse MLR and suggested that T cells recruit B cells for this purpose. However, it appears that the role of B cells in the MLR response of the mouse is fairly minor as less than 5 per cent of the cells in mitosis at the peak of the reaction are of B cell origin (Andersson, Nordling and Hayry, 1973).

An interesting experiment was carried out in rats by Johnston and Wilson (1970) to show that the majority of cells responding in the MLR are thymus-derived. They thymectomized and X-irradiated rats in order to eliminate all the immuno-

competent cells. The animals were then reconstituted with syngeneic bone marrow and thymus cells with different sex chromosome markers. When these cells were stimulated with F1 hybrid cells, it was observed that 90 per cent of the proliferating cells bore the sex chromosome marker of the thymus cells.

It has been suggested by Sheppard, Sell, Poler and Redelman (1977) that the responding cells in the rabbit MLR are also T cells. They found that the MLR of rabbit peripheral blood leucocytes was inhibited by treatment of the responding cells with anti-thymus antiserum and complement. Unfortunately, their evidence may be inconclusive since their antiserum had not been absorbed with non-thymus-derived cells and its cytotoxicity with respect to cells which were not of thymic origin was not firmly established. Consequently, it is not clear whether cells other than T cells were also killed by this treatment.

It has been demonstrated that the thymocytes of humans (Sohwartz, 1966; Han, Minowada, Subramanian and Sinks, 1976), mice (Blomgren and Svedmyr, 1971; Berman, Puryear and Argyris, 1976), and rats (Knight and Thorbecke, 1971) respond in the MLR. However, those of the rabbit do not (Chapman and Dutton, 1965; Ozer and Waksman, 1974; Milthorp and Richter, 1979).

Although this discussion is concerned primarily with the cells involved in the proliferative MLR response, it is interesting to note in passing that the cytotoxic effector cells generated in the MLR are also T cells. The cytotoxic cells of the mouse are killed by anti-thymus antiserum and complement (Hayry and Andersson, 1973). Sondel, Chess, MacDermott and Schlossman (1975) demonstrated that human blood leucocytes which did not adhere to immunoglobulin absorbent columns, and were therefore T cell-enriched, were able to develop the capacity to kill allogeneic cells to which they had been sensitized. The B cell-enriched preparations subsequently eluted from the immunoglobulin absorbent columns were unable to do this. However, as described previously (in II.1b), the cytotoxic and proliferative cells of the MLR represent entirely different clones of cells.

2d) Accessory cells in the MLR

There is much evidence that pure populations of human lymphocytes do not exhibit an MLR and that a small proportion of macrophages is required. Removal of macrophages (phagocytic and adherent mononuclear cells) from both the responder and the stimulator cell populations results in a much diminished MLR. Addition of macrophages syngeneic to either population of cells restores this response thus indicating that macrophages play a non-specific accessory role (Twomey, Sharkey, Brown, Laughter and Jordan, 1970; Rode and Gordon, 1970; Levis and Robbins, 1970; Alter and

Bach, 1970; Lohrmann, Novikovs and Graw, 1974; Sondel, Chess and Schlossman, 1975; Berlinger, Lopez and Good, 1976; Blomgren, 1977).

It has been suggested that an optimal MLR is generated when the macrophages are present in a threshold concentration; below this concentration, the magnitude of the MLR is a function of the concentration of macrophages. Macrophages present in higher concentrations have no additional effect (Twomey, Sharkey, Brown, Laughter and Jordan, 1970; Alter and Bach, 1970; Rode and Gordon, 1970). Rode and Gordon (1970) have suggested that the threshold concentration for these macrophage helper cells is approximately 1 to 2 per cent.

In humans it has been established that the MLR responder cell is a T cell and that macrophages are required to perform a non-specific helper role (Blomgren, 1977). Macrophages appear to be involved in T cell activation in many reactions. For example, it has been found that macrophages are required for T cell activation by mitogens and antigens in the human (Hersh and Harris, 1968; Lake, Bice, Schwartz and Salvaggio, 1971; Rosenstreich, Farrar and Dougherty, 1976; Mookerjee, 1977) by antigens in the guinea pig (Seeger and Oppenheim, 1970) and by mitogens in the rat (Keller, 1975) and rabbit (Kim and Herscowitz, 1978).

3. THE MIXED LEUCOCYTE CULTURE REACTION AND ALLOGRAFT IMMUNITY

3a) The genetics of transplantation reactions and the mixed leucocyte culture reaction

Soon after the discovery of the MLR in 1961 it was observed that the leucocytes of identical twins do not show an MLR (Hirschhorn, Bach, Kolodny, Firshein and Hashem, 1963; Bain, Vas and Lowenstein, 1964). Since identical twins do not reject grafts from one another, it was suggested that the MLR may be related to homograft immunity (Bain, Vas and Lowenstein, 1964). The MLR has since been used by many investigators to select kidney donors for patients with terminal renal disease.

The antigenic determinants involved in allograft rejection reactions are controlled by a group of closely linked genes known as the major histocompatibility complex (MHC) (Dupont, Hansen and Yunis, 1976). The MHC is known as the HLA system in man, the AgB system in the rat, the H-2 system in the mouse and the RL-A system in the rabbit. Some MHC differences can be defined using serological assays in which antisera are employed to detect specific antigens on the surfaces of lymphocytes (Dupont, Hansen and Yunis, 1976). Antigens identified by this method are known as serologically defined (SD) antigens. These antigens are present on practically all types of cell (Bach and Van Rood, 1976a).

In 1967, Bach and Amos showed a relationship between the MLR response exhibited by the cells of two individuals and their genetic dissimilarity as determined by SD antigen typing. Results from later experiments indicated that this relationship does not always exist: Amos and Bach (1968) and Yunis and Amos (1971) observed that an MLR response could occur between leucocytes of some pairs of individuals of the same SD antigenic type. This was also demonstrated in the rabbit (Tissot and Cohen, 1974). Because of this discrepancy, the concept of lymphocyte-derived (LD) antigenic differences evolved (Bach, Bach, Sondel and Sundharadas, 1972). These differences are reflected in the MLR and do not correlate with any known antigenic determinants (Ling and Kay, 1975).

The proliferative response in the MLR is dependent on LD differences. Cytotoxic cells are usually generated in the MLR but the target antigens recognized by these cells are not LD but SD antigens. This LD-SD collaboration was demonstrated by Schendel, Alter and Bach (1973) who used a one-way MLR with three different cell populations. In this experiment, the responder cells were stimulated initially with stimulator cells which had identical SD antigens but different LD antigens. A proliferative response ensued but no cytotoxic cells were generated. A second population of stimulator cells, which had identical LD antigens but different SD antigens with respect to the responding cells, was added to the culture. The responding

cells were unable to proliferate in response to the second population of stimulating cells. However, cytotoxic cells were generated which were specific for the second population of stimulator cells and were therefore directed against the SD antigenic determinants.

3b) Transplantation rejection reactions and the MLR

When allogeneic tissue transplants are carried out, two types of reaction can occur: the host versus graft (HvG) reaction which results in destruction and rejection of the transplanted tissue, and the graft versus host (GvH) reaction. The latter occurs when immunocompetent cells are transferred to an immunologically compromised allogeneic host. The graft"rejects" the cells of the host, causing much tissue damage and ultimately the death of the host animal. An example of this reaction is the potentially fatal situation which arises when an immunoincompetent individual receives a bone marrow transplant from a normal allogeneic donor (Bach and van Rood, 1976b). The transplanted cells proliferate in the spleen of the host causing splenomegaly. The increase in spleen size is used as a measure of the proliferative response of this reaction (Bach and van Rood, 1976b). HvG and GvH rejection reactions are considered to represent different aspects of the same physiological response and they both show many similarities to the MLR response.

The HvG reaction appears to bear a close resemblance to the MLR in that both involve the production of cytotoxic

T cells. Hayry and Roberts (1977) reported that large numbers of specific cytotoxic T cells were produced in the HvG reaction which could be found in the lymph nodes and the spleen. Contradictory reports have been published concerning the nature of the cells at the rejection site. Roberts and Hayry (1977) found that the cells infiltrating the allograft at the peak of the rejection, which occurs seven to eight days after grafting, were predominantly non-T killer cells, namely macrophages and non-T lymphocytes. However, Strom, Tilney, Paradysz, Bancewics and Carpenter (1977) found that cytotoxic T cells comprise the majority of cells at the rejection site. This discrepancy may be explained by the findings of Balch, Wilson, Lee and Feldman (1973) who observed that the infiltration of the allograft by T lymphocytes reaches its peak four days after grafting. However, by the sixth day, which is the time that Roberts and Hayry (1977) performed their assay, this infiltration is relatively diminished.

Another aspect of the relationship between the MLR and allograft rejection is the ability of the leucocytes of the allograft recipient to exhibit a modified MLR upon further exposure to cells of the allograft donor. This response is similar to the secondary MLR, described in II.1c above, as it is specific for the alloantigens of the allograft donor and occurs much more rapidly than normal. It has been observed in the rabbit (Harrison, Wei and Ahie, 1971; Milthorp, Belanger and Richter, 1979), the rat

(Wilson, Silvers and Nowell, 1967), and the mouse (Adler, Tagikuchi, Marsh and Smith, 1970).

Chapman and Dutton (1965) and Sheppard, Sell, Poler and Redelman (1977), working with the rabbit, were unable to shown an accelerated MLR by leucocytes of allograft receipients. These results may, however, be explained by the fact that these investigators evaluated the MLR blastogénic response only on or after day five 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.

The GvH also appears to be very similar to the MLR. Like the MLR it manifests itself by a proliferative response accompanied by the production of cytotoxic killer cells (Sprent and Miller, 1971). The T cells responsible for the MLR appear to be the same as the T cells responsible for the GvH reaction (Wagner, Rollinghoff and Shortman, 1974), and T cells depleted of cells which adhere to allogeneic cell monolayers retain MLR and GvH reactivity but lose specific cytotoxic activity (Mage and McHugh, 1973). A high level of correlation exists between the degree of splenomegaly in the GvH reaction and the proliferative response in the one-way MLR between leucocytes of the graft donor (responder cells) and the recipient (stimulator cells) (Livnat, Klein and Bach, 1973).

In both the GvH and the MLR the proliferative response is to LD antigenic differences (Elkins, Karathas and Bach, 1973). The role of the cytotoxic killer cells produced in the GvH reaction has not been resolved but it is conceivable that they are responsible for the tissue damage and ultimate death of the host animal.

4. CONCLUSIONS

In this chapter the MLR has been discussed in terms of its mode of action, the cells involved, and its significance as an *in vitro* counterpart of the *in vivo* allograft rejection reaction. Much of the information available at present concerning the rabbit has been discussed and it is clear that the rabbit has not been studied as thoroughly as the human and the mouse. One of the major aspects which has been almost entirely neglected in research concerning the rabbit is the function, if any, of leucocyte subpopulations in this reaction. The following experiments are designed to determine the roles of thymus-derived cells, bone marrow-derived cells, appendix-derived cells and phagocytic cells in the MLR response of rabbit cells to allogeneic stimulation.

APPENDIX

TECHNIQUES FOR SEPARATING SUBPOPULATIONS OF LEUCOCYTIC CELLS

Leucocytes can be separated from one another by techniques based on their individual properties. There are numerous methods and the ones relevant to this thesis are described below.

The polymorphonuclear cells of some animals are denser than macrophages and lymphocytes and they can be removed from a cell suspension by centrifugation over a Ficoll Hypaque solution of specific gravity 1.077 (Boyum, 1968). The polymorphonuclear cells pass through the Ficoll Hypaque and the other cells remain above it. Any red blood cells (RBCs) and dead cells present in the cell suspension are also removed by this method as they also pass through the Ficoll Hypaque solution.

Macrophages and polymorphonuclear cells have the ability, at 37°C in the presence of calcium and magnesium ions and a heat labile factor from fresh serum, to ingest and adhere to foreign particles (Tullis and Surgenor, 1956; Cassen, Hitt and Hays, 1958; Rabinowitz, 1964). These cells can be separated from lymphocytes by techniques based on these properties. Many people describe the cells which have been removed by these methods as macrophages. This is incorrect unless the original cell suspensions were initially devoid of polymorphonuclear cells (for example, from

some lymphoid organs or from Ficoll Hypaque gradient separation preparations) which would otherwise be removed with the macrophages.

Levine (1956) introduced one of the first techniques for the removal of phagocytic cells. He incubated human blood cells with starch-coated iron granules and removed the iron ingesting phagocytic cells and the residual iron with a magnet. He observed that untreated iron granules are not readily ingested by cells. Similar techniques involving the use of gum arabic-coated iron granules and carbonyl iron have been performed by Cassen, Hitt and Hays (1958) and Blomgren (1977), respectively.

The drawback of separation techniques based on phagocytosis is that they can only be used to eliminate macrophages and polymorphonuclear cells. In order to recover these cells after separation, adherence techniques must be used. Columns filled with glass beads or wool (Gordon, 1968), cotton wool (Rode and Gordon, 1974), nylon wool (Chess, MacDermott, Sondel and Schlossman, 1974; Greineder and Rosenthal, 1975), and rayon wool (Alter and Bach, 1970) have been used to remove adherent cells. The cells are incubated in the columns under the optimal conditions for adherence. Non-adherent cells are washed out with a buffered salt solution at 37°C. The adherent cells can be eluted by washing the columns with cold medium containing EDTA which chelates with the magnesium and calcium ions. Under these conditions, the adherence

properties of the cells are lost and they pass through the column. Cells adhering to plastic petri dishes have been separated from non-adherent cells in a similar manner (Greineder and Rosenthal, 1975).

The nylon wool adherence technique for the separation of macrophages and polymorphonuclear cells is not ideal as B lymphocytes also adhere to nylon wool. This technique has been successfully used for the removal of macrophages by Chess, MacDermott, Sondel and Schlossman (1974) who used cell preparations previously depleted of B lymphocytes.

Mixtures of polymorphonuclear cells and macrophages obtained by these methods can be enriched for macrophages by incubating them at 37°C for four to seven days. During this time, most of the polymorphonuclear cells die out leaving an almost pure population of macrophages (Gordon, 1968). Cells rich in macrophages can be obtained from the peritoneal cavity and these can be purified by any of the adherence techniques already mentioned.

B and T lymphocytes each have unique receptors on their surfaces. Lymphocytes of the bursa of Fabricius have surface immunoglobulins (Cooper, Lawton and Kincade, 1972). Some lymphoid cells of many species including the human (Unanue, Grey, Rabellino, Campbell and Schmidtke, 1971), the mouse (Raff, 1970), and the rabbit (Sell and Gell, 1965), have surface immunoglobulins and their presence is widely accepted as a marker for B cells. However, there is some

controversy in the rabbit concerning the nature of the immunoglobulin bearing cells. Sell and Sheppard (1973) observed that some of the rabbit peripheral blood lymphocytes which respond to T cell mitogens have surface immunoglobulins. This however was contradicted by Bell and Wigzell (1977). In their experiments, the cells of the spleen, lymph nodes and blood of the rabbit with surface immunoglobulins did not respond to T cell mitogens and the cells which did respond to these mitogens did not have surface immunoglobulins. It is conceivable that the immunoglobulins on the surfaces of the cells in the experiments of Sell and Sheppard (1973) had been adsorbed from the plasma.

Chess, MacDermott and Schlossman (1974) separated immunoglobulin-bearing cells from non-immunoglobulin-bearing cells using columns containing sephadex G-200 conjugated to anti-human immunoglobulin antibodies. The cells with surface immunoglobulins which adhered to the columns were subsequently eluted by competitive binding with a 1 per cent immunoglobulin solution.

Nylon wool adherence techniques have been used for the separation of B lymphocytes from human cells by Potter and Moore (1977) and from mouse cells by Berman, Puryear and Argyris (1976). However, as described above, macrophages are also removed by these techniques.

The thymus-derived cells of some species including humans have a receptor for untreated sheep erythrocytes and

they form erythrocyte-rosettes (E-rosettes) with them at 4°C (Coombs, Gurner, Wilson, Holm and Lindgren, 1970; Lay, Mendes, Bianco and Nussenzweig, 1971). Rosettes can be separated from non-rosette forming cells by Ficoll Hypaque density gradient centrifugation since the former is denser than the latter. Recovery of the rosette forming cells is achieved by lysis of the erythrocytes with a buffered salt solution of 0.83 per cent ammonium chloride. This method has been used to separate E-rosette forming cells by Wybran, Chantler and Fudenberg (1973). The great advantage of this technique is that all of the cells involved can be recovered. However, the possibility exists that the procedures damage the cell membranes.

T cells of the rabbit do not possess the receptor for sheep erythrocytes but they can be identified and eliminated by their susceptibility to anti-thymus antiserum in the presence of complement[1] (Colas de la Noue, Koperstych and Richter, 1972; Fradelizi, Chou, Cinader and Dubiski, 1973; Redelman, Scott, Sheppard and Sell, 1976; Fanger, Pelley and Reese, 1972; Wilson, Teodorescu and Dray, 1976). This technique has also been used to identify and eliminate thymus-derived cells of humans (Aiuti and Wigzell, 1973a, 1973b; Ablin and Morris, 1973) mice (Mosier and Cantor, 1971; Berman, Puryear and Argyris, 1976) and rats (Colley, Malakian and Waksman, 1970).

1. Complement is a system of heat labile serum proteins which interact with antibody and antigen to effect cytotoxicity.

An antiserum to cellular antigens is prepared by immunizing an animal with the foreign cells towards which the antiserum is to be cytotoxic. The animal synthesizes antibodies which are directed against the cell surface antigens. Within several weeks after the immunization the serum of the animal is usually rich in antibodies and is described as an antiserum. Incubation of the antigenic cells with the antiserum and complement results in cytotoxic lysis of these cells. Antisera generally exhibit some cytotoxicity with respect to other cells of the donor of the sensitizing antigens and must be rendered specific by absorption. This is done by incubating the serum at 37°C, in the absence of complement, with the cells to which it is to be rendered non-cytotoxic.

In addition to anti-rabbit thymus antisera, specific antisera for rabbit cells have also been prepared for bone marrow-derived cells (Colas de la Noue and Richter, 1974a), appendix-derived cells (Colas de la Noue and Richter, 1974b; Sheppard and Sell, 1977) cells with surface immunoglobulins (Wilson, Teodorescu and Dray, 1976) and macrophages (Kim and Herscowitz, 1978).

Separated subpopulations of leucocytes need to be defined not only in terms of the types of cells represented but also in terms of the techniques used for separation. This is because some overlap exists amongst these subpopulations with respect to many of their properties. When discussing depletions of subpopulations of cells using antiserum and

complement it is necessary to describe the specificity of the antiserum used in terms of the cells it does and does not kill in the presence of complement. Results expressed without these details lose much of their meaning.

CHAPTER III

MATERIALS AND METHODS

1. MATERIALS

Rabbits - outbred, 4-5 lb New Zealand White rabbits were obtained from Rockland Rabbit Ranch, Rockland, Ontario.

RPMI - 1640 with L-glutamine was obtained from Microbiological Associates, Bethesda, Maryland, and stored at 4°C.

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

Penicillin Streptomycin Mixture, containing 5,000 I.U. potassium penicillin G/ml and 5,000 µg streptomycin sulphate/ml, was obtained from Microbiological Associates and stored at -20°C.

Ficoll 400 was obtained from Pharmacia Fine Chemicals A.B., Uppsala, Sweden.

Hypaque Sodium (50% w/v, pH = 6.5-7.7) was obtained from Winthrop Laboratories, Aurora, Ontario, and stored in the dark.

Methyl Cellulose was obtained from BDH Chemicals Ltd., Poole, England.

Sterile Water (pyrogen-free) was obtained from Abbott Laboratories Ltd., Montreal, Quebec.

Dextran T 500 was obtained from Pharmacia Fine Chemicals A.B.

Heparin Sodium (10,000 U.S.P. units/ml) was obtained from Organon Canada Ltd., Toronto, Ontario, and stored at 4°C.

Saline (0.9%) in pyrogen-free sterile water was obtained from Abbot Laboratories Ltd.

Surital (Sodium Thiamylal) was obtained in powdered form from Parke, Davis and Company Ltd., Brockville, Ontario. It was dissolved in distilled water prior to use.

Trypan Blue (0.4%) in normal saline was obtained from Grand Island Biological Co., Grand Island, New York.

GAF Carbonyl Iron Powder grade SF was obtained from Chemical Developments of Canada Ltd., Toronto, Ontario.

Mitomycin-C was obtained in powdered form from Nutritional Biochemical Co., Cleveland, Ohio, and stored in the dark at 4°C. Immediately before use the powder was dissolved in RPMI-1640 to give a solution containing 1 mg/ml.

^{3}H-Thymidine (specific activity = 20 Ci/mM) was obtained from New England Nuclear, Boston, Massachussetts. This was diluted in RPMI-1640 to a concentration of 10 µCi/ml and stored at -20°C.

Ready-Solv GP Scintillation Fluid was obtained from Beckman, Fullerton, California.

Sodium Nitrite was obtained in crystalline form from Fisher Scientific Co., Fairlawn, New Jersey.

Ethylene Glycol Monomethyl Ether was obtained from Fisher Scientific Co.

Alpha-Naphthyl Acetate was obtained in anhydrous form from Sigma Chemical Co., St. Louis, Montana.

Pararosanilin was obtained in anhydrous form from Sigma Chemical Co.

Sodium Phosphate, Dibasic in anhydrous form was obtained from Fisher Scientific Co.

Potassium Phosphate, Monobasic was obtained in crystalline form from J.T. Baker Chemical Co., Phillipsburg, New Jersey.

Methyl Green was obtained from J.T. Baker Chemical Co.

Sodium Acetate in fused-anhydrous form was obtained from Fisher Scientific Co.

Hydrochloric Acid (HCl) was obtained from J.T. Baker Chemical Co.

Sodium Hydroxide electrolytic pellets were obtained from Fisher Scientific Co.

Methanol was obtained from Fisher Scientific Co.

Plastic Falcon conical centrifuge tubes (50ml) were obtained from Falcon Plastics, Oxnard, California. These were sterilized by autoclaving before use.

Plastic sterile tubes (17 x 100 mm) were obtained from Falcon Plastics.

Sterile Universal containers (20 ml) were obtained from Sterilin Ltd., Richmond, Surrey, England.

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

All glassware was obtained from Fisher Scientific Co. It was sterilized by autoclaving before use.

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

Hypodermic needles - sterile disposable Luer-Lok hypodermic needles were obtained from Becton, Dickinson Co.

Pipettes - 1, 5 and 10 ml pyrex disposable serological pipettes were obtained from Corning Glass Works, Corning, New York.

Automatic MLA Pipettes (100 µl and 50 µl) were obtained from Medical Laboratory Automation, Inc., Mount Vernon, New York. Pipette Tips were obtained from Fisher Scientific Co.

Haemacytometer Counting Chambers - AO Spencer "Bright Line" type - were obtained from Canlab, Ottawa, Ontario.

Nalgene Filters (45 µ) were obtained from Sybron Corporation, Rochester, New York.

The Coulter Counter used was the model ZBI made by Coulter Electronics, Hialeah, Florida.

The incubator - a National Incubator was obtained from Fisher Scientific Co. This was maintained at 37°C. The interior was humidified by distilled water in a tray 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.

The Liquid Scintillation Counter was a Beckman Model LSC-230 obtained from Beckman, Fullerton, California.

2. METHODS

2a) Preparation of Reagents

Rabbit serum was obtained from the laboratory rabbits by cardiac puncture: blood was allowed to clot at room temperature for approximately one hour after which the serum was separated from the clot by centrifugation at 1500g for 20 minutes. Some sera were complement-inactivated by heating at 56°C for 30 minutes. Pooled serum was composed of equal volumes of serum from at least ten different rabbits. This was divided into 5 ml aliquots and stored at -20°C until required.

RPMI - 1640 with L-glutamine and HBSS. Penicillin and streptomycin were added to these solutions to a final concentration of 100 I.U. penicillin/ml and 100 µg streptomycin/ml. This was to prevent bacterial contamination.

Tissue culture medium This was composed of RPMI-1640 medium with L-glutamine, penicillin and streptomycin supplemented with complement-inactivated pooled rabbit serum at a concentration of 2.5 per cent.

The Ficoll Hypaque density gradient solution was made by dissolving 40g of Ficoll 400 and 0.6g of methyl cellulose in 500 ml of distilled water: this was made up to a specific gravity of 1.1 at 18°C by the addition of Hypaque. This solution was sterilized by filtration through 45 μ Millipore Nalgene filter, divided into aliquots and stored in the dark at 4°C.

The dextran solution consisted of 6 per cent dextran (molecular weight 200,000 - 250,000) in saline with heparin at a final concentration of 150 units/ml.

Phosphate buffer (0.1M) of pH 7.6 was prepared by mixing 2,000 ml of 0.1M monobasic sodium phosphate with 190 ml of 0.1M dibasic potassium phosphate.

The methyl green solution consisted of 1 per cent methyl green w/v and 1.4 per cent sodium acetate w/v in distilled water. It was adjusted to pH 4.2 with concentrated acetic acid.

The pararosanilin solution was made by mixing together 1g of pararosanilin, 20 ml of distilled water and 5 ml of concentrated HCl.

2b) Preparation of cells

Rabbits were killed by the intravenous injection of Surital. Bone marrow was obtained by cracking the femur and scraping the cells into a Universal container. The spleen, thymus and appendix were rapidly removed and placed in sterile Universal containers. The appendix was slit open and rinsed several times in saline in order to remove its contents. Cells were released from these lymphoid organs by chopping with fine pointed scissors followed by shaking in HBSS. Filtration of the cell suspensions through sterile gauze removed the tissue fragments and any fine pieces of bone from the bone marrow samples. The cells were washed twice in HBSS for 10 minutes at 800g and finally re-suspended in RPMI-1640. Unless otherwise stated, white cell counts were made using a Coulter counter.

Density gradient separations of spleen cell suspensions were carried out using a Ficoll Hypaque solution of specific gravity 1.1 containing 0.001 per cent methyl cellulose. The latter constituent was added to enhance agglutination of erythrocytes. An aliquot of the cells (10 - 20 ml containing approximately 10^8 leucocytes), suspended in RPMI-1640, was layered over the Ficoll Hypaque solution (10 ml) in a 50 ml sterile glass centrifuge tube. The tubes were centrifuged at 1,000g for 30 minutes at 18°C. The supernatant was removed by aspiration and the cells at the medium-Ficoll Hypaque interface were collected. The cells were washed three times with HBSS and re-suspended in RPMI-1640.

These cells are referred to as mononuclear-enriched cell suspensions. The average recovery of spleen leucocytes was 32 per cent.

RBCs were obtained by dextran sedimentation of whole blood. Two parts of blood obtained by cardiac puncture were mixed with one part of dextran solution containing heparin. The cells were allowed to settle in a measuring cylinder at room temperature. The leucocyte-rich supernatant was discarded and the remaining RBCs were washed three times before use.

2c) Preparation of antisera

The anti-thymus antiserum and control serum were prepared by Colas de la Noue, Koperstych and Richter (1972) and the anti-bone marrow and anti-appendix antisera and their respective controls were prepared by Colas de la Noue and Richter (1974a; 1974b). Briefly, normal adult horses were immunized by the intravenous administration of the appropriate cells three times at seven-day intervals. Different horses were used for the preparation of each particular antiserum. For the preparation of horse anti-rabbit thymus antiserum (HARTAS), horse anti-rabbit bone marrow antiserum (HARBMAS) and horse anti-rabbit appendix antiserum (HARAAS), 5×10^{10} rabbit thymus cells, 5×10^{8} rabbit bone marrow cells and 5×10^{8} rabbit appendix cells respectively, were used for each injection. The animals were bled 2, 3 and 5 weeks after the last injections

of the antigens and the antisera were obtained by centrifugation of the clotted blood. The sera were stored at -20°C until used.

Control sera for each of these antisera were prepared prior to immunization from the same horses as those used to produce each antiserum.

The antisera and control sera were complement-inactivated by incubation at 56° for 30 minutes and filtered through a 45 μ Nalgene filter prior to use. All dilutions of the antisera and control sera were made with RPMI-1640.

2d) Absorption of the antisera

A specific number of the appropriate cells was suspended in the antiserum for 30 minutes at 37°C with occasional shaking. This was followed by centrifugation at 800g for 20 minutes. The absorbed antiserum was either subjected to further absorption or divided into aliquots and stored at -20°C until required.

2e) Cytotoxicity assay

The method used is based on that of Fradelizi, Chou, Cinader and Dubiski (1973). One volume of lymphoid cells (5×10^6 cells/ml) in RPMI-1640 was mixed with two volumes of the antiserum made up to an appropriate dilution. The cell-antiserum mixture was incubated at 37°C in 5 per cent CO_2 in air for 15 minutes after which two volumes of

complement (fresh undiluted autologous rabbit serum) were added and the incubation continued for a further 60 minutes.

Cells were also incubated with the control sera in place of the anti-lymphocyte antisera.

The percentage of dead cells was determined using the trypan blue dye exclusion test described below. The cytotoxic index (CI) was calculated from the formula; $CI = [(DE-DC)/(100-DC)] \times 100$, where DE is the percentage of dead cells in the sample containing the antiserum and DC is the percentage of dead cells in the sample containing the control pre-immunization serum.

2f) The trypan blue dye exclusion test

Two volumes of a cell suspension, containing cells at a concentration of 2×10^6/ml or less, were mixed with one volume of a 0.2 per cent trypan blue solution in saline. After five minutes, the cells were counted in a haemacytometer chamber. The cells which were unable to exclude the dye were considered dead. By means of this technique, the concentration of viable cells and the percentage of dead cells can be determined. At least 200 cells were counted from each sample.

2g) Depletion of lymphoid cell subpopulations

The prospective MLR responder spleen cells were treated with either anti-thymus, anti-bone marrow or anti-appendix antiserum and complement or with control serum and complement under the conditions described for the

cytotoxicity assay. After incubation, the cells were washed four times with HBSS and re-suspended in tissue culture medium. The concentration of viable cells was determined using the trypan blue dye exclusion test.

Prior to treatment of the spleen cells with the anti-bone marrow antiserum, the RBCs were removed by Ficoll Hypaque density gradient separation. This step avoided any complications which might otherwise have arisen due to the competition between the erythrocytes and the bone marrow-derived lymphocytes for the antibodies in the anti-bone marrow antiserum.

2h) Depletion of phagocytic cells

Packed spleen cells (10^7) in sterile plastic tubes were suspended in 5 ml of fresh uninactivated autologous serum diluted two-fold with RPMI-1640 containing various concentrations of carbonyl iron (See IV.2). This mixture was incubated for 60 minutes at 37°C with constant mixing provided by a rotator. The iron-ingesting phagocytic cells and the residual iron were removed by four exchanges from one test tube to another over a strong magnet. After three washes with HBSS, the cells were suspended in either RPMI-1640 and treated with mitomycin-C (stimulator cells), or in tissue culture medium (responder cells).

Control cells were treated similarly but without the addition of carbonyl iron.

Smears were stained for non-specific esterase as described immediately below in order to determine the relative proportions of lymphocytes, polymorphonuclear leucocytes and macrophages in the cell preparations.

2i) Non-specific esterase staining

This technique for the identification of cells containing the esterase enzyme was developed by Yam, Li and Crosby (1971). It involves treating slide preparations with the substrate alpha-napthyl acetate and the dye pararosanilin. The esterase enzyme splits the substrate producing a napthyl end product which becomes stained by the dye. In this method the cells are counterstained with methyl green, which allows the cytoplasm and the nucleus of the cells to be seen.

Slide preparations of cells in serum were rapidly dried using cold air from a hair dryer. The esterase staining solution was prepared as follows: equal volumes of pararosanilin solution and 4 per cent sodium nitrite were mixed; after exactly one minute 6 volumes of this mixture were added to 5 volumes of ethylene glycol monomethyl ether containing 20 mg of alpha-napthyl acetate/ml; 90 volumes of phosphate buffer of pH 7.6 were added to the mixture and the pH was adjusted to 6.1 with HCl; the buff coloured precipitate which developed was removed by filtration.

Each slide was covered with the esterase staining solution for 45 minutes at room temperature. The slides were then washed with water and dried and fixed in methanol for two minutes. After the methanol had evaporated, the slides were counterstained with a 1 per cent solution of methyl green for two minutes and then washed with water.

2j). Mitomycin-C treatment of stimulator cells

Stimulator cells at a concentration of 5×10^6/ml were treated with mitomycin-C (final concentration 50 mg/ml) for 30 minutes at 37^oC in 5 per cent CO_2 in air. Excess mitomycin-C was washed out 4 times with HBSS after which the cells were re-suspended in tissue culture medium.

2k) MLR culture

Responder and mitomycin-C treated stimulator cells (obtained from two different allogeneic rabbit spleens) were suspended in tissue culture medium. The responder cells (0.5ml) were mixed with the stimulator cells (0.5ml) in sterile plastic tissue culture tubes. In each experiment at least three different concentrations of responder cells were used, the final concentrations varying between 0.1 and 0.6×10^6/ml. Stimulator cells were cultured at a final concentration between 0.6 and 2.5×10^6/ml. The cultures were incubated at 37^oC in 5 per cent CO_2 in air for 5 days. ^{3}H-thymidine (0.1 ml containing 1 µCi: specific activity = 20 Ci/mM) was added to each of the culture tubes 18 - 22 hours prior to termination of the cultures.

Control cultures consisted of responder cells incubated with the appropriate number of mitomycin-C treated autologous cells...

The amount of ^{3}H-thymidine incorporated into the DNA was determined by liquid scintillation counting. The cells were washed twice in 3 per cent acetic acid to remove the excess tritium and the acid-soluble haemoglobin. The pellets were dissolved in 0.5N sodium hydroxide (0.5ml) at 80°C for 10 minutes and then neutralized with 5 per cent acetic acid (0.2 ml). The contents of each tube were mixed with Ready Solv GP scintillation fluid (7 ml) and the ^{3}H-thymidine incorporated into the DNA was determined by counting at ambient temperature in a liquid scintillation counter. Counting efficiency was 60 per cent in an unquenched sample.

21) Calculations

The MLR is expressed in either counts per minute (cpm) or as the stimulation index (SI) which is the cpm of the allogeneic culture divided by the cpm of the autologous control cultures. All results are based on triplicate samples.

Statistical data are based on the paired Student's T test.

CHAPTER IV

EXPERIMENTAL RESULTS

IV. 1. PREPARATION OF ANTISERA SPECIFICALLY CYTOTOXIC FOR THE THYMUS-DERIVED CELLS, BONE MARROW-DERIVED CELLS AND APPENDIX-DERIVED CELLS OF THE RABBIT

INTRODUCTION

An anti-thymus antiserum (HARTAS), an anti-bone marrow antiserum (HARBMAS) and an anti-appendix antiserum (HARAAS) have been prepared in horses for rabbit cells by Colas de la Noue, Koperstych and Richter (1972) and Colas de la Noue and Richter (1974a; 1974b). Using these antisera they demonstrated that the thymus, bone marrow and appendix of the rabbit each contains antigenically unique populations of lymphocytes. The purpose of the experiments described below is to render these antisera specific for the appropriate cells by means of absorption and dilution with the aim of using them to eliminate each of these lymphoid cell subpopulations from heterogeneous cell suspensions.

RESULTS AND DISCUSSION

Anti-thymus antiserum

The cytotoxic activity with respect to thymus cells, bone marrow cells and RBCs of the HARTAS, unabsorbed and absorbed (at a dilution of 1:5) with 0.25 ml of packed RBCs/ml of antiserum, is shown in Figure 1. The plots represent

mean values from two experiments.

The unabsorbed HARTAS, at high concentrations, was highly cytotoxic to all three cell types. Upon dilution of the antiserum (up to 1:320) much of the cytotoxic activity with respect to bone marrow cells and RBCs was lost with little effect on the cytotoxic activity with respect to thymus cells. At the dilution of 1:320, the antiserum did not lyse many RBCs but induced much RBC clumping. This clumping was probably caused by the attachment of non-complement fixing antibodies to the RBCs.

The antiserum was absorbed at a dilution of 1:5 with 0.25 ml of packed RBCs/ml of antiserum. This absorbed antiserum diluted to 1:20 was no longer cytotoxic towards RBCs and bone marrow cells but retained most of its cytotoxic activity towards thymus cells. It no longer caused RBC clumping. This preparation of the antiserum is regarded as being specific for thymus cells because it consistently gave a CI greater than 90 for thymus cells and killed less than 10 per cent of bone marrow cells and RBCs. (Table 1).

Anti-bone marrow antiserum

In these experiments, the important criteria for the definition of a specific antiserum are that the antiserum should be cytotoxic to (i) less than 10 per cent of cell populations which do not contain the cells which were used for the immunization, and to (ii) a maximum number of the cells which were used for the immunization. The thymus is

considered to be practically devoid of bone marrow-derived cells and therefore the HARBMAS cannot be considered specific unless it was cytotoxic to less than 10 per cent of thymus cells.

The cytotoxic activity with respect to bone marrow cells and thymus cells of the HARBMAS, both unabsorbed and absorbed three times with 10^7 thymus cells/ml of antiserum, is shown in Figure 2. The plots represent mean values from two experiments. It can be seen that, at high concentrations, the unabsorbed antiserum was highly cytotoxic towards both thymus cells and bone marrow cells. The unabsorbed HARBMAS gave a mean CI for bone marrow cells of approximately 46 at a dilution (1:32) which consistently killed less than 10 per cent of thymus cells (Figure 2b). The antiserum absorbed once, twice or three times with 10^7 thymus cells/ml of antiserum gave mean CIs for bone marrow cells of 58, 68 and 88, respectively, at dilutions (1:8, 1:4, 1:2, respectively) which consistently gave a CI which was less than 10 for thymus cells. The two experiments on which the above is based gave similar results.

In view of the above results, the HARBMAS absorbed three times with 10^7 thymus cells/ml of antiserum, diluted 1:2, was considered to be specific for bone marrow cells because it was cytotoxic towards approximately 90 per cent of bone marrow cells and an average of 2.5 per cent of thymus cells. (Table 1).

Anti-appendix antiserum

The unabsorbed HARAAS was cytotoxic towards appendix cells, thymus cells, bone marrow cells and RBCs at high concentrations. The anti-RBC activity of the antiserum was removed by absorption with 0.25 ml of packed RBCs/ml of antiserum.

The thymus and bone marrow are considered to be devoid of cells bearing appendix-specific antigens and therefore the HARAAS could not be considered specific unless it was cytotoxic towards less than 10 per cent of thymus cells and bone marrow cells.

Absorption of the HARAAS with thymus cells removed much of the anti-thymocyte activity and also some of the cytotoxicity towards appendix cells (Figure 3). The points on the plot in Figure 4 represent the cytotoxic activity of the anti-appendix antiserum for appendix cells under various conditions which resulted in less than 10 per cent of the thymus cells being killed. The highest possible CI with respect to appendix cells was achieved using the antiserum absorbed six or more times with 10^8 thymus cells/ml of antiserum.

The cytotoxicity with respect to appendix cells, thymus cells and bone marrow cells of the HARAAS absorbed once with 0.25 ml of packed RBCs/ml of antiserum and six times with 10^8 thymus cells/ml of antiserum can be seen in Figure 5. This absorbed antiserum, at a dilution of 1:16, was cytotoxic towards approximately 67 per cent of appendix cells and it consistently killed less than 10 per

cent of thymus and bone marrow cells (Table 1). Up to and including this dilution, this absorbed antiserum exhibited maximum cytotoxicity towards appendix cells (Figure 5). Results from cytotoxicity assays using the absorbed HARTAS and HARBMAS indicate that the appendix cells not lysed by the absorbed HARAAS were thymus-derived (approximately 12 per cent) and bone marrow-derived (approximately 22 per cent) (Table 1). It can therefore be concluded that the HARAAS absorbed once with 0.25 ml of packed RBCs/ml of antiserum and six times with 10^8 thymus cells/ml of antiserum, and diluted 1:16, is specific for appendix cells.

Control sera

The control pre-bleed sera exhibited slight cytotoxicity towards some of the cells used in the assays. This was removed by means of absorption with the appropriate cells.

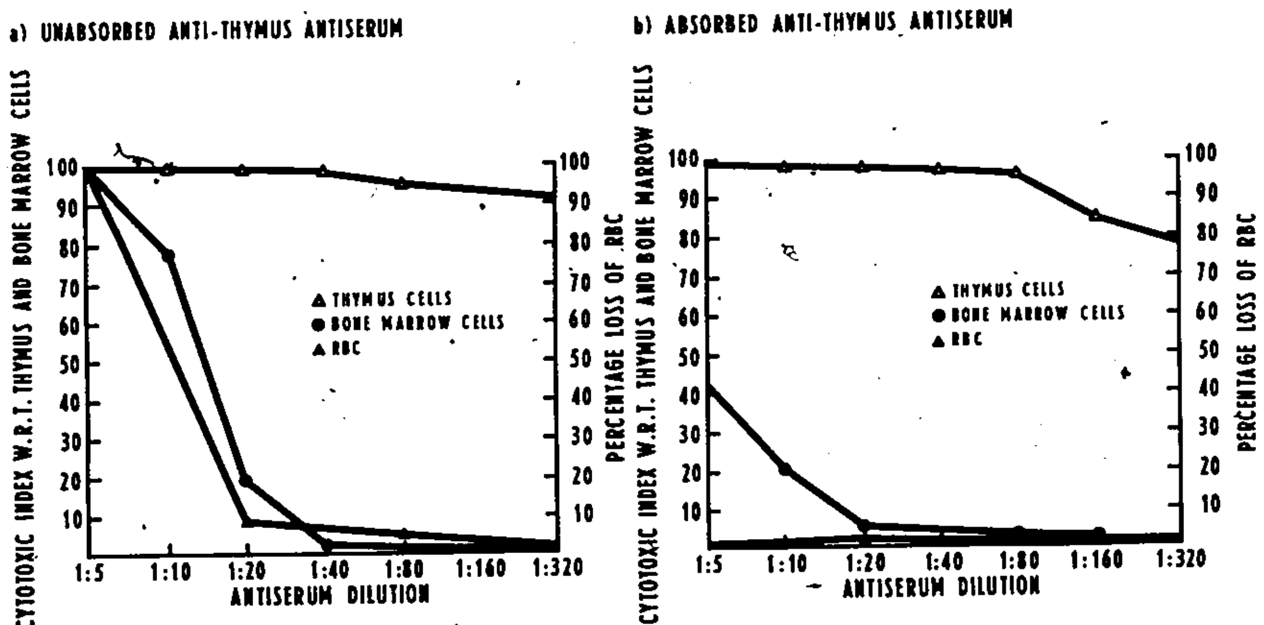

FIGURE 1

THE CYTOTOXIC ACTIVITY WITH RESPECT TO THYMUS CELLS; BONE MARROW CELLS AND RBCs OF HORSE ANTI-RABBIT THYMUS ANTISERUM, UNABSORBED AND ABSORBED (WHEN DILUTED FIVE-FOLD) WITH 0.25 ml OF PACKED RBCs/ml of ANTISERUM. n=2.

TABLE 1

THE CYTOTOXIC ACTIVITIES OF THE SPECIFIC HORSE ANTI-RABBIT THYMUS, BONE MARROW AND APPENDIX ANTISERA

Antiserum tested	n	The cytotoxic indices of the antisera with respect to the cells of the following lymphoid organs		
		Thymus	Bone marrow	Appendix
Anti-thymus	5	96.20 ± 0.66* (94-98)	2.80 ± 1.36 (0-7)	12.40 ± 2.54 (4-18)
Anti-bone marrow	8	2.50 ± 0.98 (1-9)	89.80 ± 2.15 (81-99)	21.50 ± 5.35 (5-43)
Anti-appendix	5	3.80 ± 1.39 (1-9)	3.00 ± 1.30 (1-8)	67.40 ± 4.43 (54-79)

*The results are expressed as mean values ± standard error of the mean. The figures in the brackets represent the range.

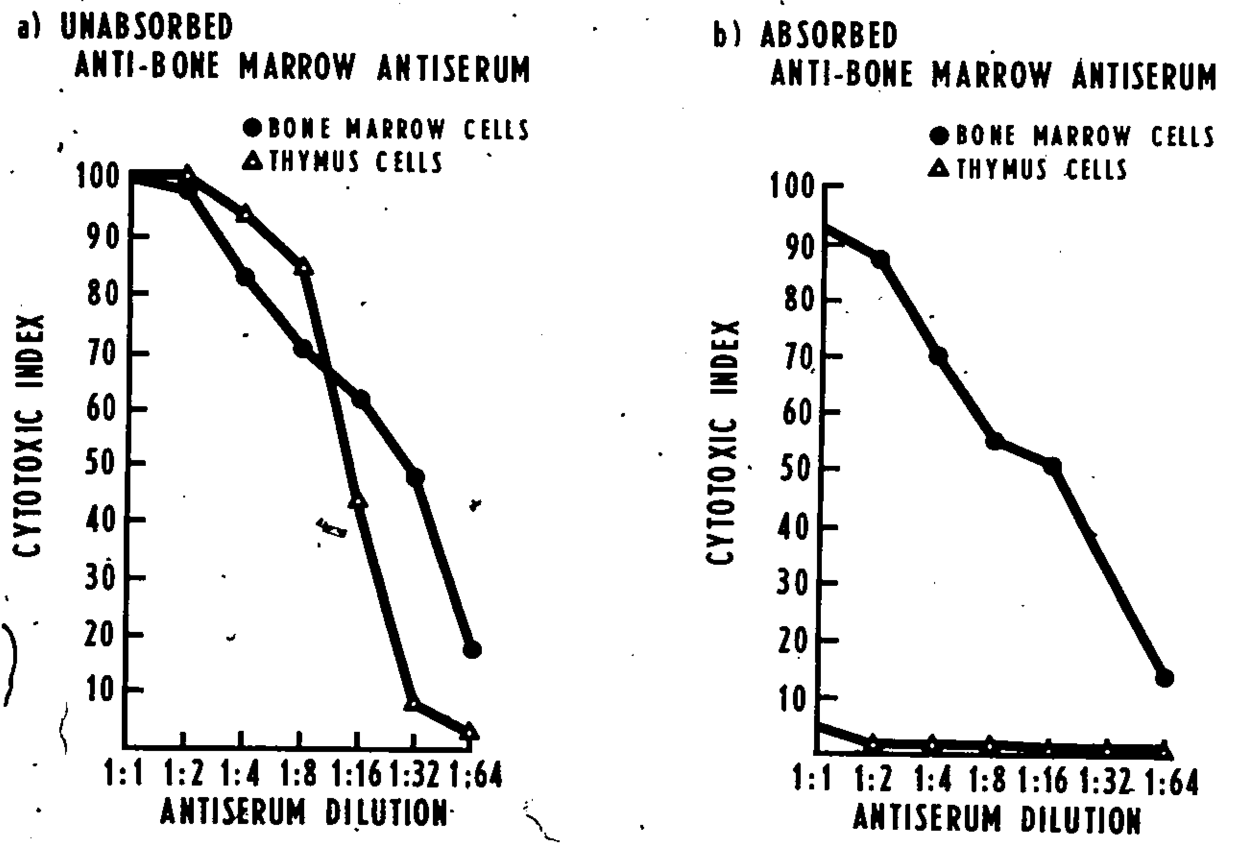

FIGURE 2

THE CYTOTOXIC ACTIVITY WITH RESPECT TO BONE MARROW CELLS AND THYMUS CELLS OF HORSE ANTI-RABBIT BONE MARROW ANTISERUM, UNABSORBED AND ABSORBED THREE TIMES WITH 5×10^7 THYMUS CELLS/ml OF ANTISERUM. n=2.

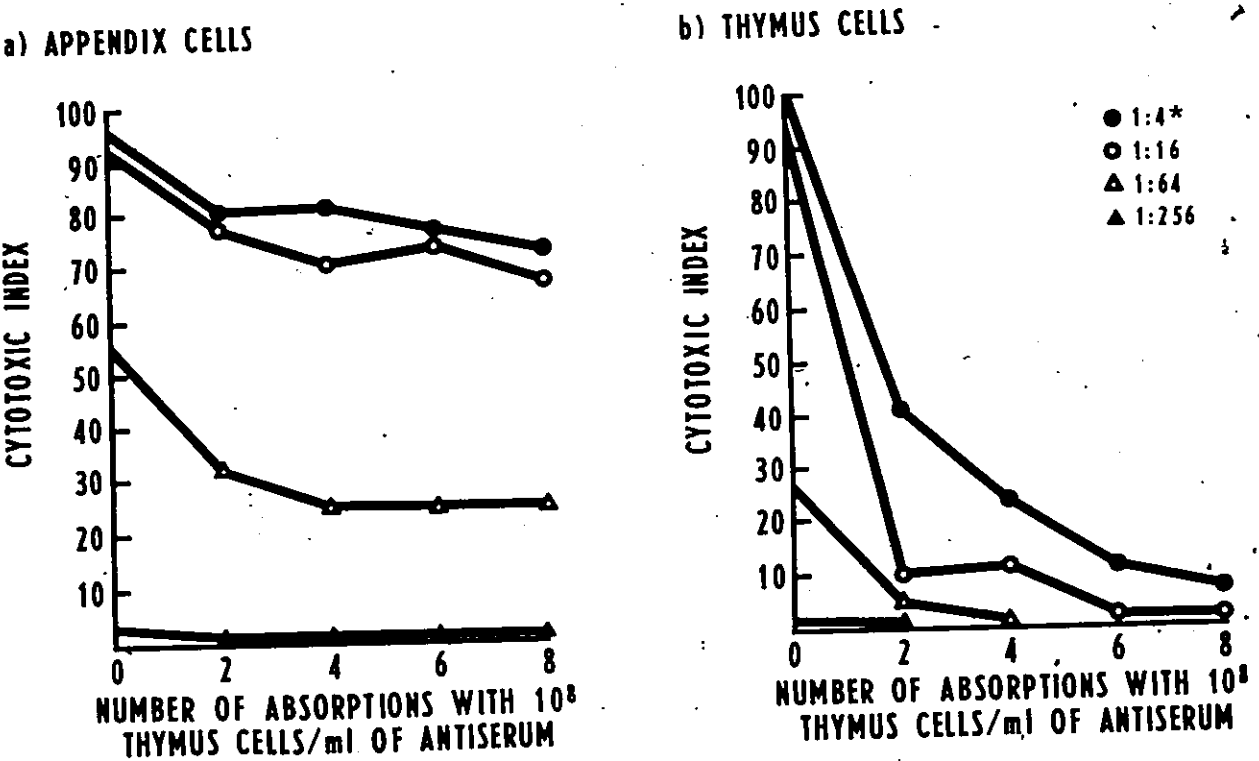

FIGURE 3

THE EFFECT OF ABSORPTION OF HORSE ANTI-RABBIT APPENDIX ANTISERUM WITH THYMUS CELLS ON THE CYTOTOXICITY OF THE ANTISERUM WITH RESPECT TO APPENDIX CELLS (a) AND THYMUS CELLS (b). n=2.

* These figures represent antiserum dilutions.

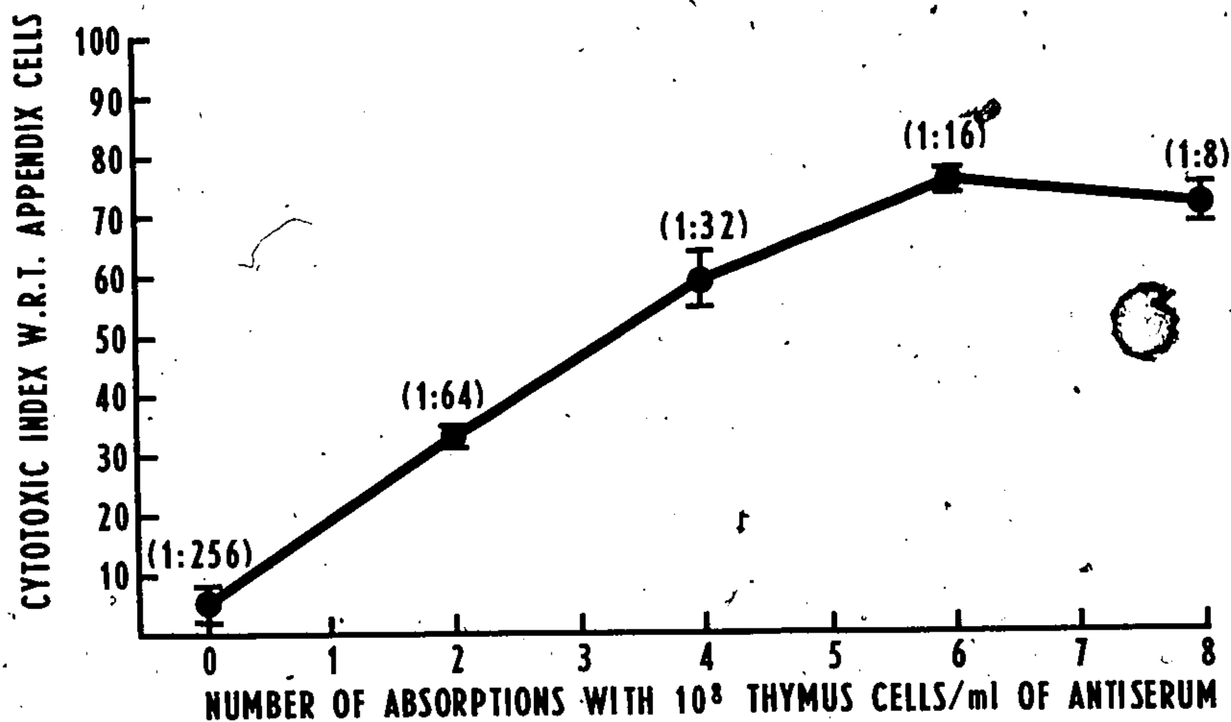

FIGURE 4

THE CYTOTOXIC ACTIVITY OF HORSE ANTI-RABBIT APPENDIX ANTISERUM AT DILUTIONS WHICH CONSISTENTLY GAVE A CYTOTOXIC INDEX FOR THYMUS CELLS OF LESS THAN 10. n=2.

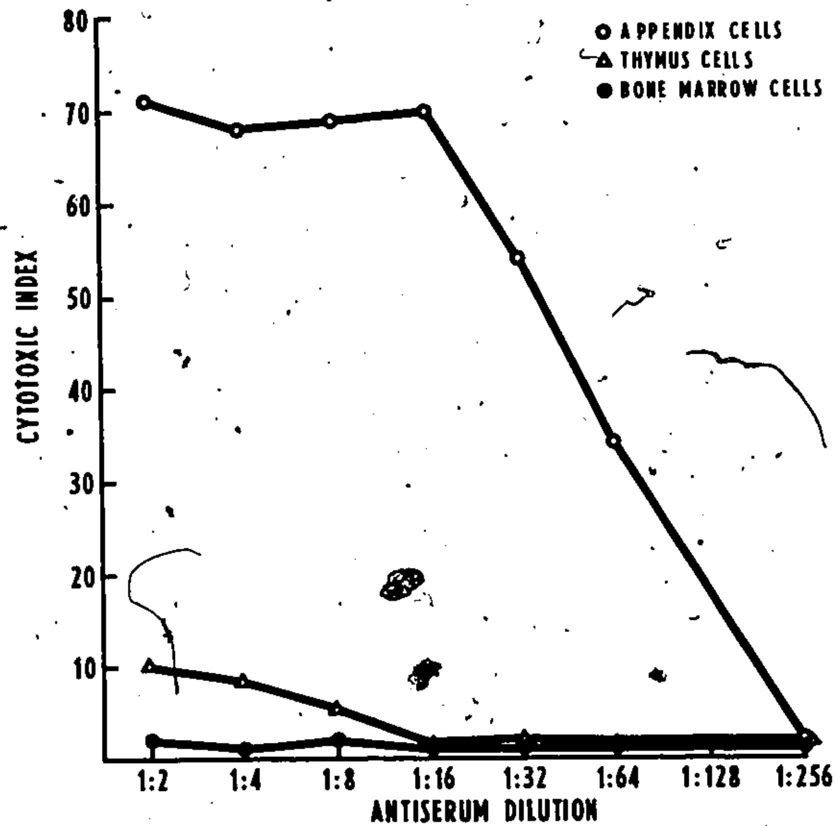

FIGURE 5

THE CYTOTOXIC ACTIVITY WITH RESPECT TO APPENDIX CELLS, THYMUS CELLS AND BONE MARROW CELLS OF HORSE ANTI-RABBIT APPENDIX ANTISERUM ABSORBED ONCE WITH 0.25 ml OF PACKED RBCs/ml OF ANTISERUM AND SIX TIMES WITH 10^8 THYMUS CELLS/ml OF ANTISERUM. n=3.

SUMMARY AND CONCLUSION

Horse antisera, obtained following immunization with rabbit thymus cells, bone marrow cells and appendix cells, were rendered specific for the respective cells by means of absorptions with the appropriate cells. The anti-thymus antiserum (diluted five-fold) required one absorption with 0.25 ml of packed RBCs/ml of antiserum. At a dilution of 1:20, this absorbed antiserum was cytotoxic to over 90 per cent of thymus cells and less than 10 per cent of bone marrow cells. It exhibited no cytotoxicity towards RBCs. The anti-bone marrow antiserum required 3 absorptions with 10^7 thymus cells/ml of antiserum. At a dilution of 1:2 this absorbed antiserum was cytotoxic to approximately 90 per cent of bone marrow cells and less than 5 per cent of thymus cells. The anti-appendix antiserum required one absorption with 0.25 ml of packed RBCs/ml of antiserum and six absorptions with 10^8 thymus cells/ml of antiserum. At a dilution of 1:16, this absorbed antiserum was cytotoxic to approximately 67 per cent of the cells found in the appendix. Approximately 4 per cent of thymus cells and 3 per cent of bone marrow cells were lysed by this antiserum. It was not cytotoxic to RBCs. The appendix cells not lysed with the anti-appendix antiserum were thymus-derived cells and bone-marrow derived cells.

These three antisera, suitably absorbed and diluted, can therefore be used for the elimination of cells bearing the antigenic markers of each of these three unique rabbit lymphoid cell subpopulations, namely, thymus-derived cells, bone marrow-derived cells and appendix-derived cells.

IV. 2. THE ELIMINATION OF PHAGOCYTIC CELLS FROM RABBIT SPLEEN CELLS

INTRODUCTION

It has been demonstrated by many investigators that no MLR occurs between populations of human stimulator and responder cells when both have been depleted of macrophages (Gordon, 1968; Alter and Bach, 1970; Twomey, Sharkey, Brown Laughter and Jordan, 1970; Levis and Robbins, 1970; Blomgren, 1977). The purpose of these experiments is to determine whether the depletion of phagocytic cells (i.e. macrophages and polymorphonuclear cells) from rabbit spleen responder and stimulator cells prevents them from exhibiting an MLR as is observed with macrophage-depleted human responder and stimulator cells.

In the experiments described below, the phagocyte depletions carried out are based on the ability of these cells to ingest carbonyl iron and, as a result, to adhere to a magnet. The efficiency of the procedures for the depletion of phagocytes from the MLR-responder and the MLR-stimulator cells is measured by the study of cell preparations stained for non-specific esterase. In the rabbit, macrophages become heavily stained whereas most polymorphonuclear cells and lymphocytes exhibit a weak stain. Furthermore, the polymorphonuclear cells can be identified by their characteristically shaped nuclei.

RESULTS

The basic method for the removal of phagocytic cells is outlined in Chapter 3. The results of attempts to remove these cells from unseparated spleen cell suspensions by means of a magnet after incubation with 4 mg and 8 mg of carbonyl iron/ml under the optimal conditions for phagocytosis are shown in Table 2.

A study of cell preparations stained for non-specific esterase revealed that, of the cells remaining in the responder and stimulator cell populations after phagocyte depletions using 4 mg of carbonyl iron/ml, an average of 1.5 per cent were macrophages and an average of 1.2 per cent were polymorphonuclear cells. These responder and stimulator cells did not exhibit a modified MLR in two out of three experiments.

After treatment with 8 mg of carbonyl iron/ml, a lower proportion of phagocytic cells remained. On average, 0.6 per cent were macrophages and 1.1 per cent were polymorphonuclear cells. However, this treatment also had no effect on the MLR response.

The next attempt was carried out using mononuclear-enriched cells obtained from the medium-Ficoll Hypaque interface following density gradient centrifugation. These cell suspensions contained a lower percentage of phagocytic cells than unseparated cells but could nevertheless

exhibit an MLR. Phagocyte depletions of these mononuclear-enriched responder and stimulator cells, using 4 mg of carbonyl iron/ml, severely limited their ability to exhibit an MLR. Of the cells remaining after treatment, an average of 0.6 per cent were macrophages and 0.8 were polymorphonuclear cells.

TABLE 2

THE EFFECT ON THE ONE-WAY MLR OF THE DEPLETION OF PHAGOCYTIC CELLS FROM UNSEPARATED RABBIT SPLEEN RESPONDER AND STIMULATOR CELLS.

Phagocyte depletions were carried out using a magnet after incubation of the cells with carbonyl iron.

Exp. no.	MLR response (cpm) after treatment with the following concentrations of carbonyl iron			Number of phagocytic cells remaining after treatment with the following concentrations of carbonyl iron											
	0 mg/ml	4 mg/ml	8 mg/ml	0 mg/ml				4 mg/ml				8 mg/ml			
	Rc + Sc[1]	Rd + Sd	Rd + Sd	Rc		Sc		Rd		Sd		Rd		Sd	
				M%[2]	P%[3]	M%	P%	M%	P%	M%	P%	M%	P%	M%	P%
1	42,600	2,990		5.5	5.0	3.5	2.5	1.0	1.0	0.5	0.5				
2	3,420	8,420		N.D.[4]	N.D.	N.D.	N.D.	0.5	3.0	1.0	<0.5				
3	13,000	12,500		3.0	3.0	3.5	2.5	2.5	0.5	3.5	2.0				
4	1,040		500	2.5	3.5	2.0	3.0					<0.5	2.0	0.5	0.5
5	34,700		49,200	2.0	3.5	1.0	3.0					<0.5	0.5	1.5	0.5
6	17,300		11,400	5.0	3.0	2.0	4.0					1.0	3.0	1.0	<0.5

[1] Rc and Sc refer to responder and stimulator (control) cells respectively which were not depleted of phagocytic cells.
Rd and Sd refer to responder and stimulator cells respectively which were depleted of phagocytic cells.

[2] M% = percentage of macrophages.

[3] P% = percentage of polymorphonuclear cells.

[4] N.D. = Not done.

TABLE 3

THE EFFECT ON THE ONE-WAY MLR OF THE DEPLETION OF PHAGOCYTIC CELLS FROM MONONUCLEAR-ENRICHED RABBIT SPLEEN RESPONDER AND STIMULATOR CELLS.

Phagocyte depletions were carried out using a magnet after incubation of the cells with carbonyl iron.

Exp. no.	MLR response (cpm) after treatment with the following concentrations of carbonyl iron.		Number of phagocytic cells after treatment with the following concentrations of carbonyl iron.							
	0 mg/ml	4 mg/ml	0 mg/ml				4 mg/ml			
	Rc + Sc[1]	Rd + Sd	Rc		Sc		Rd		Sd	
			M%[2]	P%[3]	M%	P%	M%	P%	M%	P%
1	9,860	517	3.0	2.0	3.0	2.0	N.D.[4]	N.D.	1.0	1.0
2	6,590	2,460	3.0	1.0	1.5	1.0	0.5	0.5	<0.5	<0.5
3	96,500	61,100	2.0	2.0	2.5	2.0	<0.5	1.0	<0.5	<0.5
4	20,100	3,170	3.0	2.0	4.5	0.5	1.0	1.0	1.0	<0.5
5	11,400	1,920	4.0	1.0	N.D.	N.D.	1.0	0.5	N.D.	N.D.
6	65,600	14,700	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
7	20,000	7,090	2.0	2.0	2.0	1.0	0.5	1.0	<0.5	2.0
8	20,800	3,590	6.0	3.0	3.5	1.5	1.0	1.0	N.D.	N.D.
9	12,900	3,540	4.5	1.0	N.D.	N.D.	0.5	0.5	N.D.	N.D.
10	46,000	16,800	2.5	2.5	1.5	3.0	<0.5	<0.5	0.5	1.0
11	43,100	28,300	3.5	3.0	4.5	1.5	1.0	<0.5	1.0	2.5

[1] Rc and Sc refer to responder and stimulator (control) cells respectively which were not depleted of phagocytic cells.
Rd and Sd refer to reponder and stimulator cells respectively which were depleted of phagocytic cells.

[2] M% = percentage of macrophages.

[3] P% = percentage of polymorphonuclear cells.

[4] N.D. = Not done.

SUMMARY AND CONCLUSION

The removal of phagocytic cells from mononuclear-enriched rabbit spleen cell suspensions, by means of a magnet after incubation with 4 mg of carbonyl iron/ml under the optimal conditions for phagocytosis, resulted in a much reduced MLR. On the other hand, for reasons which are not understood, a reduced MLR could not be achieved by phagocyte depletions of unseparated rabbit spleen cells.

IV. 3. THE ROLES OF THYMUS-DERIVED CELLS, BONE MARROW-DERIVED CELLS, APPENDIX-DERIVED CELLS AND PHAGOCYTIC CELLS IN THE RESPONSE OF RABBIT SPLEEN CELLS IN THE MIXED LEUCOCYTE REACTION

INTRODUCTION

The purpose of this set of experiments was to determine the roles of thymus-derived cells, bone marrow-derived cells, appendix-derived cells and phagocytic cells in the MLR response of the rabbit to allogeneic stimulation. This investigation was carried out with suspensions of spleen cells depleted of each of the above-mentioned subpopulations by means of the techniques described in IV.1 and IV.2.

RESULTS

The role of thymus-derived cells in the MLR response

The removal of thymus-derived cells from the MLR-responder spleen cell population resulted in virtual elimination of the MLR response by these cells. A typical plot of the MLR using increasing concentrations of spleen cells depleted of thymus-derived cells and control spleen cells is shown in Figure 6. The experiment was repeated four times and a comparison of the stimulation indices is shown in Table 4. The values presented in Table 4 and in all other tables concerning the MLR are taken from the ascending portions of the MLR response plots.

The specific anti-thymus antiserum in the presence of complement killed an average of 24 per cent of the spleen cells (Table 5). The results of simultaneous cytotoxicity assays carried out with thymus cells and bone marrow cells, isogeneic to the responder cells, with the same source of complement and batch of antiserum or control serum as used for the depletion procedure, are shown in Table 5. It can be seen that the conditions used for the elimination of thymus-derived cells from the responder spleen cell suspensions were such that practically all thymus-derived cells but few bone marrow-derived cells were killed.

<u>The role of bone marrow-derived cells in the MLR response</u>

Treatment of the responder spleen cell suspension with the specific anti-bone marrow antiserum and complement killed an average of 18 per cent of the spleen cells (Table 5). However, this loss did not affect the MLR-responding capacity of the residual cells. In fact, a higher MLR was exhibited by these cells reflecting an increase in the proportion of thymus-derived cells as a result of the elimination of bone marrow-derived cells (Figure 7, Table 6). The results of parallel cytotoxicity assays indicate that the conditions used for the elimination of bone marrow-derived cells from responder spleen cell suspensions were such that practically all bone marrow-derived cells but few thymus-derived cells were killed (Table 5).

The role of appendix-derived cells in the MLR response

The specific anti-appendix antiserum killed a larger proportion of spleen cells than either of the other two antisera. In the presence of complement it was cytotoxic towards an average of 32 per cent of spleen cells (Table 5). However, the elimination of these appendix-derived cells from the MLR-responder cell population had little effect on the capacity of the remaining cells to exhibit an MLR (Figure 8, Table 7). The results of simultaneous cytotoxicity assays, shown in Table 5, indicate that the conditions used for the elimination of spleen cells bearing appendix-specific antigens were such that few thymus-derived cells and bone marrow-derived cells were killed.

The role of phagocytic cells in the MLR response

The effect on the MLR of the removal of phagocytic cells from the responder and/or the stimulator cell populations can be seen in Table 8. The elimination of phagocytic cells from the MLR-responder cells resulted either in only partial or no reduction in the MLR response, indicating that the MLR responding cells are not phagocytic. However, the MLR response was significantly reduced (by 34-95 per cent) following elimination of the phagocytic cells from both the responder and the stimulator cell populations ($p= 0.02$) (Figure 9). The poor MLR response observed when both stimulator and responder cells were depleted of phagocytes cannot be attributed to the elimination of phagocytic stimulating cells since the MLR was neither greatly nor consistently affected when only the stimulator cells were depleted of phagocytic cells (Table 8).

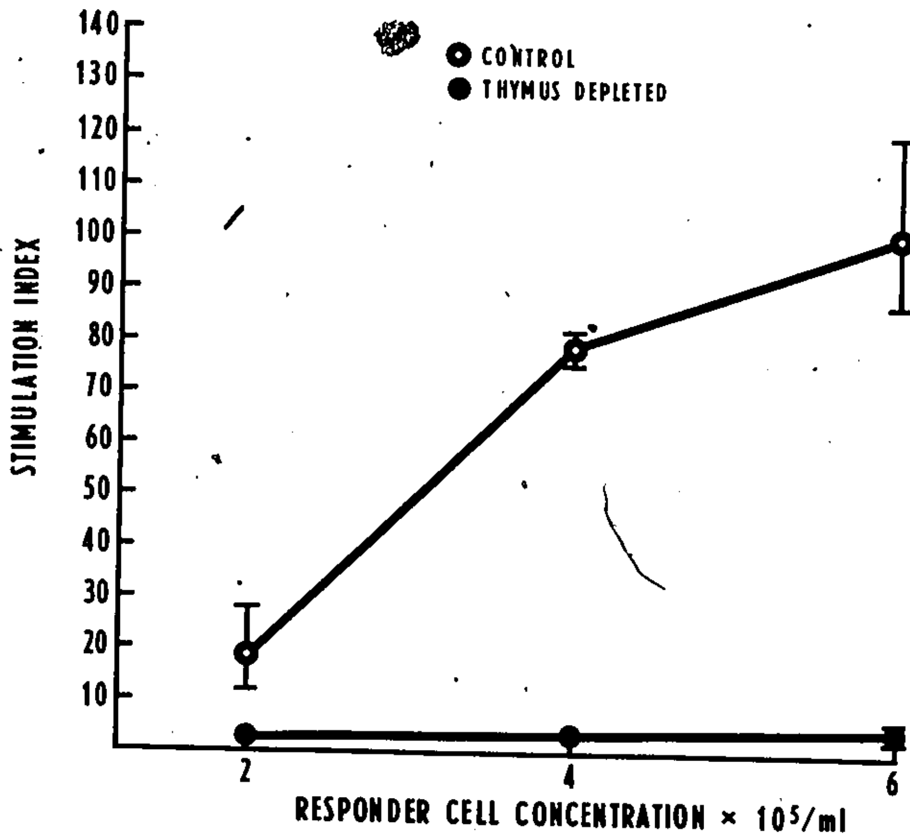

FIGURE 6

THE EFFECT ON THE ONE-WAY MLR OF THE DEPLETION OF THYMUS-DERIVED CELLS FROM RABBIT SPLEEN RESPONDER CELLS.

TABLE 4

THE EFFECT ON THE ONE-WAY MLR OF THE DEPLETION OF THYMUS-DERIVED CELLS FROM RABBIT SPLEEN RESPONDER CELLS.

Experiment number	Concentration of responder cells	MLR response (stimulation index)	
		Control	Thymus depleted
1	4×10^5/ml	13.60	1.11
2	4×10^5/ml	78.60	2.49
3	4×10^5/ml	44.70	2.34
4	4×10^5/ml	6.06	1.24

TABLE 5

THE CYTOTOXIC ACTIVITIES OF THE HORSE ANTI-RABBIT THYMUS, BONE MARROW AND APPENDIX ANTISERA WHICH WERE USED, IN THE PRESENCE OF COMPLEMENT, FOR THE ELIMINATION OF SPECIFIC SUBPOPULATIONS OF SPLEEN MLR-RESPONDER CELLS.

Antiserum tested	n	The cytotoxic indices (mean ± S.E.M.) of the antisera with respect to the cells of the following lymphoid organs			
		Thymus	Bone marrow	Appendix	Spleen
Anti-thymus	4	95.80 ± 0.63	3.50 ± 1.50	N.A.[1]	23.80 ± 3.42
Anti-bone marrow	4	1.50 ± 0.49	91.30 ± 3.75	N.A.	18.30 ± 3.50
Anti-appendix	4	5.50 ± 2.53	1.75 ± 0.48	69.00 ± 5.34	31.80 ± 4.01

1 N.A. means not applicable

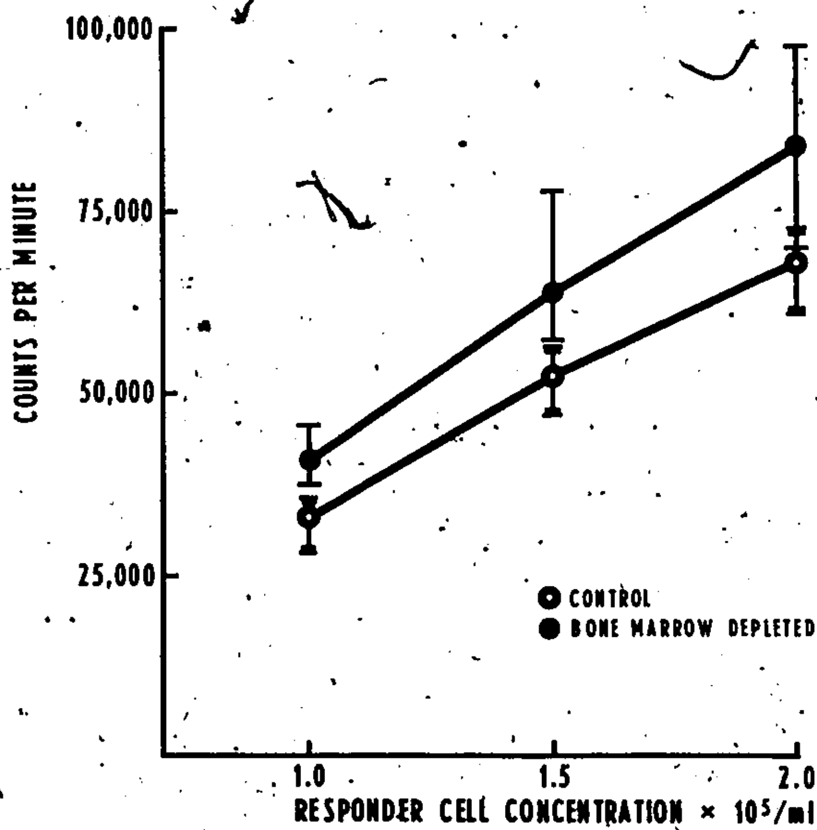

FIGURE 7

THE EFFECT ON THE ONE-WAY MLR OF THE DEPLETION OF BONE MARROW-DERIVED CELLS FROM RABBIT SPLEEN RESPONDER CELLS.

TABLE 6

THE EFFECT ON THE ONE-WAY MLR OF THE DEPLETION OF BONE MARROW-DERIVED CELLS FROM RABBIT SPLEEN RESPONDER CELLS.

Experiment number	Concentration of responder cells	MLR response (cpm)	
		Control	Bone marrow depleted
1	3.3×10^5/ml	74,000	86,200
2	1.6×10^5/ml	1,570	2,560
3	1.6×10^5/ml	52,800	64,700
4	3.0×10^5/ml	14,900	24,400

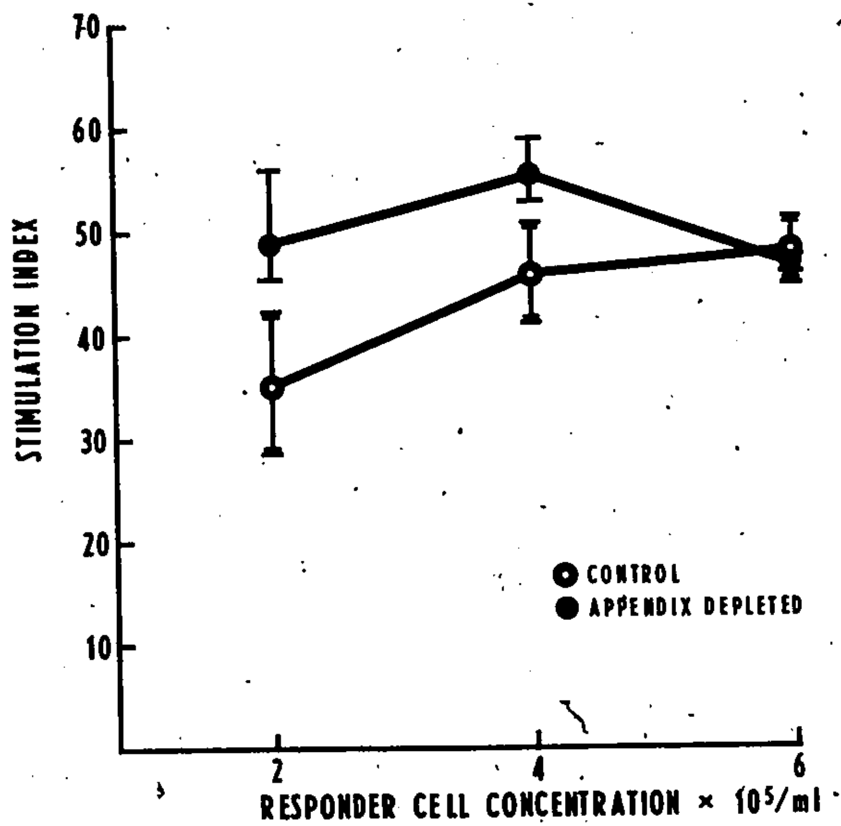

FIGURE 8

THE EFFECT ON THE ONE-WAY MLR OF THE DEPLETION OF APPENDIX-DERIVED CELLS FROM RABBIT SPLEEN RESPONDER CELLS.

TABLE 7

THE EFFECT ON THE ONE-WAY MLR OF THE DEPLETION OF APPENDIX-DERIVED CELLS FROM RABBIT SPLEEN RESPONDER CELLS.

Experiment number	Concentration of responder cells	MLR response (stimulation index)	
		Control	Appendix depleted
1	4×10^5/ml	2.10	2.88
2	2×10^5/ml	35.50	46.10
3	4×10^5/ml	90.50	81.80
4	2×10^5/ml	7.85	9.04

TABLE 8

THE EFFECT ON THE ONE-WAY MLR OF THE DEPLETION OF PHAGOCYTIC CELLS FROM RABBIT SPLEEN RESPONDER AND/OR STIMULATOR CELLS.

Exp. No.	MLR response (cpm)				Percentage of phagocytic cells							
	Rc+Sc[1]	Rd+Sc	Rd+Sd	Rc+Sd	Rc		Sc		Rd		Sd	
					M%[2]	P%[3]	M%	P%	M%	P%	M%	P%
1	138,000	116,000			N.D.[4]	N.D.	N.D.	N.D.	N.D.	N.D.		
2	108,000	51,700			6.0	1.0	2.0	3.5	0.5	0.5		
3	52,400	51,200			2.0	3.5	5.0	2.0	1.0	<0.5		
4	9,860		517		3.0	2.0	3.0	2.0	N.D.	N.D.	1.0	1.0
5	6,590		2,460		3.0	1.0	1.5	1.0	0.5	0.5	<0.5	<0.5
6	96,500		61,100		2.0	2.0	2.5	2.0	<0.5	1.0	<0.5	<0.5
7	20,100		3,170		3.0	2.0	4.5	0.5	1.0	1.0	1.0	<0.5
8	11,400		1,920		4.0	1.0	N.D.	N.D.	1.0	0.5	N.D.	N.D.
9	65,600		14,700		N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
10	67,800			76,800	4.0	1.0	N.D.	N.D.			N.D.	N.D.
11	97,600			58,200	2.0	2.0	3.0	2.0			1.5	1.5
12	19,200			7,090	2.0	5.0	2.0	0.5			<0.5	<0.5
13	23,200			30,400	2.5	1.5	1.0	4.0			<0.5	0.5
14	20,000	13,100	7,090	17,200	2.0	2.0	2.0	1.0	0.5	1.0	<0.5	2.0
[illegible]	20,800	14,900	3,590	7,230	6.0	3.0	3.5	1.5	1.0	1.0	N.D.	N.D.
16	12,900	14,900	3,540	11,300	4.5	1.0	N.D.	N.D.	0.5	0.5	N.D.	N.D.
17	46,000	36,100	16,800	29,500	2.5	2.5	1.5	3.0	<0.5	<0.5	0.5	1.0
18	43,100	40,300	28,300	26,300	3.5	3.0	4.5	1.5	0.5	<0.5	1.0	2.5

[1] Rc and Sc refer to responder and stimulator (control) cells respectively which were not depleted of phagocytic cells. Rd and Sd refer to responder and stimulator cells respectively which were depleted of phagocytic cells.

[2] M% = percentage of macrophages. [3] P% = percentage of polymorphonuclear cells. [4] N.D. = Not done.

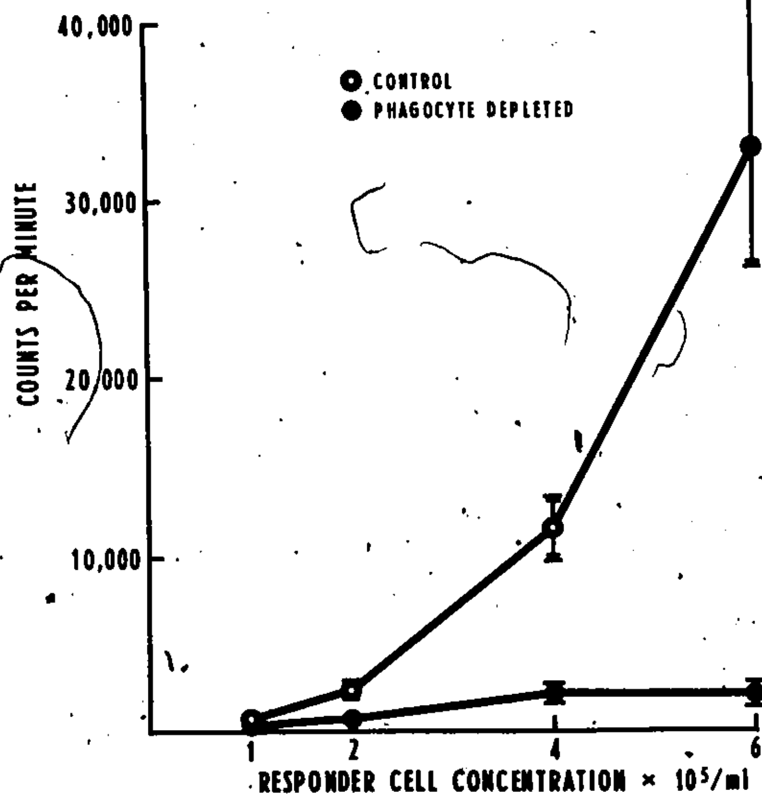

FIGURE 9

THE EFFECT ON THE ONE-WAY MLR OF THE DEPLETION OF PHAGOCYTIC CELLS FROM RABBIT SPLEEN RESPONDER AND STIMULATOR CELLS.

SUMMARY AND CONCLUSIONS

The roles of thymus-derived cells, bone marrow-derived cells, appendix-derived cells and phagocytic cells in the MLR response of the rabbit were determined using spleen cells depleted of each of these cell subpopulations. The elimination of thymus-derived cells from the MLR-responder cells abolished the response whereas no detrimental effect on the MLR was observed when bone marrow-derived cells and appendix-derived cells were eliminated from the responder cell population. It is concluded that thymus-derived cells are the primary responding cells in the MLR and that bone marrow-derived cells and appendix-derived cells are not essential for this reaction.

The elimination of phagocytic cells from both the responder and the stimulator cell populations resulted in a much reduced MLR response. This was not due to the removal of responding or stimulating phagocytic cells since their elimination from only the responder or only the stimulator cells resulted in only a partial or no decrease in the MLR response. These experiments demonstrate that phagocytic cells are essential for the MLR of rabbit spleen cells. They play a non-specific accessory role in this reaction since they can be provided by either the responder or the stimulator population of cells.

CHAPTER V

DISCUSSION

In the last two decades the allograft rejection phenomenon has become a major area of interest as a result of the overwhelming evidence in support of the immunological basis for allograft rejection. Much progress has been made towards understanding the allograft rejection reaction and as a result tissue transplants have met with increasing success. Surgical techniques have been developed to such an extent that the function and survival of implanted allografts are no longer subject to surgical limitations. Today it is recognized that the primary cause for the cessation of function and ultimate rejection of the graft is the immune response of the host to the allograft.

Laboratory assays have been established and developed for predicting graft survival and results from these tests have been used to select appropriate donors for designated recipients. The two assays most frequently used are the lymphocytotoxicity assay, which is also known as tissue typing, and the MLR, which is considered to be the _in vitro_ counterpart of the _in vivo_ allograft rejection reaction. The lymphocytotoxicity assay, which is a serological test, identifies antigenic differences between the cells of the potential allograft donor and the cells of the designated recipient. However, many of the serologically defined (SD)

antigens (over 90 have now been identified) are "lesser" or "weak" antigens and the absence of a complete match between SD antigens of the donor and the recipient does not necessarily indicate potential rejection. Furthermore, allograft rejection sometimes occurs when the host and donor exhibit indentical SD antigens. The advantage of this assay is that it only takes a few hours to perform.

The MLR, unlike the lymphocytotoxicity assay, measures functional properties of the cells concerned. In the MLR, the cells of the potential allograft recipient are cultured with the cells of the potential donor, the latter having been treated with either mitomycin-C or X-irradiation in order to prevent their proliferation. The degree of the proliferative response is considered to be indicative of the antigenic dissimilarity between the responder and stimulator cells and therefore the absence of a response indicates a good match. The survival of allografts in patients generally corresponds to expectations based on the results of the MLR.

The main disadvantage associated with the MLR assay is its 4 to 5 day duration. This is considered unsatisfactory on occasions when a delay in the implantation of the allograft cannot be tolerated. However, these occasions are rare since in practice the majority of transplants involve kidneys from living donors and the recipient can be kept alive by dialysis while awaiting the results of the MLR assays.

The immunologically-mature outbred rabbit was used for the experiments carried out for this thesis. This animal is exceedingly useful for the investigation of immune phenomena for many reasons. Since the rabbit is a large animal it is possible to obtain large numbers of cells from each of the lymphoid organs and from the blood and therefore to carry out a large number of assays with the cells of one animal. It is also possible to perform a large number of assays on the circulating cells of the rabbit over a period of time. Furthermore, since the rabbit, unlike the inbred laboratory mouse, rat and guinea pig, is an outbred animal, results obtained with the former should be more representative of the entire species than results obtained with the latter. In addition, the rabbit is more closely related to the human on a physio-anatomic basis than the mouse, rat and guinea pig, since neither the spleen nor the lymph nodes are haemopoietic in the mature human and rabbit, in contrast to those of the mouse, rat and guinea pig.

Since the MLR of the rabbit was first described by Chapman and Dutton in 1965, a number of attempts have been made to correlate the rabbit MLR with skin graft survival (Harrison, Wei and Ahie, 1971; Chai and Lerner, 1975; Milthorp, Belanger and Richter, 1979). It has been shown that the cells of allograft recipients exhibit an accelerated and enhanced MLR upon further exposure to cells bearing the antigens of the allograft which is comparable to the secondary MLR described in II.1c (Harrison, Wei and Ahie, 1971; Milthorp, Belanger and Richter, 1979). The

ability of the circulating cells and the cells of each of the lymphoid organs of the rabbit to respond or stimulate in the MLR has been determined but the roles of the leucocyte subpopulations in this reaction have not yet been systematically studied.

The purpose of this investigation was to determine the roles of thymus-derived cells, bone marrow-derived cells, appendix-derived cells and phagocytic cells in the response of rabbit cells in the MLR. The results of this investigation indicate that: (i) thymus-derived cells are essential for the blastogenic response in the MLR; (ii) the response is not given by bone marrow-derived or appendix-derived cells; and (iii) phagocytic cells are required for the MLR and they can be supplied by either the responder of the stimulator cells, which can be either untreated or mitomycin-C treated.

The cells which respond in the MLR of the rabbit were shown in this investigation to be thymus-derived. Similar results were reported by Sheppard, Sell, Poler and Redelman (1976) who observed that the MLR-responding cells of rabbit peripheral blood were killed by treatment with anti-thymocyte antiserum (ATS) and complement. However, their results are subject to some doubt because the cytotoxic activity of their ATS towards cells of non-thymic origin was not defined. Their ATS, which was cytotoxic to "virtually all thymocytes", was not cytotoxic to 50 per cent of spleen cells which included most (80 to 100 per cent)

of the splenic plaque-forming (B) cells to sheep red blood cells subsequent to immunization with these antigens. The horse anti-rabbit thymus antiserum (HARTAS) used in the experiments described in this thesis, which is cytotoxic to virtually all thymocytes and only 4 per cent of the cells of the bone marrow, was cytotoxic to only 24 per cent of spleen cells. Despite this, the MLR response of spleen cells was almost obliterated following treatment with HARTAS and complement. It therefore seems likely that the ATS used by Sheppard and his colleagues killed not only thymus-derived cells but other types of cells as well.

The results of the majority of studies carried out with other animal species implicate T cells as the primary, if not the only, cells capable of responding with blastogenesis and mitosis in the MLR. The results reported in this thesis concerning the rabbit are in agreement with those obtained with the human (Lohrmann, Novikovs and Graw, 1974; Chess, MacDermott, Sondel and Schlossman, 1974; Potter and Moore, 1977; Blomgren, 1977), mouse (Mosier and Cantor, 1971; Gorczynski and Rittenberg, 1974; Rollinghof, Pfizenmeier, Trostman and Wagner, 1975; Berman, Puryear and Argyris, 1976), rat (Wilson, Silvers and Nowell, 1967; Johnston and Wilson, 1970) and chicken (Alm, 1971; Weber, 1970) which indicate that the cells which respond in the MLR are thymus-derived (T) cells and not B cells.

It is interesting to note that although rabbit thymus-derived cells respond well in the MLR, the endogenous thymus cells of the rabbit do not respond in this reaction (Chapman and Dutton, 1965; Ozer and Waksman, 1974; Milthorp and Richter, 1979). The thymocytes of the human (Schwartz, 1966; Han, Minowada, Subramanian and Sinks, 1976), the mouse (Blomgren and Svedmyr, 1971; Berman, Puryear and Argyris, 1976), and rat (Knight and Thorbecke, 1971) are able to respond in the MLR. It would therefore appear that the thymus cells of the rabbit may be unable to respond because they are less mature than the thymus cells of the human, mouse and rat.

Bone marrow-derived cells and appendix-derived cells were shown in this investigation not to be essential for the rabbit MLR since no detrimental effect on the MLR was seen when these cells were eliminated from the responder cell population. In fact, the blastogenic response of the residual cells was usually greater than that obtained with the untreated cells, a finding which can be attributed to the an actual increase in the proportion of T cells in the responder cell population.

A number of investigators have presented evidence favouring some involvement of B cells in the MLR. A proportion of lymphoblasts in the human MLR were shown to be immunoglobulin-bearing cells by Phillips and Weisrose (1974). These cells were assumed to be B cells. Wagner (1972) reported that spleen cells of congenitally athymic

mice proliferated in the MLR although to a much lesser degree than did spleen cells of normal mice and attributed this response to splenic B cells. von Boehmer (1974) observed that mouse spleen cells depleted of T cells are able to proliferate in the MLR in the presence of non-replicating mitomycin-C treated cells. Piquet and Vassalli (1972) also reported B cell proliferation in the mouse MLR. von Boehmer (1974) and Piquet and Vassalli (1972) both suggested that T cells recruit some B cells to proliferate in the mouse MLR, probably via the release of yet undefined lymphokines. However, since less than 5 per cent of the cells in mitosis at the peak of the mouse MLR reaction are B cells (Andersson, Nordling and Hayry, 1973), it may be concluded that B cells play only a minor role in the MLR response.

In this investigation, both polymorphonuclear cells and macrophages were removed by the magnet after carbonyl iron ingestion and for this reason the procedure is described as a phagocyte depletion and not as a macrophage depletion. The elimination of phagocytic cells or adherent cells from heterogeneous human cell suspensions has usually been carried out following depletion of polymorphonuclear cells using the Ficoll Hypaque density gradient separation technique. Therefore the phagocytic or adherent cells removed from these mononuclear-enriched cell preparations are mainly macrophages. It was desirable, for comparative purposes, to eliminate polymorphonuclear cells from rabbit spleen cells.

Unfortunately, it was not possible to eliminate the polymorphonuclear cells of the rabbit using Ficoll Hypaque. Differential counts of cell preparations, stained for non-specific esterase, of unseparated cells and cells obtained at the medium-Ficoll Hypaque interface are shown in Table 9. It can be seen that using this technique over two-thirds of the polymorphonuclear cells were removed but many macrophages were also removed. The ratio of these cells to one another in the cell fraction obtained at the interface was only marginally changed in favour of macrophages.

Unseparated spleen responder and stimulator cells subjected to phagocyte depletion procedures consistently responded in the MLR. However, mononuclear-enriched cell preparations, subjected to identical phagocyte depletion procedures, exhibited a markedly reduced MLR. The reason for these dichotomous results is not understood since essentially the same number of esterase positive cells were observed following phagocyte depletion in the preparations of unseparated cells as in the preparations of mononuclear-enriched cells. It is of interest that Kim and Herscowitz (1978) encountered similar problems with the depletion of macrophages in their investigation of the response of rabbit spleen cells to T cell mitogens. In their experiments the blastogenic response was not affected by the removal of adherent cells; however, the response was abrogated following elimination of macrophages using an anti-macrophage antiserum in the presence of complement.

TABLE 9

DIFFERENTIAL CELL COUNTS OF UNSEPARATED SPLEEN LEUCOCYTES AND SPLEEN LEUCOCYTES OBTAINED FROM THE MEDIUM-FICOLL HYPAQUE INTERFACE FOLLOWING DENSITY GRADIENT CENTRIFUGATION.

Experiment number	Unseparated cells			Interface cells		
	Lymphocytes (%)	PMN[1] cells (%)	Macrophages (%)	Lymphocytes (%)	PMN cells (%)	Macrophages (%)
1	88.0	9.5	2.5	95.0	2.5	2.5
2	90.5	5.5	4.0	95.5	2.5	2.0
3	92.5	1.5	6.0	98.0	1.0	1.0
4	91.5	3.0	5.5	94.0	1.0	3.0
5	92.0	3.0	5.0	98.5	<0.5	1.5
Mean ± SEM[2]	91.10 ± 0.80	4.50 ± 1.41	4.60 ± 0.62	96.20 ± 0.87	1.40 ± 0.48	2.00 ± 0.35

1. PMN cells = polymorphonuclear cells.
2. SEM = standard error of the mean

The results of this investigation indicate that phagocytic cells are essential for the rabbit MLR since the removal of these cells from both the responder and the stimulator cell populations resulted in a much reduced MLR. This was not due to the removal of responding or stimulating phagocytic cells since phagocyte depletion from only the responder or only the stimulator populations resulted either in a partial or in no decrease in the MLR response. It is concluded that the phagocytic cells of the rabbit play a non-specific helper role in this reaction. These results are in agreement with those of Twomey, Sharkey, Brown, Laughter and Jordan (1970), Berlinger, Lopez and Good (1970), Levis and Robbins (1970) and Blomgren (1977) concerning the role of macrophages in the human MLR.

The magnitude of the human MLR response is independent of the number of macrophages when they are present above a threshold concentration (Twomey, Sharkey, Brown, Laughter and Jordan, 1970; Alter and Bach, 1970; Rode and Gordon, 1970). Rode and Gordon (1970) have suggested that the threshold concentration for these cells is approximately 1 to 2 per cent. The results of the experiments described here indicate that the threshold concentration for phagocytic cells is the same in the rabbit MLR.

The requirement for phagocytic accessory cells in the rabbit MLR may explain the recent findings of Milthorp and Richter (1979). These investigators noted that the

circulating white blood cells (WBC) of a minority of rabbits (18 per cent) failed to respond in the MLR upon stimulation with allogeneic WBC. These cells cannot be described as "non-responders" since they responded very well when stimulated with allogeneic spleen cells. Their results suggest that all rabbits possess circulating MLR-responding cells but that a minority of them lack the necessary accessory cells in the circulation.

Macrophages have been shown to be highly stimulatory in the MLRs of many species. Greineder and Rosenthal (1975) and Oehler and Herberman (1977) suggested that macrophages are the predominant stimulators in the MLRs of the guinea pig and rat, respectively. In their experiments, stimulator cells depleted of macrophages were unable to stimulate even in the presence of macrophages syngeneic to the responder cells. Macrophages are also highly stimulatory in the MLR of the mouse but they are not solely responsible for stimulation in this species (Schirrmacher, Pena-Martinez and Festenstein, 1975). There appears to be some controversy concerning the ability of human macrophages to stimulate in the MLR. Rode and Gordon (1974) have reported that macrophages are ten times more stimulatory than lymphocytes in the human MLR. If this were the case, the removal of macrophages from the stimulating cell population would result in a decrease in the MLR response. This was not observed in the experiments of Twomey, Sharkey, Brown, Laughter and Jordan (1970), Berlinger, Lopez and Good (1976) and Blomgren (1977). The results of the investigation described in this

thesis do not indicate that the phagocytic cells of the rabbit are stimulatory but the results are not sufficiently consistent for conclusions concerning this to be drawn.

Macrophages appear to be involved in T cell activation in many reactions. For example, it has been observed that macrophages are required for the T cell response to mitogens, antigens, and allogeneic cells of the human (Hersh and Harris, 1968; Lake, Bice, Schwartz and Salvaggio, 1971; Lohrmann, Novikovs and Graw, 1974; Rosenstreich, Farrar and Dougherty, 1976; Mookerjee, 1977; Blomgren, 1977) to antigens in the guinea pig (Seeger and Oppenheim, 1970) and to mitogens in the rat (Keller, 1974) and rabbit (Kim and Herscowitz, 1968). The results of this investigation indicate that macrophages are also required for the activation of T cells in the rabbit MLR.

In summary, the cells involved in the blastogenic response to allogeneic stimulation in the rabbit MLR, i.e. thymus-derived cells, are ontogenically analogous to those involved in the human, mouse, rat and chicken MLRs. Bone marrow-derived cells and appendix-derived cells do not participate in the rabbit MLR to any significant degree. Phagocytic cells do not in themselves respond in the rabbit MLR but exhibit an essential though non-specific accessory function which permits the reaction to occur.

CHAPTER VI

CONTRIBUTION TO KNOWLEDGE

1. The candidate has demonstrated that the cells which respond in the MLR of the rabbit are thymus-derived (T) cells.

2. The candidate has demonstrated that neither bone marrow-derived cells nor appendix-derived cells are essential for the MLR response of the rabbit.

3. The candidate has demonstrated that phagocytic cells are essential for the rabbit MLR and that these cells play a non-specific accessory role in this reaction.

CHAPTER VII

BIBLIOGRAPHY

Ablin, R.J., and Morris, A.J., (1973). Transplantation 15, 415.

Adler, W.H., Takiguchi, T., Marsh, B., and Smith, R.T., (1970). J. Immunol. 104, 984.

Aiuti, F., and Wigzell, H., (1973a). Clin. Exp. Immunol. 13, 171.

Aiuti, F., and Wigzell, H., (1973b). Clin. Exp. Immunol. 13, 183.

Alm, G.V., (1971). Int. Arch. Allergy 41, 345.

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

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

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

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

Asantila, T., Vahala, J., and Toivanen, P., (1974). Immunogenetics 2, 272.

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

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

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

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

Bach, F.H., Segall, M., Zier, S.A., Sondel, P.M., Alter, B.J., and Bach, M.L., (1973). Science 180, 403.

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, 927.

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

Bain, B., Vas, M.R., and Lowenstein, L., (1963). Fed. Proc. 22, 428.

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

Balch, C.M., Wilson, C.B., Lee, S., and Feldman, J.D., (1973). J. Exptl. Med. 138, 1584.

Behelak, Y., and Richter, M., (1972). Cell. Immunol. 4, 197.

Bell, C., and Wigzell, H., (1977). Eur. J. Immunol. 7, 726.

Berlinger, N.T., Lopez, C., and Good, R.A., (1976). Nature 260, 145.

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

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

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

Boyum, A., (1968). Scand. J. Clin. Lab. Invest. Supplement 21, 97.

Cassen, B., Hitt, J., and Hays, E.F., (1958). J. Lab. Clin. Med. 52, 778.

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

Chai, C.K., (1974). Immunogenetics 1, 126.

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

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

Cheers, C., and Sprent, J., (1973). Transplantation 15, 336.

Chess, L., MacDermott, R.P., and Schlossman, S.F., (1974). J. Immunol. 113, 1113.

Chess, L., MacDermott, R.P., Sondel, P.M., and Schlossman, S.F., (1974). In Progress in Immunology II, Vol. 3, L. Brent and J. Holborow, Eds. North Holland Publishing Company, Amsterdam, p. 125.

Claman, H.M., Chaperon, E.A., and Triplett, R.F., (1966). Proc. Soc. Exp. Biol. Med. 122, 1167.

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.

Colley, D.G., Malakian, A., and Waksman, B.H., (1970). J. Immunol. 104, 585.

Coombs, R.R.A., Gurner, B.W., Wilson, A.B., Holm, C., and Lindgren, B., (1970) Int. Arch. Allergy 39, 658.

Cooper, M.D., Lawton, A.R., and Kincade, P.W., (1972). Clin. Exp. Immunol. 11, 143.

Cooper, M.D., Perey, D.Y., Gabrielsen, A.E., Sutherland, D.E.R., McKneally, M.F., and Good, R.A., (1968). Int. Arch. Allergy 33, 65.

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

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

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

Fanger, M.W., Pelley, P.P., and Reese, A.L., (1972). J. Immunol. 109, 294.

Fathman, C.G., Handwerger, B.S., and Sachs, D.H., (1974). J. Exptl. Med. 140, 853.

Fradelizi, D.P., Chou, C.T., Cinader, B., and Dubiski, S., (1973). Cell. Immunol. 7, 484.

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

Glick, B., Chang, T.S., and Jaap, R.G., (1954). Poultry Science 35, 224.

Golub, E.S., (1977). In the Cellular Basis of the Immune Response. Sinauer associates, Inc., Sunderland, Mass. p. 34.

Good, R.A., and Gabrielson, A.E., (Ed.) (1964). The Thymus in Immunobiology. Harper and Row, New York.

Gorczynski, R.M., and Rittenberg, M.B., (1974). J. Immunol. 112, 47.

Gordon, J., (1968). Proc. Soc. Exp. Biol. Med. 127, 30.

Greaves, M.F., Owen, J.J.T., and Raff, M.C., (1973). In T and B Lymphocytes. Excerpta Medica, Amsterdam.

Greineder, D.K., and Rosenthal, A.S., (1975). J. Immunol. 114, 1541.

Guilano, V.J., Jasin, H.E., Hurd, E.R., and Ziff, M., (1974). J. Immunol. 112, 1494.

Haasz, R., and Richter, M., (1972). Path. Microbiol. 38, 313.

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

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

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

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

Hayry, P., (1976). Immunogenetics 3, 417.

Hayry, P., and Andersson, L.C., (1973). Transplant. Proc. 5, 1967.

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

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

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

Hirschhorn, K., Bach, F.H., Kolodny, R.L., Firschein, I.L., and Hashem, N., (1963). Science 142, 1185.

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

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

Jones, A.L., (1966). Transplantation 4, 337.

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

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

Keller, R., (1975). Cell. Immunol. 17, 542.

Kim, K.J., and Herscowitz, H.B., (1978). J. Reticuloendothelial. Soc. 23, 75.

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

Kuntz, M.M., Innes, J.B., and Weksler, M.E., (1976). J. Exptl. Med. 143, 1042.

Lake, W.W., Bice, D., Schwartz, H.J., and Salvaggio, J., (1971). J. Immunol. 107, 1745.

Lay, W.H., Mendes, N.F., Bianco, C., and Nussenzweig, V., (1971). Nature 230, 531.

Levine, S., (1956). Science 123, 185.

Levis, W.R., and Robbins, J.H., (1970). Transplantation 9, 515.

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

Livnat, S., Klein, J., and Bach, F.H., (1973). Nature New Biol. 243, 42.

Lohrmann, H.P., Novikovs, L., and Graw, R.G., (1974). J. Exptl. Med. 139, 1553.

Mage, M.G., and McHugh, L.L., (1973). J. Immunol. 111, 652.

Mardiney, M.R., Bock, G.N., and Chess, L., (1972). Transplantation 14, 274.

Miller, J.F.A.P., Marshall, A.H.E., and White, R.G., (1962). Advan. Immunol. 2, 111.

Miller, J.F.A.P., and Mitchell, G.F., (1968). Proc. Nat. Acad. Sci. U.S.A. 59, 296.

Milthorp, P., Belanger, R., and Richter, M., (1979). Immunology. In press.

Milthorp, P., and Richter, M., (1979). Immunology. In press.

Mookerjee, B.K., (1977). Transplantation 23, 22.

Mosier, D., and Cantor, H., (1971). Eur. J. Immunol. 1, 459.

Mueller, A.P., Wolfe, H.R., and Meyer, R.K., (1960). J. Immunol. 85, 172.

Oehler, J.R., and Herberman, R.B., (1977). J. Immunol. 119, 1785.

Ozer, H., and Waksman, B.H., (1974). J. Immunol. 113, 1780.

Phillips, B., Weisrose, E., (1974). Clin. Exp. Immunol. 18, 49.

Piquet, P.F., and Vassalli, P., (1972). J. Exptl. Med. 136, 962.

Plate, J.M.D., and McKenzie, I.F.C., (1973). Nature New Biol. 245, 247.

Potter, M.R., and Moore, M., (1977). Immunology 32, 359.

Rabinowitz, Y., (1964). Blood 23, 811.

Raff, M.C., (1970). Immunology 19, 637.

Redelman, D., Scott, C.B., Sheppard, W., and Sell, S., (1976). Cell. Immunol. 24, 11.

Richter, M., Colas de la Noue, H., and Hamdy, H., (1975) Immunology 29, 103.

Roberts, P.J., and Hayry, P., (1977). Cell. Immunol. 30, 236.

Rode, H.N., and Gordon, J., (1970). J. Immunol. 104, 1453.

Rode, H.N., and Gordon, J., (1974). Cell. Immunol. 13, 87.

Rollinghof, M., Pfizenmeier, K., Trostmann, H., and Wagner, H., (1975). Eur. J. Immunol. 5, 560.

Rosenstreich, D.L., Farrar, J.J., and Dougherty, S., (1976). J. Immunol. 116, 131.

Rubin, A.L., Stenzel, K.H., Hirschhorn, K., and Bach, F.H., (1964). Science 143, 815.

Schendel, D.J., Alter, B.J., and Bach, F.H., (1973). Transplant. Proc. 5, 1651.

Schirrmacher, V., Pena-Martinez, J., and Festenstein, H., (1975). Nature 225, 155.

Schrek, R., and Donnelly, W.J., (1961). Blood 18, 561.

Schwartz, M.R., (1966). Immunology 10, 281.

Seeger, R.C., and Oppenheim, J.J., (1970). J. Exptl. Med. 132, 44.

Sell, S., and Gell, P.G.H., (1965). J. Exptl. Med. 122, 423.

Sell, S., and Sheppard, H.W., (1973). Science 182, 586.

Shellekens, P.T.A., and Eijsvoogel, V.P., (1970). Clin. Exp. Immunol. 7, 229.

Sheppard, H.W., Sell, S., Poler, S.M., and Redelman, D.D., (1977). J. Immunol. Methods 16, 185.

Simpson, E., (1975). Eur. J. Immunol. 5, 456.

Sondel, P.M., Chess, L., MacDermott, R.P., and Schlossman, S.F., (1975). J. Immunol. 114, 982.

Sondel, P.M., Chess, L., and Schlossman, S.F., (1975). Cell. Immunol. 18, 351.

Sprent, J., and Miller, J.F.A.P., (1971). Nature New Biol. 234, 195.

Strom, T.B., Tilney, N.L., Paradysz, J.M., Bancewics, J., and Carpenter, C.B., (1977). J. Immunol. 118, 2020.

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., Thomsen, M., (1974). Tissue Antigens 4, 507.

Tissot, R.G. and Cohen, C., (1974). Transplantation 18, 142.

Tullis, J.L., and Surgenor, D.M., (1956). Ann. New York. Acad. Sci. 66, 386.

Twomey, J.J., Sharkey, O., Brown, J.A., Laughter, A.H., and Jordan, P.H., (1970). J. Immunol. 104, 845.

Unanue, E.R., Grey, H.M., Rabellino, E., Campbell, P., and Schmidtke, J., (1971). J. Exptl. Med. 133, 1188.

von Boehmer, (1974). J. Immunol. 112, 70.

Wagner, H., (1972). J. Immunol. 109, 630.

Warner, N.L., Szenberg, A., and Burnet, F.M., (1962). Aust. J. Exp. Biol. Med. Sci. 40, 373.

Weber, W.T., (1970). Clin. Exp. Immunol. 6, 919.

Wilson, D.B., (1967). J. Exptl. Med. 126, 625.

Wilson, D.B., Silvers, W.K., and Nowell, P.C., (1967). J. Exptl. Med. 126, 655.

Wilson, B.S., Teodorescu, M., and Dray, S., (1976). J. Immunol. 116, 1306.

Wybran, J., Chantler, S., and Fudenberg, H.H., (1973). Lancet i, 126.

Yam, L.T., Li, C.Y., and Crosby, W.H., (1971). Amer. J. Clin. Path. 55, 283.

Yunis, E.J., and Amos, D.B., (1971). Proc. Natl. Acad. Sci. U.S.A. 68, 3031.