Ph.D. EXPERIMENTAL MEDICINE

SHARWAN KUMAR SINGHAL

THE IMMUNE RESPONSE BY LYMPHOID CELLS IN VITRO

ABSTRACT

Bone marrow cells obtained from normal unimmunized rabbits were stimulated to undergo blastogenesis and mitosis and to incorporate tritiated thymidine upon incubation with various protein antigens in vitro. Cell suspensions of other lymphoid organs, obtained from normal rabbits, failed to respond in this fashion. This in vitro response did not occur with bone marrow cells of immunized rabbits, although it was observed with lymph node and spleen cells obtained from previously immunized rabbits. The immune nature of the bone marrow blastogenic response was demonstrated by the detection of specific antibody, by the fluorescent antibody technique, within the stimulated cells.

The response of the bone marrow cells was localized to the lymphocyte-enriched cell fraction following centrifugation of the whole bone marrow in a linear sucrose density gradient. The findings suggest that the bone marrow lymphocyte plays an important role during the induction of a primary immune response.

IMMUNE RESPONSE BY LYMPHOID CELLS IN VITRO

S.K. SINGHAL

THE IMMUNE RESPONSE

BY

LYMPHOID CELLS IN VITRO

SHARWAN KUMAR SINGHAL

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Department of Experimental Medicine,
McGill University,
Montreal, Canada

March, 1968

ACKNOWLEDGEMENTS

It is my sincere pleasure to record and express my gratitude to Dr. Maxwell Richter for his understanding, guidance, stimulating encouragement and criticism, both in the performance of the experiments and the preparation of this thesis and to Dr. B. Rose for his continued interest and encouragement.

I gratefully acknowledge my thanks to Miss B. Drechsler for her competent and conscientious assistance in carrying out the various experimental procedures. I also wish to thank Mrs. J. Aubray and Mrs. P. Pardo for technical assistance in some of the experiments and Miss L. Kines for her assistance in typing the manuscript.

I also wish to thank Dr. D. Osmond of the department of Anatomy, McGill University for his assistance in the fractionation of bone marrow cells and Dr. I. Watson and Mrs. F. Shenker for their kind assistance in experiments involving the fluorescent antibody technique.

I am especially indebted to my wife for her encouragement during the past four years.

This work was supported by a grant from the Medical Research Council, Ottawa, Canada.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

TABLES

TABLE OF CONTENTS (Continued)

TABLES

TABLE OF CONTENTS (Continued)

TABLES

TABLES

TABLE OF CONTENTS (Continued)

FIGURES

CHAPTER I

GENERAL INTRODUCTION

"Immunology has a long past but only a short history." (J. Freund, 1958, Claude Bernard Lecture, University of Montreal, Canada). Dr. Jules Freund, in making the above statement, implied that although the synthesis of antibody following antigenic stimulation has been known since the middle of the 19th century, the mechanism (s) of antibody formation and the cell site (s) of antibody synthesis are even today only little understood.

We know that an injection of an antigen into the normal intact animal triggers off a series of events marked by cellular proliferation in various lymphoid organs accompanied or followed by the production of antibody. There is a constant flow of cells through the lymphoid organs and, at any one time, only a small number of cells are engaged in the synthesis of antibody. In order to study, under controlled conditions, the sequence of cellular events characteristic of the immune response, tissue culture techniques have been resorted to.

The induction and maintenance of the secondary immune response has been accomplished in vitro by culturing tissue fragments, slices or cell suspension of lymph nodes and spleens of previously immunized animals in the presence of the

immunizing antigen. However, the induction of a primary immune response in vitro has been accomplished with great difficulty.

It has been demonstrated by a large body of investigators that the lymphocyte-plasma cell series is responsible for mediating both cellular and humoral immunity. More specifically, only lymphoid cells have been shown capable of mediating cellular or delayed immune reactions, whereas the plasmablast-plasma cell series has been held chiefly responsible for the production of humoral antibody, especially following hyper and secondary immunization. The in vitro experiments of Nossal and others (See Chapter 2.1.2) suggest a direct transformation of the large lymphocyte into the plasma cell. The cellular events leading to plasma cell differentiation in vivo are not as well documented.

In order to better elucidate the relationship between cellular events and antibody formation the investigator resorted to tissue culture procedures, which, although incapable of attaining a milieu as optimal as in vivo conditions, nevertheless do permit for a greater degree of flexibility in the experimental design and for a continuous analysis of cell morphology during the period of culture.

Undoubtedly, though rich in lymphoid cells, the complex nature and cellular heterogeniety of the bone marrow has deterred serious investigation into the role of this organ in the immune response. To attempt to better understand the role

of the bone marrow in antibody formation, the techniques of fragment and cell culture have been utilized. The results of the experiments reported upon in this thesis suggest that the bone marrow lymphocytes occupy a unique position in the events leading to the initiation of the primary immune response.

CHAPTER 2

HISTORICAL REVIEW

2.1 ORGAN AND CELL SITE OF ANTIBODY FORMATION. IN VIVO STUDIES

2.1.1 CELLS MEDIATING THE PRIMARY IMMUNE RESPONSE

The numerous studies pertaining to cellular alterations during the primary immune response in vivo are difficult to compare and evaluate in view of the different routes of antigen administration, the different antigens used (particulate or soluble), number of antigen injections and species of animal used. Multiple injections have often been used to produce a maximum cellular response, but sequential cellular changes are difficult to interpret in such a study.

Marshall and White (1) and White (2) administered either one, two or multiple intravenous injections into rabbits using several particulate and non-particulate antigens. They described the appearance of large, basophilic cells in the regions of the arterioles of the splenic red pulp three to four days after injection of the antigen. This cell was named the "activated reticulum cell" and its later differentiation was presumed to give rise to the plasma cell, but only after multiple injections of the antigen. These cells were not present in the germinal centers. Fagraeus (3), in a classical work with hyper-

immunized rabbits during the primary response, described the formation of plasma cells in the splenic red pulp as the main feature following the injection of an antigen. The precursor cell had a morphology similar to the "activated reticulum cell" described by Marshall and White (1) and was named the "transitional cell".

Wissler et al (4) reported that after the intravenous injection of a particulate antigen, into the rat, mitotic activity was observed to commence in the red pulp thirty six hours after the injection of the antigen and reach a peak at days three to four while the serum antibody titer attained peak levels at day six. No changes were noticed in the splenic white pulp. On the other hand, Ward et al (5) observed enlargement of the germinal centers in rabbit splenic white pulp and appearance of blast cells, but not plasma cells, three days after the intravenous injection of a single dose of bovine gamma-globulin. Congdon and Makinodan (6) also observed marked cellular alterations in the splenic white pulp of mice given sheep red cells before circulating antibody could be detected. The most striking observation was the loss of germinal centers in the spleen accompanied by extensive proliferation of antibody forming cells throughout the white pulp. This process lasted about three days and was followed by a regeneration of germinal centers. The sequence of histological events suggested that the large, newly formed cells in the white pulp gave rise to antibody forming

cells of the plasmacytic series later seen in the red pulp. Thorbecke et al (7) observed blast cells formed in the rabbit splenic white pulp during the primary immune response to protein antigen. They could not detect antibody in these cells. Some antibody-forming plasma cells were detected in the red pulp. Gunderson et al (8) noted changes in the splenic follicles in the rat spleen two days after a single intravenous injection of a bacterial antigen but not after the injection of sheep red cells. They described the red pulp as the site of antibody-forming cells.

Keuning et al (9) irradiated rabbits immediately following a single injection of paratyphoid-B antigen and observed complete disruption and loss of splenic germinal centers. Since the humoral immune response was not effected by the irradiation, they concluded that the germinal centers play no part during the primary immune response.

Langevoort (10) investigated the histological changes in rabbit spleen after a single intravenous injection of horse gamma-globulin. He found that plasma cells arose among the lymphocytes of the periarteriolar lymphocyte sheaths of the white pulp and suggested that the sheath lymphocyte was the precursor of the plasma cell. The plasma cell proliferation commenced twenty-four hours after injection of the antigen, was maximal at day three to four and by day seven the periarteriolar lymphocyte sheaths had resumed their normal appearance. Four days after

the antigen injection blast cells appeared in the germinal centers, giving rise to numerous medium sized lymphocytes.

Bramms (11) injected a single dose of horse γ-globulin intravenously into rabbits and utilized electron microscopy to evaluate histological changes in the spleen. He noticed an increase in the number of lymphocytes and the appearance of plasma cells containing ribonucleoprotein particles in the white pulp. He suggested that plasma cells are antibody-producing cells and that they arise from lymphocytes in the splenic white pulp. These findings of Langevoort (10) and Bramms (11) are in contradiction to those of other investigators reported above (4, 8, 9) who concluded that antibody forming cells develop in the red pulp.

Histological and radioautographic studies by Hanna (12, 13) on the germinal center changes in the mouse spleen revealed proliferation following an injection of sheep red blood cell. He noticed that an increase in the number of immature lymphocytic cells coincided with germinal center dissociation. These cells migrated into the red pulp where they presumably transformed to plasma cells. On the other hand, Craddock et al (14) studied the response of rats to the injection of the same antigen and reported the development of new germinal centers in the spleen, with prominent phagocytic activity and appearance of antibody-forming cells which were not the products of germinal center proliferation.

Ehrich and Harris (15) observed an increase in the number of lymphocytes in the popliteal lymph nodes of rabbits following an injection of a particulate antigen into the hind foot pad. Hall and Morris (16) described a similar finding in sheep and later (17) showed that very large doses of x-irradiation given locally to the popliteal lymph nodes did not reduce the output of cells in the efferent lymph nor did it markedly affect the antibody response to an injection of antigen into the foot pad. They concluded that the immunocompetence of the lymph node was not affected by the irradiation due to the constant inflow of small lymphocytes from the blood.

Smith (18) observed the presence of reticulum cells and large lymphocytes in the lymph nodes of human babies following primary immunization with typhoid vaccine. He noticed no plasma cells.

2.1.2 CELLS MEDIATING THE SECONDARY IMMUNE RESPONSE

Studies on the cell types involved in mediating the secondary immune response have produced evidence implicating several, rather than a single, cell type.

The classical observations of Fagreus (3) and Coons et al (19) have strongly suggested that most of the antibodies produced in rabbits during the hyperimmune and secondary immune responses are synthesized by plasma cells, which arise from precursor reticular cells and go through cellular division and

differentiation prior to antibody synthesis. Leduc et al (20), White et al (21) and Keuning and Vander Slikke (22) induced secondary immune responses in rabbits using protein antigens and described the appearance of large, basophilic cells in the splenic red pulp which appeared to give rise to plasma cells. They utilized the immunofluorescent staining technique to demonstrate specific antibody within the plasma cells.

Nossal and Makela (23) and Makela and Nossal (24) induced a secondary immune response in rats by the foot pad administration of the purified flagella antigen from Salmonella adelaide. A large dose of tritiated thymidine was given, as a pulse label, two hours before the second injection of the antigen and it was found that almost all the antibody-forming cells from the popliteal lymph nodes were labelled and that these cells were all members of the plasma cell series. They obtained identical results in rats reinjected with antigen either four or forty weeks following the primary immunization. On the assumption that any cell which began to divide after the reinjection of the antigen could not have become labelled, they concluded that immunological memory was carried by a continually-dividing large lymphocyte which appeared subsequent to the primary antigen injection and which could divide and differentiate into the plasma cell in response to a second exposure to the antigen.

Ward et al (25) and Cottier et al (26) investigated the histological changes in rabbit lymph node after two intravenous

injections of a protein antigen. They reported that the second injection stimulated cell division in the germinal centers and led to the rapid formation of new germinal centers. White et al (2) showed the presence of specific antibody in blast cells in the lymph node germinal centers during the secondary immune response in rabbits. It was suggested but not claimed explicitly that the antibody-forming cells were derived from small lymphocytes.

Thorbecke et al (7) suggested that some of the dividing blast cells found in the rabbit splenic white pulp during the primary immune response might be unable to synthesize antibody because of a lack of antigen. They suggested that these dividing cells produce secondary germinal centers in the spleen and respond to a second injection of antigen by migrating towards the splenic red pulp as they divide and differentiate into plasma cells. Thorbecke et al (7) did not consider germinal centers as important sites of antibody synthesis during the secondary immune response but rather as generative areas from which the plasma cells of the red pulp are derived. Sainte-Marie and Coons (27) investigated the origin of plasma cells in rabbit spleen and lymph node during a secondary immune response to a protein antigen. They stated that the plasma cells arise from primitive reticular cells in the splenic red pulp and lymph nodes. They further suggested, as a result of their electron microscopic studies, that the cells which Nossal etc.

and Makela (23) and Makela and Nossal (24) classified as large lymphocytes were, in fact, activated reticulum cells.

Hall et al (28) investigated the origin of antibody-forming cells in sheep lymph nodes during the secondary immune response to a protein antigen. They found a large number of blast cells with free ribosomes which contained antibody. Plasma cells were noticed only in vigorously hyperimmunized sheep. Cunningham and Mercer (29) studied the histologic charges in the sheep popliteal lymph nodes during the secondary immune response to the Salmonella somatic antigen. They reported that the majority of the antibody-forming cells were large and basophilic and contained ribosomes and small amounts of endoplasmic reticulum. A few typical plasma cells with large amounts of endoplasmic reticulum were also noted.

Attardi et al (30) and Vazquez (31) utilized the immunofluorescent staining technique to demonstrate specific antibody in small lymphocytes in the lymph nodes of rabbits producing a secondary immune response to protein antigens.

2.1.3 CELL TRANSFER STUDIES

2.1.3.1 CELL TRANSFER EXPERIMENTS WITH LYMPHOCYTES

To better understand the relationship between cell type and antibody formation, a large number of investigators have turned to cell transfer studies. The first to transfer

antibody-producing capacity by means of cells was Deutsch (32) who showed that spleen cells from rabbits infected with typhoid organisms could be transferred to normal homologous recipients with the subsequent development of circulating agglutinins. Chase (33, 34) and Landsteiner and Chase (35) successfully transferred a hypersensitivity to simple chemicals, such as picryl chloride or o-chlorobenzoyl chloride, in guinea pigs with lymph node or spleen cells.

Harris and Harris (36, 37, 38) injected Shigella paradysenteriae or Salmonella typhosa antigen into the hind foot pads of rabbits and succeeded in obtaining agglutinin formation in recipients of the immune popliteal lymph node cells.

This type of study gained momentum as a result of the studies of Harris et al (39) who observed antibody formation in heavily irradiated recipients who were given normal lymph node cells incubated with soluble Shigella antigen in vitro. The lymph node cells, of which 95 to 99 percent were large, medium and small lymphocytes, were most efficient in mediating a primary immune response in the recipients. They also showed that peritoneal exudates consisting mainly of monocytic cells were also effective in similar cell transfer studies, while granulocytic exudate cells were not. Circulating blood leucocytes did transfer some activity, but much less than the lymph node cells. The transferred cells were capable of conferring a secondary immune response in the recipients upon injection of

antigen, which could be effectively inhibited by the simultaneous administration of homologous antiserum prepared against the transferred cells. All of these studies strongly suggest that living homologous lymphoid cells can transfer antibody-producing capacity.

McGregor and Gowans (40) showed that previously unimmunized rats, depleted of small lymphocytes by drainage of a thoracic duct fistula, were able to respond only minimally to a single injection of either sheep erythrocytes or tetanus toxoid. This marked depression of the immune response could be reversed by the intravenous administration of isologous small lymphocytes. Depletion of small lymphocytes prior to a second injection of the antigen did not affect the secondary antibody response. They (41) also showed that the immune response to sheep erythrocytes by rats severely depressed by prior x-irradiation could be restored by the injection of a suspension of isologous small lymphocytes. The small lymphocytes from rats tolerant to sheep erythocytes were incapable of restoring antibody formation to sheep erythrocytes in previously x-irradiated rats. They concluded that the immune response was strongly dependent on and mediated by the small lymphocyte.

Gowans and Uhr (419) noted that recipients of lymphocytes obtained from the thoracic ducts of rats immunized to bacteriophage ϕ x 174 and incubated in vitro for 24 hours before transfer in order to destroy, selectivity, the large dividing lymphocytes

gave higher and more rapid antibody responses, following antigenic challenge, than rats which received unincubated immune thoracic duct cells. Naspitz et al (43) have demonstrated that immune rabbit lymphoid cells, incubated in vitro for three days with or without phytohemagglutinin (PHA), are capable of initiating antibody synthesis in recipient rabbits. Furthermore, these cells appear to possess greater immunocompetence than unincubated cells. Incubation of immune cells with PHA did not effect the immunological capacity of these cells subsequent to transfer although approximately eighty percent of the cells had transformed to blast cells. They concluded that the blast cell is not a terminal stage in the life-span of the cell but that it can differentiate into other immunocompetent cells in vivo.

Dixon et al (44, 45), compared the primary antibody transferring capacity of peritoneal exudates, containing an average of seventy one percent macrophages, eleven percent lymphocytes and fifteen percent polymorphs, with lymph node cell suspensions containing ninety percent lymphocytes and eight percent macrophages obtained from rabbits previously immunized with bovine serum albumin. These two cell populations produced comparable antibody responses in x-irradiated recipients. The ability of peritoneal exudate cells to transfer a secondary antibody response has been confirmed by Konda (46) and by Perkins et al (47)

2.1.3.2 CELL TRANSFER EXPERIMENTS WITH THYMUS CELLS

Stoner and Hale (48) successfully transferred tetanus antitoxin formation in mice with thymus cells. However, Rosenberg et al (49) and Dixon et al (45) reported that rabbit and guinea pig thymic cells, taken from previously immunized donors, produced little or no antibody in the homologous recipients. Harris et al (39) reported similar negative results when rabbit thymus cells were incubated in vitro with Shigella antigen and subsequently transferred into homologous recipients.

2.1.3.3 CELL TRANSFER EXPERIMENTS WITH BONE MARROW CELLS

Taliaferro and Taliaferro (50) reported that the transfer of bone marrow cells from rabbits three to four days after the primary immunization with sheep red cells to homologous x-irradiated recipients did not result in antibody formation in the recipients, but bone marrow cells from repeatedly injected rabbits during primary immunization could continue to synthesize antibody in x-irradiated recipients. The transferred cells were also capable of conferring a secondary immune response in the recipients upon injection of the antigen. Doria et al (51) showed that the injection of normal mouse bone marrow cells into irradiated isologous or homologous recipients could confer the capacity to initiate a primary immune response to an injection of sheep red blood cells. This immune response was noted to be greater in isologous than in homologous irradiated recipients.

Claman et al (52) have recently demonstrated that the injection of isologous marrow and thymus cells into irradiated host mice subsequently challenged with antigen resulted in more centers of hemolytic activity in the spleen than were observed in mice receiving cells of either type alone. In a recent communication, Miller and Mitchell (53) reported that the administration of normal mouse bone marrow cells to x-irradiated isologous mice resulted in the appearance of hemolytic plaque forming cells in the spleen within a week following an injection of sheep erythocytes.

2.1.3.4 CELL TRANSFER EXPERIMENTS USING NEONATAL ANIMALS

Dixon and Weigle (54, 55) showed that cells from neonatal rabbits, which were themselves immunologically incompetent, could form antibody when transferred to x-irradiated adult recipients although lymphoid cells from normal adult animals appeared to be incapable of initiating antibody formation in neonatal rabbits following the injection of the antigen. Trnka (56) and Harris et al (57) were unable to successfully repeat these experiments. Harris et al (58) later reported that neonatal rabbits could form antibody following the transfer of homologous adult spleen cells, but the response was markedly lower than that observed in adult irradiated recipients. The transfer of neonatal spleen lymphocytes to x-irradiated adult rabbits did not result in the restoration of immunocompetence.

Nossal (59) showed that when spleen cells obtained from adult immune mice were incubated with the antigen in vitro and transferred to neonatal isologous mice, a typical secondary immune response with high antibody titers was initiated, presumably mediated by the transferred cells.

2.1.3.5 EFFECTS OF X-IRRADIATION ON CELL TRANSFER

In the intact animal it has not been possible to determine whether the immunosuppressive effect of x-irradiation is wholly caused by direct injury to the antibody-forming cell(s) or whether secondary humoral factors are also involved. Dixon et al (60) presented evidence to support the latter possibility by comparing the amount of antibody produced in irradiated and normal recipient rabbits following the transfer of spleen cells, obtained from previously immunized rabbits, which were x-irradiated in vitro. A large number of irradiated recipients failed to support antibody synthesis by cells given 400 r irradiation or more whereas normal recipients of irradiated cells formed antibody without the administration of antigen. In addition, no detectable primary antibody response to bovine gamma globulin occurred following the transfer of normal lymphoid cells and antigen to irradiated recipients (60). Weigle and Dixon (61) found that the transfer of normal lymphoid cells to unirradiated recipients previously made tolerant to bovine gamma globulin

did permit the induction of a primary immune respone. This finding suggested that the irradiated recipient either harbors some sort of inhibitor, circulating or sessile, or is in some way immunologically deficient, and that the immunosuppressive effect of irradiation must be accounted for by more than just the direct injury to the lymphoid cells.

2.2 DEVELOPMENT OF TISSUE CULTURE METHODS

The in vitro tissue culture methods currently used evolved quite naturally from some of the basic and early techniques of embryology which were used during the latter half of the last century. Whilhelm Roux (62) in 1885 performed the first successful tissue culture experiment by maintaining the medullary plate of a chick embryo for a few days in warm saline. Jolly (63), in 1903, observed that salamander leucocytes could survive and divide in vitro for a period of at least thirty days. Three years later, in 1906, Beebe and Ewing (64) cultured, with some success, an infectious canine lymphosarcoma in medium containing blood from resistant and non-resistant animals. The results of these experiments were generally unsatisfactory with the cells surviving for only a few days.

A true and genuine demonstration of tissue survival under culture conditions was that of Harrison (65) in 1907 who observed a continuation of normal function and survival of fragments of the medullary tube region of frog embryos cultured in clots of frog lymph. These fragments were maintained in aseptic condition and growth of axones (nerve fibers) in these fragments was observed.

Burrows (66), in 1912, successfully used plasma clot as culture medium instead of lymph clot. Shortly afterwards, Burrows and Carrel (67) studied the effects of tissue extracts on

growth of tissues in culture and observed that embryo extracts had a strong growth-enhancing effect on certain cells. Henceforth plasma clot was supplemented with embryo extracts in tissue culture experiments by many investigators. Thompson (68) in 1914, cultured small fragments of embryonic chick tissue fragments, placed very close to each other, and observed continuous cellular differentiation.

The greatest difficulty in tissue culture technique at the turn of the century was to avoid contamination. Carrel (69) was largely responsible for the development of the methods for culturing tissues under sterile conditions. He was able to keep strains of cells in active multiplication for several years in vitro. However the tedious and meticulous surgical technique used by Carrel discouraged many investigators from employing tissue culture techniques.

It was during the period of 1914 to 1925 that tissue culture began to receive widespread attention and gained many international disciples. Carrel (63), Ebling (70) and the Lewises (71, 72) in the United States, Strangeways (73) in England, Fischer (74) in Denmark, Champy (75) in France and Choplin (76) in Russia were the major investigators during this period who employed tissue culture techniques for various studies. It was Strangeways (73) who provided the first complete description of the process of cell division and inspired Canti (77) to initiate experiments which culminated in the preparation of the most exciting and informative cinematographic films of cells migrating and dividing

in culture that had been taken to that day. Photographic evidence of cell division had already been shown in 1913 in France by Comadou et al (78) but Canti's films were superior technically and in content.

Carrel and Baker (79) investigated the composition of the medium employed in tissue culture in order to identify the important constituents required for optimal growth conditions. This work was carried further by Fischer (80), Parker (81), White (82, 83), Waymouth (84, 85) and Eagle et al (22). Their investigations resulted in the development of the synthetic culture media currently used.

In 1933 Gey (86) and his co-workers developed the roller tube, which was found to be superior to the stationary culture tube since more tissue could be placed in it, more medium could be used and conditions could be better controlled due to constant mixing. This culture technique permitted the initiation of studies concerned with the nucleoprotein content of growing cells and allowed for a study of biological activity on a per cell basis.

In 1941, Medawar (87) used tryptic digestion to separate epidermis from dermis in connection with grafting procedures for wound healing. About ten years later, in 1952, Moscona and Moscona (88) developed a more reliable and practical method for dispersing viable cells with the help of trypsin. Shortly afterwards Rinaldini (89), Dulbecco (90) and Younger (91)

applied the Moscona technique to enable the growth of cells as monolayers in culture.

The improvements of the basic tissue culture procedures have permitted the development of mass cell culture techniques which have opened doors for further studies at the cellular level with very obvious and practical applications.

2.3 IMMUNOLOGICAL STUDIES IN VITRO

2.3.1 EARLY STUDIES

Pfeiffer and Marx (92) in 1898 prepared cell-free extracts of tissues from immunized animals and found that lymph nodes, spleen and bone marrow contained antibody before it could be detected in the serum. In 1912, Carrel (69) claimed to have shown antibody production by adult guinea pig lymph node and bone marrow fragments in tissue culture. He mixed fragments of these tissues in the presence of goat erythrocytes in culture dishes. After three to five days he prepared extracts from the fragments which produced hemolysis of goat red cells to a degree greater than that of extracts prepared from control tissues incubated in the absence of goat erythrocytes. It was also in 1912 that Ludke (93) produced evidence of antibody production in vitro by cultures of spleen and bone marrow fragments of rabbits or guinea pigs which had been actively immunized with killed salmonella or shigella bacteria a few days previously. He claimed that his culture supernatants had agglutinating antibody titers up to 1:160. It was not until 1925 that Kuczynski et al (94) attempted a similar experiment by culturing rabbit spleen cells in the presence of sheep erythrocytes but they failed completely to show production of any detectable amount of antibody. On the assumption that the antigen may not have diffused adequately into the cells, they injected sheep erythrocytes into the rabbits before sacrifice but

again obtained negative in vitro results. Meyer and Lowenthal (95), in 1928, were the first to claim the demonstration of actual secretion of measurable antibody into the medium in vitro by lymph node fragments obtained from rabbits previously immunized with salmonella typhi vaccine. They reported hemagglutinatination titers up to 1:320, tissue growth and inhibition of antibody formation when an excess of manganese ionswere added to the medium. This work was criticized in 1937 in a review article by Salle and McOmie (96), who suggested that the results could be attributed to the gradual autolysis of cells and release of intracellular antibody which had already been produced in the intact immunized animal.

2.3.2 RECENT STUDIES

2.3.2.1 GENERAL CULTURE CONDITIONS

(A) ANTIGENS USED

There seems to have been no special thought given to the choice of antigen used in in vitro studies. As can be seen from Tables I-IV, a large number of antigens-soluble, particulate, bacterial and tissue antigens-have been used, the choice apparently depending on their availability and convenience of methods for detecting the corresponding antibody.

(B) PREPARATION OF TISSUES FOR CULTURE

Various tissues of the rabbit, rat, mouse, guinea pig, monkey, frog, chicken and man have been used to study the

immunological response in vitro (Tables I-IV). These include lymph node, spleen, thymus, bone marrow, appendix, illeum, colon, retina, liver, lung, kidney, adrenal, thymus and peyers patches.

Several different techniques have been utilized in the preparation of tissue for culture. Most commonly used are perfused organ cultures or tissue slices in which metabolic activity appears to be favourable for antibody formation, probably attributable to the retention of the original architecture of the tissue.

The technique of fragment culture involves the cutting of the tissue into small pieces approximately 1 mm^3 and placing them into a suitable media. Although necrotic degeneration sets in rapidly in the centers of these fragments, some cellular population (s) are capable of migrating and multiplying. There is no substantial and detailed histological study available of fragments cultured in vitro.

The technique of culturing cell suspensions has also been used recently in the study of the immune response in vitro. The original organization of the tissue is completely destroyed but the total cell population is preserved and exposed to the culture medium. Rapid changes in this preparation are noticed during the first 48 hours of culture due to differential death and proliferation. There is evidence of antibody synthesis with all of the above mentioned tissue preparations. Quite high rates of antibody synthesis have been obtained with perfused organ cultures

or with tissue slices obtained from previously immunized animals when incubated for short periods of time (97,). . Fragments of tissues of previously immunized animals have been found to be more suitable for studying long term antibody formation in culture in vitro (99-106). Cell suspensions have been used, though with less success, in the demonstration of antibody synthesis (107-120).

It has also been shown by Nossal and others (24, 121-130) that single cells incubated in microdrops can synthesize antibody detectable by bacterial agglutination, adherence or neutralization techniques

A technique for detecting hemolytic antibody produced by a single cell has also recently been developed by Jerne and co-workers (136, 137). Hemolytic antibody released by a cell becomes bound to the erythrocytes present in a semi-solid medium. When complement is added, a small area of lysis or plaque is formed around the cell. This method has been successfully used by many investigators (114, 115, 131-142).

(C) CULTURE CONTAINERS

Tissue slices, fragments and suspensionshave been cultured in petri dishes, glass or plastic culture tubes, Leighton tubes and prescription bottles. These containers have been maintained in a stationary, straight or angledposition or have been rotated at various

speeds. The main variables include glass versus plastic tubes, shaking versus stationary culture, depth of medium over the cells, surface area of culture tube, composition of the gas phase and changing and composition of the medium.

(D) MEDIUM AND NUTRIENT CONDITIONS

A wide range of media has been used for cell and fragment culture. Dilute cell suspensions may require factors not required by dense cell suspensions or fragments, since essential metabolites are released into the medium. For long incubation periods, addition of serum or some other nutrient has in general been used. In most cases, as seen in Tables I-IV, the medium has contained five to fifty percent homologous normal serum.

The addition of purine and pyrimidine bases, vitamins and essential amino acids did not produce an enhancing effect (143) in cultures of immune rabbit spleen fragments maintained in Eagle's medium containing ten percent normal rabbit serum. High concentrations of amino acids, purine and pyrimidine bases has inhibitory effects. The optimal concentration of amino acids in the medium has been investigated in detail (144, 109, 99).

Ambrose (145, 146) has demonstrated that lymph node fragments of previously immunized rabbits synthesized more antibody when the serum was replaced by 0.01 to 1 uM hydrocortisone, provided that the medium was supplemented with serine (0.1 mM). The medium was further improved by the addition of insulin and vitamin B_{12}, but this finding was not consistent. The presence of

hydrocortisone was found to be more critical during the early phase of culture and could be removed after eight to ten days of culture (145, 146, 102).

Heterologous serum has been used by various workers in place of autologous serum in the medium. Presumably it can supply the components necessary for the maintenance of antibody synthesis. Dresser (147) observed a considerable variation in the ability of heterologous sera to support antibody synthesis by guinea pig lymph node fragments. Fetal calf serum has also been shown to support antibody synthesis (116, 148, 149). Dutton (152) has shown that fetal calf serum could induce DNA formation in rabbit spleen cells in vitro. Holm et al (153) and Moller (154) showed that fetal calf serum contains a factor which can agglutinate mouse spleen cells. Patterson et al (155) showed that cultures of spleen cells from chickens immunized with bovine serum albumin and horse spleen ferritin cultured in medium 199 supplemented with two different nutrients produced cells with different morphology. Cultures in homologous normal chicken serum presented with cells with the appearance of macrophages, large and small lymphocytes, plasma cells and monocytes. Cultures of cells of the same spleen in medium containing egg albumin contained small cells with the appearance of either small lymphocytes or small plasma cells.

(E) CELL DENSITY

An increase in cell concentration in culture seems to obviate some difficiences in the medium (156). The proliferation of mouse spleen cell suspensions in modified Eagle's medium is particularly dependent on cell density as 1.5×10^6 cells per ml was found to be optimal for DNA synthesis (152). Our own studies suggest that 10^6 cells per ml is optimal for maximal and long term DNA synthesis in rabbit lymphoid cells (157). Moorhead et al (158), in a recent quantitive study of in vitro stimulated blood leucocytes, have shown that DNA synthesis is affected by variation in cell number, duration of culture and area of the surface on which the cells are cultured. They demonstrated, in both mixed leucocyte and antigen stimulated cultures, that as cell density increases, the synthesis of DNA increases proportionally. The optimal results were always obtained between the fifth and seventh day of culture. Using horizontally and vertically placed Leighton tubes they clearly demonstrated that the activity in the vertical position greatly exceeded the activity in the horizontal position. They demonstrated that there was a progressive increase in activity as the cells were more closely approximated in the tube.

2.3.2.2 FRAGMENT CULTURE IN VITRO

A. PRIMARY ANTIBODY RESPONSE

(ii) Antigen Administered In Vivo

There have been some attempts to study the primary immune response in vitro by culturing lymph node and spleen fragments from animals injected with antigen a few hours or a few days prior to sacrifice. Stevens and McKenna (159) incubated bovine gamma globulin for one hour with splenic fragments from rabbits immunized with Salmonella typhosa endotoxin twenty four hours previously. After washing off excess antigen, the fragments were placed in a synthetic medium. A small amount of hemagglutinating antibody appeared in the medium after only one hour incubation but larger amounts of antibody appeared with longer incubation. Mountain (143, 160), Kong and Johnson (161), and Schoenberg et al (162) injected rabbits with S. typhosa, bovine gamma globulin and diphtheria toxoid antigens, respectively, in order to demonstrate the capacity of spleen fragments to release antibody into the culture medium in vitro. Kong and Johnson (161) showed that antibody synthesis could be inhibited by the addition of 5- fluro- 2- deoxyuridine which could be partially restored by the addition of thymidine.

La Via et al (163) administered S. typhosa antigen into rats and cultured their splenic fragments in synthetic medium. They added labelled amino acids to the medium which resulted in the demonstration of radioactively-labelled antibody in the supernatants

of these cultures. Similar results have been reported by Pernis et al (164) and Grabar and Corrazier (165).

Berglund et al (166) immunized mice with sheep erythrocytes and three days later they cultured sections of spleen in agarose medium on glass slides in the presence of sheep erythrocytes and complement. Plaque forming cells were observed in the splenic sections.

Leibowitz and Parks (167) injected bovine serum albumin into the cornea of rabbits and cultured corneal fragments in vitro. They demonstrated that antibody formed in vitro could induce a passive cutanous anaphylaxis reaction in guinea pigs.

The above results are summarized in Table I-A.

(ii) Antigen Incubated In Vitro

Saunders and King (148) cultured normal mouse spleen and thymus fragments together in the presence of coliphage R17 antigen and claimed to have shown induction of a primary immune response which was measured by the phage neutralization technique. Tao and Uhr (168) described similar results using rabbit lymph node fragments.

Globerson and Auerbach (169, 170) injected phytohemagglutinin into mice, cultured splenic and lymph node fragments seperately in the presence of sheep erythrocytes and demonstrated the appearance of hemagglutinins and hemolysins in the culture supernatants. They failed to show any detectable amount of anti-

body when thymus fragments were cultured under similar conditions,

For a summary of the experiments referred to above see Table I-B.

B. SECONDARY ANTIBODY RESPONSE

(i) Antigen Administered In Vivo

A summary of the published experiments demonstrating the capacity of tissues of immunized animals to continue synthesizing antibody in vitro is presented in Table II-A. Therefore only the relevant and best documented experiments will be discussed.

Stavitsky (111) showed that lymph node and spleen fragments from rabbits previously immunized with either diphtheria or tetanus toxoid could continue to produce diphtheria or tetanus antitoxin when cultured in synthetic media. The addition of cyanide or dinitrophenol resulted in complete inhibition of antibody production. He stated that the in vitro synthesis of antibody appeared to parallel its in vivo synthesis.

Keuning and Van der Slike (22), Thorbecke and Keuning (171) and McKenna and Stevens (172) have also reported similar results when rabbit lymph node or spleen fragments were cultured in medium. They utilized the hemagglutination technique to show specific antibody released into the media.

Askonas et al (173), Ranney and London (174), Askonas and Humphrey (97), Wolf and Stavitsky (144) Keston and Ketchen (176), Lazda and Starr (177) and Thorbecke (149) employed

the labelled amino acid incorporation technique to demonstrate continuation of antibody synthesis by lymph node and splenic fragments of rabbits producing a secondary immune response to various protein antigens.

(ii) Antigen Incubated In Vitro

A summary of the published experiments related to the induction of a secondary immune response in vitro is presented in Table II-B.

Michaelides (100) reported upon experiments with lymph node fragments, obtained from rabbits previously immunized with diphtheria toxoid, cultured in Eagle's medium in the presence of the immunizing antigen. He was able to show a small increase of antibody production by the fragments which had been exposed to the antigen. Michaelides and Coons (99) reported that, with diphtheria toxoid as the antigen, lymph node fragments maintained their capacity to produce a secondary immune response for several days after the beginning of culture. With bovine serum albumin as antigen, the in vitro immune response lasted for four weeks. O'Brien et al (101) studied the morphological changes taking place in the stimulated fragments by means of the immunofluorscent technique. They reported that no antibody-containing cells could be found two days after in vitro stimulation. By day three, a few cells with cytoplasm stained with the fluorescein conjugate could be seen and by the fourth day many antibody containing plasma cells,

immature as well as mature, were widely distributed throughout the fragments. By the eighth day and thereafter, most of the stained cells were mature plasma cells.

Stavitsky (178), Stavitsky and Wolf (179), Haliday and Garvey (102), Kritzman and Harper (180), Obrien and Coons (101), Stevens and McKenna (182), Ambrose and Coons (183), Juhasz and Rose (104), Juhasz and Richter (105) and Richter et al (106) have all demonstrated the induction and maintenance of a secondary immune response by lymph node fragments from rabbits previously immunized with various protein antigens using the passive hemagglutination technique.

Stavitsky (178), Stavitsky and Wolf (179), Richards et al (184) and Dresser (185) utilized the labelled-amino acid incorporation assay to demonstrate the induction of a secondary antibody response in vitro.

Tao (103) cultured lymph node fragments from rabbits previously immunized with bovine serum albumin and bovine gamma-globulin. Upon the addition of phytohemagglutinin to these cultures, a "non-specific" secondary immune response was induced.

2.3.2.3 CELL CULTURE IN VITRO

A. PRIMARY ANTIBODY RESPONSE

(i) Antigen Administered In Vivo

Roberts et al (186) cultued lymph node and spleen cell suspensions from rats injected with sheep erythrocytes several days previously and demonstrated the release of hemagglutinating antibody into the culture medium. Langevoort et al (187) cultured spleen cell suspensions from rabbits immunized with bovine gamma-globulin and demonstrated the release of hemagglutinating antibody into the culture medium.

Jerne and Nordin (188), Strander (189) and Tannenberg (190) cultured spleen cells from mice previously immunized with sheep erythrocytes and demonstrated the release of hemolytic antibody in the presence of sheep erythrocytes and complement. Strander (189) showed that the plaque-forming capacity of spleen cells could be inhibited by puromycin and actinomycin-D. Massie and Frick (191) injected mice with penicillin-G which was conjugated to KLH. The spleen cell suspensions from these animals showed hemolytic plaque-forming capacity when cultured in the presence of antigen-sensitized sheep cells and complement.

Kern and Eisen (192) cultured lymph node cells from guinea pigs previously immunized with bovine gamma-globulin and demonstrated an increase in the incorporation of phosphate by the cells. They did not measure antibody synthesis.

In all of the experiments referred to above, the animals were injected with the antigen only several days prior to

sacrifice and culture of the cells. Therefore any antibody detected by the cultures represents the continuation of the primary immune response initiated in vivo. A summary of these experiments is presented in Table III-A.

(ii) Antigen Incubated In Vitro

Many attempts have been made to induce the primary immune response entirely in vitro. Fishman (193) incubated macrophages from rat peritoneal exudates with bacteriophage virus particles. After thirty minutes, the excess antigen was washed off and the macrophages were homogenized. The soluble macrophage extract was incubated with normal lymph node cells from adult rats for twelve days. The medium was changed at various intervals of time and tested for phage neutralizing activity. One experiment in five showed phage neutralizing activity. This activity was inhibited by rabbit anti-rat globulin. No antibody was detected when heterologous macrophages were used. Fishman (194) showed that when lymph node cells exposed to the macrophage extract were placed in diffusion chambers and incubated in the peritoneal cavities of x-irradiated homologous rats, a large amount of antibody was detected in the culture media within the diffusion chambers. Friedman et al (195) have successfully repeated Fishman's experiments and demonstrated the synthesis of antibody by the complement fixation test.

McKenna and Stevens (196) injected rabbits intraperitoneally with sterile mineral oil and cultured peritoneal

exudate cells in vitro in the presence of bovine gamma-globulin and egg albumin. They claimed that the supernatants collected from these cultures showed hemagglutinating antibody titers of up to 1:28.

A large number of investigators have reported the in vitro induction of synthesis of hemolytic antibody by cultures of normal spleen cells incubated with sheep erythrocytes and complement. Mishell and Dutton (107), Marbrook (197), Zalberg et al (198), Bussard and Anderson (199), Mosier (200), Hege and Cole (201) and Robinson et al (202) have all demonstrated hemolytic plaque formation by " normal " mouse spleen cells incubated with sheep erythocytes in vitro. Robinson et al (202) demonstrated that the antibody synthesizing capacity of mouse spleen cells could be inhibited by the addition of colchicin, actinomycin-D and rabbit anti-mouse globulin. Hege and Cole (201) claimed that the addition of phytohemagglutinin could enhance the plaque formation and that neonatal thymectomy and x-irradiation of adult animals did not inhibit the plaque forming capacities of normal mice. Bussard and Lurie (203), Bendinelli and Wedderbern (204) and Bussard (205) utilized mouse peritoneal exudate cells to demonstrate hemolytic plaque formation induced by prior incubation with antigen in vitro.

A short summary of these results, in tabular form, is presented in Table III-B.

B. SECONDARY ANTIBODY RESPONSE

(i) Antigen Administered In Vivo

Experiments related to the maintenance of a secondary immune response in vitro following antigenic stimulation in vivo are summarized in Table IV-A. In the majority of the investigations, the animal was sacrified and the spleen and lymph nodes were extirpated at the time when the donor animal was synthesizing antibody at a maximal rate during the secondary immune response induced in vivo.

Thorbecke et al (151) and Cremer (206) immunized rabbits with bovine gamma-globulin, ovalbumin, bovine serum albumin or hemocyanin and cultured splenic cell suspension in synthetic medium. They demonstrated hemagglutinating activity in the supernatant culture fluids. Roberts et al (186) utilized rat spleen and lymph node cell suspensions to demonstrate similar results in vitro.

Fleishman (207) induced a secondary immune response in rabbits with bacteriophage antigen and demonstrated the capacity of their lymph node cell suspensions to continue synthesizing antibody which was detected by the labelled amino acid incorporation technique followed by co-precipitation. Dutton et al (208), Dutton and Pearce (209), Smiley et al (110), Dutton et al (210), Steiner and Anker (112), Vaughan et al (109) and Smiley and Jasin (211) have all demonstrated the synthesis of specific antibody in vitro, directed against various protein antigens, by the method of labelled amino acid incorporation followed by co-precipitation. Dutton (212)

described that the specific antibody synthesized in his cultures could be completely inhibited by high concentration of H^3-labelled thymidine. Hulliger and Sorkin (119, 120) cultured thoracic duct lymphocytes and peripheral leukocytes obtained from rabbits after secondary immunization with human serum albumin. The synthesis of antibody was detected by incubation of the cultures with radioactive amino acid followed by co-precipitation of the supernatants.

Sussdorf (213) showed that subcellular preperations of spleen obtained from rabbits previously immunized with sheep erythrocytes could continue to produce small amounts of hemolytic antibody in vitro.

Krueger and Twedt (118) demonstrated the antibody forming capacity of spleen cells of frogs immunized previously with S. typhosa antigen. The technique of bacterial immobilization was utilized. Ainis (214) demonstrated precipitable amounts of antibody which had been synthesized by spleen cell suspensions from rabbits which had been hyperimmunized against hemocyanin.

(ii) Antigen Incubated In Vitro

Less successful results have been obtained in investigations dealing with the induction of the secondary immune response in vitro. The majority of investigators have utilized a relatively sensitive isotope incorporation assay to demonstrate the induction of the secondary immune response in vitro.

Helmreich et al (114, 115) hyperimmunized rabbits with dinitrophenol, bovine gamma-globulin and ovalbumin. When lymph node cell suspensions were incubated for a short period of time in the presence of the immunizing antigens, antibody synthesis was demonstrated by the amino acid incorporation technique.

Scharff and Uhr (116), Bussard and Huyny (215) and Popisil (216) cultured lymph node cells obtained from rabbits previously injected with T2 phage, sheep erythrocytes and Brucella antigens, respectively. The labelled amino acid incorporation technique was employed to demonstrate a small amount of antibody synthesized following an exposure to the specific immunizing antigen in vitro. Medzon and Vas (217) showed that the secondary immune response by rabbit lymph node cell suspensions could be inhibited by the addition of New Castle disease virus.

Capalbo et al (218) showed that spleen cells from rats and mice previously immunized with sheep erythrocytes could initiate a secondary immune response following incubation with sheep erythrocytes in diffusion chambers implanted in the peritoneal cavity of x-irradiated homologous animals. Hemagglutinating antibody was demonstrated in the culture medium within the chambers.

Van der Meer and Koningsberger (219) cultured spleen cell homogenates obtained from rats previously immunized with phage x-174. They claimed that a secondary immune response was initiated in the cultures following an exposure to the immunizing antigen. The presence of antibody synthesized by the subcellular

preparation was demonstrated by the phage nutralization assay. Richardson and Dutton (117) hyperimmunized rabbits with sheep erythrocytes. When the spleen cells were subsequently incubated with sheep erythrocytes and complement in vitro plaque forming cells were detected, thus demonstrating antibody formation in vitro.

A brief summary of the experiments presented above is presented in Table IV-B.

(iii) Blastogenic and Mitogenic Response To Specific Antigen (s) In Vitro

Human Cells

Various antigens have been shown to possess the capacity to stimulate peripheral blood lymphocytes obtained from the specifically-immunized individual to undergo blastogenesis and mitosis. Specific antigens stimulate the enlargement of 5 to 40 per cent of the cells after 5 to 10 days of incubation. The mechanism of this transformation, which provides an in vitro method for the diagnosis of immunity and hypersensitivity, will be discussed in Chapter 2.3.2.7.

Timofejewsky and Benewolenskaja (220), in 1926, suggested that it might be possible to differentiate leukocytes derived from immune and non-immune donors by a study of the reaction of the cells to microbes and toxins in an in vitro system. They (221) also reported that human lymphocytes and monocytes transformed into polyblasts and epithelioid cells in the presence of tubercle

bacilli. Pearmain et al (222), Schrek (223), Hirschhorn et al (224), Marshall and Roberts (225) and Cowling et al (226) all showed that the addition of the purified protein derivative (PPD) of tuberculin to cultures of lymphocytes from individuals sensitive to tuberculin induced the appearance of blast cells and mitosis. The in vitro mitogenic action of PPD on lymphocytes of tuberculin-positive individuals was further confirmed by Coulson and Chalmers (227), Hartog et al (228), Caron and Sarkany (229), Gump et al (230), Rauch (231), McFarland and Heilman (232), Caron (233), Heilman and McFarland (234, 235), Fireman et al (236), Hirschhorn et al (237), Vischer (238) and Mayron and Baram (239). Aspergren and Rorsman (240) observed that tuberculin in culture medium stimulated mitosis in leukocytes of 14 of 20 tuberculin-positive patients and 4 of 10 tuberculin-negative persons. Heilman and McFarland (234) and Ricci et al (241) showed that cultures of peripheral lymphocytes obtained from patients with active tuberculosis were not transformed after the addition of PPD, thus confirming the earlier findings of Pearmain et al (222). Heilman and McFarland (234) suggested that the inhibitory activity resided in the tuberculosis serum or plasma, based on their findings that tuberculous serum or plasma depressed the mitogenic action of tuberculin on lymphocytes from healthy tuberculin-positive donors.

Lycette and Pearmain (242) cultured lymphocytes from individuals immunized with poliomyelitis antigen. The

addition of Sabin vaccine to the cultures produced blast transformation and mitosis in all cases. Elves et al (243, 244) observed that tetanus toxoid, typhoid-paratyphoid vaccine (TAB), Hemophilus pertussis vaccine, diphtheria toxoid, poliovirus vaccine and smallpox vaccine were able to induce blastogenesis when added to cultures of lymphocytes obtained from patients sensitized to these antigens. Hirschhorn et al (245) reported similar results using diphtheria toxoid, pertussis vaccine and penicillin as antigens. Ling and Husband (246) added a number of antigens (TAB, Tetaxus toxoid, PPD and endotoxin) to cultures of lymphocytes from sensitized patients and observed no blastogenesis.

Cowling and Quaglino (247) determined the optimal concentrations for various antigens (old tuberculin, diphtheria toxoid, TAB vaccine and tetanus toxoid) to induce blastogenesis in cultures of human peripheral lymphocytes after seven days of incubation. Mixtures of antigens showed no summation of effect and the transforming activity of PHA was not inhibited by any of the antigens tested.

Caron (233) incubated lymphocytes from a donor sensitized to both tetanus toxoid and smallpox vaccine with mixtures of varying concentrations of these antigens. When both antigens were present at their optimal concentrations, there was no summation of the effects produced by each antigen separately The only circumstance in which a summation effect was detected was that in which both antigens in the mixture were at concen-

trations considerably below their optimal. Girard et al (248), using lymphocytes of patients allergic to ragweed, alternaria and penicillin, also observed no cumulative stimulatory effect when two or more antigens were added simultaneously to the culture.

Lycette and Pearmain (242) cultured lymphocytes obtained from five patients with severe grass hay fever. A polyvalent extract of grass pollens, which gave strongly positive skin tests, was added to the cultures. Blast cells and mitoses were observed after four to six days of incubation although much lower in number when compared to cultures of PHA stimulated cells. Wiener and Brasch (249) reported that the addition of grass-pollen extracts to lymphocytes from sensitized individuals resulted in significant increase in the number of mitoses. This mitogenic effect was inhibited when leukocytes of desensitized patients were used. Zeitz et al (250) observed blast transformation in cultures of peripheral lymphocytes obtained from timothy-sensitive individuals when timothy pollen was added to the lymphocyte cultures. The response was not changed with hyposensitization treatment, thus not supporting the findings of Wiener and Brasch (249). Girard et al (248) were also able to obtain blastogenesis using ragweed, alternaria and penicillin as antigens. The results in all of these investigations were based upon a morphological analysis of samples of the cell suspensions. However, this technique of estimating the blastogenic response is highly subjective and, as stated by Girard et al (248), it is sometimes very difficult to differentiate blast cells from other large mononuclear cells, such as macrophages, after several days in culture. Richter and Naspitz (251) cultured lymphocytes of ragweed allergic patients and normal controls with the allergen and measured the blastogenic response by the incorporation of radioactive thymidine into the entire cell population. The results reflect the behaviour of the entire cell population and therefore

circumvents the errors inherent in a purely morphological analysis. Eighty per cent of the cultures obtained from the allergic patients incorporated thymidine specifically to a degree 4-18 times that of control cultures.

On the other hand, Ricci et al (252) were unable to induce blastogenesis in lymphocyte cultures from patients sensitive to Graminacea pollens when this antigen was added to the cultures. Similarly, Mayron and Baram (239) observed only a slight increase in the rate of nucleic acid synthesis when ragweed pollen extracts were incubated with lymphocytes of individuals with ragweed allergy.

The fact that a large number of antigens possess the capacity to stimulate specific blastogenesis in vitro prompted several investigators to use this technique as an in vitro diagnostic tool for drug hypersensitivity. Hirschhorn et al (245) reported that lymphocytes from patients with allergy to penicillin, when cultured with this antibiotic, presented with blastogenesis and mitosis. Similar results were also obtained by Heitman (253) and Gotz and Heitman (254). Holland and Mauer (255) reported blastogenesis and mitosis induced in lymphocyte cultures of a patient with a sensitivity reaction to diphenylhydantoin by diphenylhydantoin. Similar findings were reported by Caron and Sarkany (256) with cells of a patient with sulphonamide sensitivity. Visher (238) examined lymphocytes of patients sensitive to penicillin, phenylbutazone, isoniazid and p-aminosalicylic acid. Using the technique of incorporation of labelled nucleic acid precursors, he

was able to demonstrate stimulation of the lymphocytes of only 2 of 13 penicillin-sensitive patients. With the other drugs all cultures were negative. Girard et al (248) added penicilloyl-polylysine to cultures of lymphocytes from penicillin allergic patients and found that the number of blast cells was greater than in control cultures.

Ripps and Hirschhorn (258) found that the addition of penicillin to cultures of lymphocytes from penicillin-allergic patients induced the formation of antibodies capable of binding penicillin.

Ripps anf Fellner (259) found that lymphocytes of only eleven of seventeen penicillin-sensitive individuals manifested a blastogenic in vitro response to penicillin. These patients reacted to the intra-dermal injection of penicillin or penicilloyl-polylysine. Similar results were reported by Ripps et al (260) in one patient sensitive to tetracycline. Gill (261) also obtained blast transformation using penicillin as an antigen. However, he reported that the spontaneous transformation of the "allergic" lymphocytes in vitro, in the absence of any stimulant, was greater than with lymphocytes of non-allergic controls. Halpern et al (262, 263), using several drugs including penicillin as stimulants in leukocyte cultures from allergic patients, reported blast transformation after four days of incubation with the lymphocytes of most of the patients tested. However, Woodliff and Onesti (264) could not reproduce these findings with cells of a single patient sensitive

to penicillin and Sarkany (265) tested 22 different drugs on cultures of lymphocytes from 40 patients sensitive to these drugs and was able to obtain blastogenesis in only seven cultures. Evans (266) using penicillyl-polylysine and potassium benzyl penicillin G as antigens, found no difference in response between the lymphocytes of control and antibiotic sensitive patients.

Hashem and Barr (267) obtained blastogenesis in cultures of lymphocytes from seven of nine patients with degenerative nervous desease by incubating the lymphocytes with rabies vaccine (rabbit brain) or with a cell-free brain extract preparad from human embryos. Fowler et al (268) incubated cultures of peripheral blood lymphocytes, obtained from patients with multiple sclerosis, with cerebrospinal fluid, the latter serving as the source of antigens. They found a significant increase in the percentage of lymphocyte transformation of cells of patients in comparison to normal controls. Hashem et al (268a) studied lymphocytes from children with eczema using extracts of autologous and homologous skin as antigens. The number of blast cells obtained was greater in lymphocyte cultures of the patients as compared to controls. Fjelde and Kopecka (269), in similar studies, showed that autologous skin extracts induce a slight but definite blastogenic response in leukocytes from atopic patients. Pass et al (270) using a much more objective method to detect blast transformation and mitosis, by determining the uptake of tritiated thymidine, were unable to reproduce the results reported by both Hashem et al (268a) and Fjelde and Kopecka (269).

Patrucco et al (271) cultured lymphocytes from patients with systemic lupus erythematosus (SLE) with DNA. Blast transformation was obtained with the lymphocytes of 11 of 12 patients with SLE. No relation could be demonstrated between the degree of in vitro response to DNA and the titer of circulating antinuclear antibody.

Hirschhorn et al (272) reported that lymphocytes from patients with sarcoidosis undergo transformation to blast cells in vitro when cultured for eight days in the presence of Kveim antigen. Cowling et al (273) failed to observe stimulation of lymphocytes from patients with sarcoidosis cultured in the presence of Kveim antigen.

Animal Cells

Aspergren and Rorsman (274) sensitized guinea pigs with tuberculin and observed no blast transformation when this antigen was added to cultures of peripheral blood lymphocytes obtained from the sensitized animals. Tuberculin did not inhibit the stimulating effect of PHA on the lymphocytes. On the other hand, in similar studies, Zweiman et al (274, 276) obtained opposite results in that they observed blastogenesis and mitosis after addition of PPD to the cultures. Mills (277) immunized guinea pigs with tuberculin, egg albumin and bovine gamma globulin. Only lymphocytes obtained from animals which presented a delayed reaction to these antigens underwent transformation in vitro when cul-

tured in the presence of the specific antigens. Negative results were obtained in cultures of lymphocytes from guinea pigs with circulating antibodies. Similar results were obtained by Oppenheim et al (278) using PPD and guinea pig albumin conjugated to orthanilic acid as antigens.

Opposite results to those of Mills (277) and Oppenheim et al (278) were reported by Visher et al (279) and by Visher and Stastny (280). They immunized rabbits with keyhole limpet hemocyanin and found that cultures of peripheral lymphocytes from these animals, all of whom had demonstrable circulating antibody, proliferated and incorporated tritiated thymidine in the presence of the antigen. Similar results were reported by Dutton and Eady (281), Dutton and Bulman (281a), Chapman et al (282), Dutton and Page (384), Benezra et al (283) and Harris (284, 285). Zavaleta and Stastny (286) obtained blastogenesis and mitosis, in the presence of specific antigen, with lymphocyte cultures obtained from rabbits previously immunized to thyroglobulin and incubated with this antigen.

2.3.2.4 SINGLE CELL CULTURE IN VITRO

PRIMARY AND SECONDARY RESPONSES

Nossal and Lederberg (121), Nossal (122-125), Makela and Nossal (24, 126, 127) and Nossal and Makela (23, 128, 129) immunized rats with various Salmonella antigens, isolated lymph node cells by micro manipulation and incubated these cells in microdrops of Eisen's medium containing a known number of Salmonella organisms. They observed agglutination and immobilization of the bacteria microscopically and concluded that most anti body-producing cells belong to the plasma cell series. Occasionally, they observed lymphocytes with abundant cytoplasm and described this cell as an "aberrant type of plasma cell".

Using the single cell culture technique, Nossal and Makela (23) and Makela and Nossal (24) ascribed the difference between a primary and a secondary immune response to the greater number of antibody-forming plasma cells proliferating in the lymph nodes after the secondary injection of the antigen. They also concluded that, during both the primary and secondary immune responses to multiple antigens, antibody producing rat lymph node cells could form antibody of one specificity only. Less than two percent of the cells demonstrated antibody formation towards more than one antigen. They suggested that antibody-forming plasma cells are the descendants of primitive lymphocytes which proliferate rapidly even in the unimmunized animal and that the exposure to antigen provides

a stimulus for some of these cells to differentiate into plasma cells.

On the other hand, Attardi et al (30, 130) immunized rabbits with whole phage particles and later isolated lymph node cells by free hand manipulation and incubated them in droplets of medium for 48 hours. They observed these cultures for phage neutralizing activity and stated that antibody production was by no means confined to the plasma-cell during both the primary and secondary immune responses. Though a greater proportion of plasma cells than lymphocytes were observed to form antibody, the total number of antibody-forming small lymphocytes was greater than the total number of antibody-forming plasma cells. Each lymphocyte contained approximately as much antibody as a plasma cell. They further claimed that fifteen to twenty-two per cent of the plasma cells obtained from rabbit popliteal lymph nodes following immunization with two unrelated phage antigens were capable of forming equal amounts of antibody directed to the two antigenically-distinct antigens. This work, therefore, contradicts that of Nossal and colleagues referred to above.

2.3.2.5 BLASTOGENIC AND MITOGENIC RESPONSE OF LYMPHOCYTES TO PHYTOHEMAGGLUTININ IN VITRO

A. HUMAN CELLS

(I) Peripheral Lymphocytes

There is good evidence from the reports of Marshall and Roberts (287), MacKinney et al (288), Hastings et al (289) and Tanka et al (290) that the proliferating blast cells in cultures of human peripheral blood in the presence of phytohemagglutinin (PHA) originates from small lymphocytes. Robbins (291) and Lindahl-Kiessling and Book (292) reported a normal response to PHA in cultures of peripheral lymphocytes obtained from foetuses and newborns. Pisciotta et al (293) analyzed the PHA response in peripheral lymphocytes obtained from healthy individuals ranging in age from 1 to 98 years. The ability of the lymphocyte to undergo blast transformation in response to PHA stimulation appears to reach a maximum at puberty and to decline uniformly with age thereafter. The data suggest that the diminished in vitro blastogenic response with age may be attributed to a cellular, rather than a humoral, factor.

(II) Thoracic Duct Cells

Cooper (294), Cooper et al (295) and Lindahl-Kiessling and Werner (296) obtained an appreciable degree of cell transformation by incubating human thoracic duct cells with PHA in vitro.

(III) Lymph Node Cells

Rubio and Zajicek (297) reported that 70 to 90 percent of the cells aspirated from human lymph nodes and cultured in vitro with PHA transformed to blast cells after three days of incubation. Similar results were reported by Winkenstein and Craddock (298) and McIntyre and Segal (299).

(IV) Thymus Cells

Bain and Gould (300), Lischner and Punnett (301) and Wilson (302) observed blastogenesis of human thymus cells in the presence of PHA. McIntyre and Segel (303) and Strosselli et al (304), however, obtained negative results when human thymus cells were cultured in vitro with PHA. Claman (305) reported that normal human thymus contains at least two populations of lymphoid cells: a major component which shows antonomous and unsustained proliferative activity and does not respond to PHA and a second minor cellular component which transforms and proliferates in response to PHA.

(V) Tonsillar Cells

Oettgen et al (306) studied the in vitro behaviour of human tonsillar lymphocytes. In comparison with peripheral blood lymphocytes, these cells showed a higher degree of transformation in the presence of PHA. On the other hand, Goldfarb and Ullal (307) did not obtain blast cell transformation upon incubating human tonsillar lymphocytes with PHA.

(VI) Spleen Cells

Bain and Gould (300) and Conen and Erkman (308) obtained a satisfactory blastogenic response when spleen cells from foetuses and infants were stimulated with PHA in vitro.

(VII) Bone Marrow Cells

In the absence of any communications in the literature dealing with the effect of PHA on bone marrow cells, it may be assumed that this aspect of bone marrow activity has not been investigated.

B. ANIMAL CELLS

(I) Peripheral Lymphocytes

RATS

Knight et al (309) reported that rat peripheral lymphocytes survived poorly in the presence of PHA in vitro. Marshall and Roberts (225) and Williams and Ray (310) were unable to show transformation of these cells in the presence of PHA. Elrod and Schreck (311), however, found that rat peripheral lymphocytes responded better to PHA when incubated in the presence of rabbit serum. Metcalf (312) showed that these cells did not respond to PHA when cultured with rat plasma, but were able to transform well in the presence of rat or human serum. Johnson et al (313) showed that diffusion chambers placed in the ventral abdominal wall of rats provided optimal conditions to obtain a blastogenic response to PHA with rat peripheral lymphocytes.

MICE

Knight et al (309) reported poor survival with only occasional transformed cells in cultures of mouse lymphocytes in the presence of PHA. Neither Elves (314), Marshall and Roberts (225) nor Willard et al (315) were able to show survival of mouse lymphocytes in culture. However, using diffusion chambers placed into isologous mice, Willard et al (315) obtained some transformation in the presence of PHA. Macario (316) reported a good degree of blast transformation when these cells were cultured in the presence of PHA.

GUINEA PIGS

Knight et al (309), Sabesin (317), Aspergren and Rorsman (274) and Nichols and Levan (318) reported that guinea pig lymphocytes could be stimulated to transform into blast cells by PHA in vitro. Marshall and Roberts (225), however, could not demonstrate such transformation with guinea pig peripheral lymphocytes.

RABBITS

Knight et al (309), Sabesin (317), Nicholas and Levan (318), Fikrig et al (319) and Sell et al (320) observed a marked stimulation of rabbit peripheral lymphocytes incubated in vitro with PHA. Williams and Ray (310) and Marshall and Roberts (225), however, did not observe blast transformation of rabbit lymphocytes after in vitro incubation with PHA.

MONKEYS

Knight et al (309) reports that peripheral lymphocytes obtained from rhesus monkeys responded markedly to the in vitro stimulation by PHA to a degree comparable to that obtained with human lymphocytes.

HAMSTERS

Knight et al (309) were unable to show blast transformation by incubating hamster peripheral lymphocytes with PHA in vitro.

(II) Thoracic Duct Cells

RATS

Rieke and Schwartz (321), Rieke (322) and Metcalf (312) were able to show blast transformation in cultures of rat lymphocytes derived from the thoracic duct lymph in response to PHA. They reported that blastogenesis was inhibited if the rat serum was replaced by rat plasma in the culture medium.

RABBITS

Fikrig et al (319) demonstrated that rabbit lymphocytes obtained from the mesenteric lymph transformed into blast cells when incubated in vitro in the presence of PHA.

(III) Lymph Node Cells

RATS

Metcalf (312) and Metcalf and Osmond (323) incubated

rat lymph node cells with PHA in the presence of either rat serum or human plasma. They obtained a large number of blast cells after three days of culture.

RABBITS

Foft and Romero (324) reported blast cell transformation in cultures of lymphocytes from rabbit lymph nodes following incubation with PHA in vitro.

(IV) Spleen Cells

RATS

Elrod and Schrek (311), Metcalf (312) and Metcalf and Osmond (323) observed blast transformation in cultures of rat splenic lymphocytes in the presence of PHA.

RABBITS

Harris and Littleton (325) and Schrek and Batra (326) showed that rabbit splenic lymphocytes could transform into blast cells in the presence of PHA in vitro.

CHICKENS

Weber (327) cultured spleen cells from four to ten weeks old White Leghorn chickens in the presence of PHA and demonstrated a marked blast transformation after 48 hours.

(V) Thymus Cells

RATS

Schwartz and Rieke (328) showed that small lymphocytes

from rat thymus cultured in vitro with PHA were transformed into large blast cells. Thymic cells from neonatal animals failed to respond to PHA. Metcalf (312) and Metcalf and Osmond (323) also reported a good response of rat thymic lymphocytes to PHA in vitro.

PIGS

Weber (327) studied the in vitro response to PHA of lymphocytes obtained from pig thymus. He observed that within the first hour of culture six to eighteen percent of the lymphocytes were synthesizing DNA. During the following 24 hours a sharp decrease in the number of DNA synthesizing cells was observed. This was followed by a definite peak in DNA synthesis and mitotic response in a minority of the cells between 48-54 hours of culture. Weber (329) claimed that the lymphocytes capable of responding to PHA are located predominently in the medulla of the thymus.

RABBITS

Schrek and Batra (326) reported that PHA induced the transformation of rabbit thymic lymphocytes into lymphoblastoid cells.

CHICKENS

Weber (327) demonstrated that lymphocytes obtained from four to ten week old chickens transformed to blast cells when cultured with PHA in vitro.

(VI) Appendix Cells

RABBITS

Schrek and Batra (326) failed to demonstrate transformation in rabbit appendix lymphocytes cultured with PHA in vitro.

(VII) Bone Marrow Cells

There has thus far been no report as to the capacity of bone marrow lymphocytes to transform to blast cells in the presence of PHA.

2.3.2.6 BLASTOGENIC AND MITOGENIC AGENTS OTHER THAN PHYTOHEMAGGLUTININ AND ANTIGENS

A. ALLOGENEIC CELLS

Schrek and Donnelly (330) observed a number of large cells with large nuclei and nucleoli in a culture consisting of a mixture of bloods from two patients with hemochromatosis. However, they did not elaborate on this observation. Bain, Vas and Lowenstein (331) reported that when leukocytes from two normal, unrelated subjects were mixed together and cultured, a number of them transformed into large basophilic cells that could synthesize DNA and undergo mitosis. The blast cell obtained in the mixed leukocyte reaction was morphologically similar to that obtained following incubation with PHA although it was a less intense reaction and involved changes in a smaller proportion of the lymphocytes. Erythrocytes, plasma or platelets had no effect in the mixed leukocyte reaction. These authors were the first to suggest a relationship of the mixed leukocyte reaction to homograft immunity. Hashem and Carr (332) obtained similar results when they incubated lymphocytes of two unrelated individuals.

Jones (333) observed that the presence of large numbers of polymorphonuclear leukocytes resulted in higher levels of blastoid transformation than when polymorphs were absent.

Kasakura and Lowenstein (334) and Gordon and Maclean (335) reported that the cell-free medium obtained from single or mixed leukocyte cultures was able to induce blastogenesis when added

to homologous leukocytes. Kasakura and Lowenstein (336) studied the physical and chemical properties of this blastogenic material and suggested that it may contain transplantation antigens. It has also been demonstrated that blastogenesis can be obtained when extracts of cells from one individual are mixed with intact cells from another individual (337). Hashem (338) noted that ribosomal RNA extracted from peripheral lymphocytes which had been incubated in vitro with the specific antigens to which the donor had been sensitized was also capable of promoting transformation and mitosis when added to cultures of autologous lymphocytes. In this case, the RNA appeared to be acting as the antigen to stimulate autologous cells to undergo blastogenesis. Hashem and Rosen (339) fractionated cell extracts and added the fractions to cultures of homologous lymphocytes. They found that blastogenesis was induced by the whole cell extract, cell-wall nuclear and ribosomal fractions but not by the mitochondrial fraction.

Bain and Lowenstein (340) carried out genetic studies with the mixed leukocyte reaction. By mixing leukocytes in culture from 15 sibling pairs, they noted that the leukocytes of most individuals reacted less strongly with those of their siblings than with the cells of unrelated subjects. Bach and Hirschhorn (337) observed a correlation between the degree of the blastogenic response in a culture of lymphocytes from two unrelated individuals and the degree of cross-reactivity of grafts from the two individuals placed on a third unrelated recipient. Oppenheim et al (341) and Moynihan

et al (342) examined mixed leukocyte cultures of skin graft recipients and a normal donor before, during and after skin transplantation and observed an increase in lymphocyte proliferation and blastogenesis at the time of graft rejection. Silvers et al (343) reported that mixed leukocyte cultures of rats showed blastogenesis only when the donors of the leukocytes differed at the important Ag-B histocompatibility locus.

In order to determine whether both donor cells in the mixed leukocyte reaction are responding equally, attempts have been made to inactivate the cells of one of the donor pairs. The treatment of the cells of one individual with mitomycin-C (344). On with x-irradiation (345) has been shown to result in the loss of their capacity to undergo blastogenesis in mixed leukocyte reactions; however these cells are able to stimulate homologous untreated cells to undergo blastogenesis.

B. ANTI-LEUKOCYTE SERUM

Grasbeck et al (346, 347) immunized rabbits by the intravenous injection of a concentrate of human peripheral leukocytes. All the rabbits produced antisera which were mitogenic in cell cultures of human peripheral leukocytes. The antisera had a lytic effect on the cells which was related to the presence of complement. Absorption of the antiserum with leukocytes removed the mitogen. Holt et al (348) also reported that rabbit anti-human leukocyte antisera had a blastogenic effect on human lymphocytes in vitro.

Knight and Ling (349) reported that two out of nine rabbits immunized with homologous leukocytes produced antisera which caused blast formation and stimulated DNA synthesis in leukocytes of the donor rabbits in vitro. Heterologous antisera obtained by injecting rabbit leukocytes into rats and guinea pigs were cytotoxic to rabbit leukocytes in vitro. These antisera caused leukoagglutination but did not stimulate DNA synthesis and blast formation.

C. ANTI-PROTEIN SERUM

Sell and Gell (350) reported that rabbit lymphocytes may be stimulated in vitro with specific rabbit anti-allotype sera to transform into blast cells and to synthesize DNA. This transformation only occurred when the donor cells were obtained from a given gamma-globulin allotype (As4) and cultured in the presence of an antiserum prepared against the given allotype (anti-As4). Heterologous anti rabbit Y-globulin sera (sheep, goat and guinea pig) also induced significant blast transformation and DNA synthesis in rabbit lymphocytes. Gell and Sell (351) stated that this effect could be obtained with specific antisera directed against all six of the well characterized allotype determinants of rabbit IgG (As 1, 2, 3, 4, 5 and 6) and with sheep anti-rabbit whole serum (320). Sell and Gell (353) showed that lymphocytes from the peripheral blood of newborn rabbits heterozygous for IgG allotype As4 and As5, or As5 and As6, obtained at an age when only the maternal allotypic determinants are detectable in the serum, could be stimulated to

transform into blast cells when incubated with antiallotype sera directed against the determinants controlled by both the maternal and paternal chromosomes. The in vitro blastogenic response of rabbit lymphocytes incubated with sheep antisera to rabbit IgG subunits, with sheep antisera to rabbit IgA and IgM and with the $F(ab)^2$ and F(ab) fragments of sheep antibody to rabbit IgG was demonstrated by Sell (354-356).

Oppenheim et al (357) immunized monkeys with purified preparations of human immunoglobulins: IgG, IgA, IgM and Bence Jones protein. Each of the antisera incubated with washed human peripheral lymphocytes stimulated DNA synthesis.

D. SERUM FACTORS

Sabesin (358) cultured small lymphocytes from rabbits and guinea pigs in vitro and observed blast transformation in 40-70 percent of the peripheral lymphocytes on the fifth day of cultures in the presence of either fetal calf or autologous serum. On the other hand, Johnson and Russel (359) found that fetal calf serum, but not autologous serum, in the culture medium was blastogenic to lymphocytes. Similar findings were reported by Woodliff and Onesti (360). However, Caron (361) observed no spontaneous blastoid transformation when the cells were cultured in the presence of autologous or fetal calf serum. Pulvertaft and Pulvertaft (362) reported the absence of blastogenesis by fetal calf serum but noted spontaneous blastogenesis in cultures of lymphocytes obtained from the umbilical

cord-vein. They suggested that this latter transformation is probably due to the mixture of maternal and infant lymphocytes. Gill (363) showed spontaneous transformation in cultures of lymphocytes obtained from penicillin-sensitive patients during the allergic drug reaction.

E. POKEWEED

Farnes et al (364) reported that extracts of the plant phytolacca-Americana, pokeweed, induced transformation of human lymphocytes in vitro. Borjesson et al (365) documented certain biological properties of pokeweed (hemagglutinating and mitogenic). Chessin et al (366) showed that pokeweed and PHA stimulated lymphocytes behave in a similar fashion in vitro with RNA synthesis preceding the onset of DNA synthesis by 24 hours.

F. STREPTOLYSIN-S

Hirschhorn et al (367) have demonstrated that a non-antigenic preperation of the B-hemolytic streptococcus, streptolysin S (SLS), has the property of inducing blastogenesis when incubated with normal human peripheral lymphocytes. This effect of SLS was absent with lymphocytes obtained from patients with untreated rheumatic fever or from an occasional patient with streptococcal infection.

G. STAPHYLOCOCCAL FILTRATE

Ling and Husband (246) found that a staphyloccocal filtrate (SF) induced transformation in most of the peripheral lymphocytes from normal human donors.

H. ENZYMES

Mazzèi et al (368) demonstrated that trypsin, chymotrypsin and papain have mitogenic action on peripheral lymphocytes from normal humans in vitro.

I. MICROWAVE IRRADIATION

Stodolnik-Baranska (369) showed that microwave irradiation induces blastogenesis and mitosis in cultures of human peripheral lymphocytes.

2.3.2.7 MECHANISM OF BLAST CELL TRANSFORMATION INDUCED BY ANTIGEN(S) AND OTHER MITOGENIC AGENTS IN VITRO

The mechanism whereby antigens and PHA induce blastogenesis and mitosis of lymphocytes in tissue culture is still highly controversial in spite of the many studies which have been carried out over the past few years. (See Chapters 2.3.2.3 and 2.3.2.5).

The morphology of the blast cell obtained after PHA stimulation is very similar, both by light and electron microscopy, to that of the blast cell induced as a consequence of incubation which specific antigen (287, 290, 295, 370, 371). Furthermore, blast cells induced by PHA are capable of synthesizing gamma-globulin (337, 372, 373, 374) similar to cells stimulated by antigen in vitro (244). The protection to x-irradiation afforded by PHA in vitro (375-377) may appear to be a unique property not possessed by antigens in general. However, it has recently been noted that old tuberculin is also capable of imparting radioprotection when incubated with lymphocytes obtained from PPD-positive patients but not when incubated with cells of PPD-negative patients (378). The authors observed a relation between old tuberculin concentration, blastoid transformation and radioprotection and suggested that the radioprotective effect of old tuberculin has an immunologic basis.

The fact that it is essentially the small lymphocyte which is transformed by antigen and PHA in vitro and that the small lymphocyte possesses all the attributes required for immunologic competence (379) cannot be ignored. This cell is capable of under-

going blast transformation under a variety of antigenic stimuli, both in vivo and in vitro. In vivo, blast transformation has been observed in contact hypersensitivity reactions (380, 381), graft-versus-host reactions (379) and skin homograft rejection reactions (382). The morphology of the blast cell arising in each of these immune reactions has been found to closely resemble that of the PHA-induced blast cell in vitro (379, 383).

If the specific blast transformation of sensitized lymphocytes after exposure to specific antigens, by its high degree of specificity, is immunological in nature, a summation of effects should be obtained when lymphocytes from an individual sensitized to more than one antigen are incubated in the presence of these antigens. However, summation of action was not observed by Caron (233), Cowling and Quaglino (247) and by Girard et al (248). Ling and Husband (246) suggested that they obtained an apparent summation of effects with two different antigens, but they did not present any data.

Harris (284, 285) showed that spleen and peritoneal exudate cells from rabbits previously immunized to heterologous protein antigens could be stimulated to synthesize DNA when incubated with the same antigen in vitro. He observed that macrophages were surrounded by non-dividing lymphocytes during the early period of the culture and postulated that this cell-cell interaction resulted in enhancement of DNA synthesis by responding cells, with the small lymphocytes acting as messengers between cells containing

antigen and those dividing in response to its presence. It is impossible to avoid the interpretation that the close contact which develops between macrophage and lymphocyte is relevant since the cells which aggregated around the macrophages did not incorporate tritiated thymidine while most of the labelling was observed in other dividing large cells. The possibility exists that cells which have left the vicinity of the macrophages went on to divide. On the other hand, Dutton and Eady (281) and Dutton and Page (384) claimed that the antigen stimulated an already dividing population of cells to divide more rapidly.

In various human diseased states, a good correlation has been found between impaired delayed hypersensitivity in vivo and depressed in vitro transformation of lymphocytes with PHA (341, 385), suggesting that PHA is measuring the immunocompetence of the lymphocytes. Blastogenesis has also been shown to occur when lymphocytes are cultured in the presence of anti-lymphocyte and anti-immunoglobulin antisera or when they are mixed in cell culture with lymphocytes of an unrelated, but not from a genetically-identical, individual (See Chapter 2.3.2.6). The presence of blast cells in all of these reactions strongly suggests that this cell plays an important role in immunological processes.

In PHA stimulated cultures the percentage of transformed cells is 70-90 per cent after three days of incubation whereas only 5-30 per cent of the cells incubated with antigen are transformed

after 7-10 days of culture (370). Furthermore, the in vitro transformation with PHA does not require the presence of macrophages (314, 386), whereas macrophages are necessary for the antigen-induced blastogenic response (386-388). Recently, however, it has been shown that blastogenesis can be induced upon incubation of PPD with a highly purified suspension of small lymphocytes obtained from PPD-positive patients (227). It has also been demonstrated that PHA can stimulate lymphocytes, previously immunized to various protein antigens, to secrete greater amounts of the specific antibody than can the specific antigens in vitro (103, 389).

The non-immunological mechanism of PHA transformation is further supported by the findings that PHA can stimulate epithelial cells in the skin (390), free-living amoeba (391, 392) and non-lymphoid cell lines (393) to undergo mitosis.

An alternative hypothesis as to the mechanism of PHA action was proposed by Hirschhorn et al (245). They suggested that PHA exerts its action by attaching to some immunologically nonspecific surface structure on the lymphocyte. This idea was fostered by the observation that PHA causes agglutination of lymphocytes and that both this property and the blastogenic activity of PHA are lost by absorption of PHA with lymphocytes. A similar view was expressed by Grasbeck et al (346, 347), who demonstrated that rabbit anti-human leukocyte antiserum agglutinated and transformed a high percentage of the cultured human lymphocytes. The probability that PHA

attaches to the cell surface was suggested by Vassar and Culling (394) who showed that the electrophoretic mobility of lymphoid cells was changed after treatment with PHA. On the other hand, the studies on the cell localization of PHA (395-397), although not conclusive, suggest that PHA is present within the cell, and is not absorbed onto the surface of the cell.

The above hypothesis is, however, unlikely since Borjesson et al (398) showed that coating human peripheral lymphocytes with Vi polysaccharide completely suppressed the agglutination of these cells when PHA was added to the culture. Nevertheless the normal complement of blast cells was observed at the termination of the culture.

In conclusion although a great deal of work has been reported with respect to the action of various mitogenic agents on lymphoid cells in vitro, the exact mechanism of their action is not understood.

TABLE I-A

PRIMARY ANTIBODY RESPONSE BY FRAGMENT CULTURE IN VITRO. ANTIGEN ADMINISTERED IN VIVO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
Stevens and McKenna (159)	Salmonella typhosa	Rabbit Spleen fragments	Medium	Antibody synthesis shown by hemagglutination.
Mountain (143, 160)	Salmonella typhosa	Rabbit Spleen fragments	Medium with 10 per cent rabbit serum	Antibody synthesis shown by hemagglutination.
Kong and Johnson (161)	Bovine gamma-globulin	Rabbit Spleen fragments	Medium with 20 per cent rabbit serum	Antibody synthesis shown by hemagglutination. 5-fluro-2'-deoxyuridine inhibited antibody synthesis which could be reversed partially by thymidine.
Schoenberg et al (162)	Diphtheria toxoid	Rabbit Spleen fragments	Medium with 20 per cent rabbit serum	Antibody synthesis shown by labelled amino acid incorporation and hemagglutination.

TABLE I-A (Continued)

PRIMARY ANTIBODY RESPONSE BY FRAGMENT CULTURE IN VITRO. ANTIGEN ADMINISTERED IN VIVO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
La Via et al (163)	Salmonella typhosa	Rat spleen fragments	Eagle's medium with 30-50 per cent rat serum	Demonstration of antibody synthesis by labelled amino acid incorporation
Pernis et al (164)	Diphtheria toxoid	Rabbit lymph node fragments	Hanks solution with 0.5 per cent ovalbumin	Antibody synthesis measured by labelled amino acid incorporation and hemagglutination
Berglund et al (166)	Sheep erythrocytes	Mouse spleen-frozen section	Medium in gel	Demonstration of hemolysis in gel (plaque formation) up to 113 days following immunization
Leibowitz and Parks (167)	Bovine gamma-globulin injected into cornea	Rabbit cornea fragments	Medium with 10 per cent rabbit serum	Demonstration of antibody formed in vitro by capacity to induce PCA reaction in guinea pigs

TABLE I-B

PRIMARY ANTIBODY RESPONSE BY FRAGMENT CULTURE IN VITRO. ANTIGEN INCUBATED IN VITRO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
Saunders and King (148)	Coliphage R17	Mouse spleen and thymus fragments cultured together	Eagle's medium with 10 per cent calf serum	Antibody response measured by phage neutralization
Tao and Uhr (168)	Coliphage ϕ X 174	Rabbit lymph node fragments	Eagle's medium with 20 per cent rabbit serum	Antibody response measured by phage neutralization
Globerson and Auerbach (169)	Sheep erythrocytes	Mice spleen fragments	Eagle's medium with 10 per cent horse serum in millipore chambers	Administration of PHA or adjuvant in vivo prior to culture resulted in appearance of hemagglutinins and hemolysins in vitro
Globerson and Auerbach (170)	Sheep and chick erythrocytes	Mice spleen, lymph node and thymus fragments	Eagle's medium with 10 per cent horse serum in millipore chambers	Lymph node and spleen gave agglutinin titers whereas thymus failed to give any response

TABLE II-A

SECONDARY ANTIBODY RESPONSE BY FRAGMENT CULTURE IN VITRO. ANTIGEN ADMINISTERED IN VIVO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
Stavitsky (111)	Diphtheria tetanus toxoid	Rabbit lymph node and spleen fragments	Fisher's medium	Antibody synthesis shown by hemagglutination
Keuning and Van der Slikee (22)	Paratyphi antigen	Rabbit spleen fragments	Medium with 20 per cent rabbit serum	Antibody synthesis shown by hemagglutination. Role of various cell types in antibody formation (See Chapter 2.1.2)
Thorbecke and Keuning (171)	Paratyphi H antigen	Rabbit spleen and lymph node fragments	Medium with 20 per cent rabbit serum	Antibody synthesis shown by hemagglutination
McKenna and Stevens (172)	Bovine serum albumin and bovine gamma-globulin	Rabbit spleen fragments	Modified Trowell's medium	Antibody synthesis shown by hemagglutination
Askonas et al (173)	Ovalbumin	Rabbit spleen, lymph node and bone marrow slices	Medium	Demonstrated labelled amino acid incorporation into Y-globulin fractions

TABLE II-A(Continued)

SECONDARY ANTIBODY RESPONSE BY FRAGMENT CULTURE IN VITRO. ANTIGEN ADMINISTERED IN VIVO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
Ranney and London (174)	Pneumococcal polysaccharide	Rabbit spleen slices	Medium	Antibody synthesis shown by labelled amino acid incorporation
Askonas and Humphrey (97)	Penumococcal polysaccharide and ovalbumin	Rabbit lymph node spleen, bone marrow, lung, liver and kidney slices	Medium	Antibody synthesis by lymph node and spleen fragments. Shown by labelled amino acid incorporation.
Wolf and Stavitsky (144)	Diphtheria toxoid, ovalbumin, bovine gamma-globulin and bovine serum albumin	Rabbit lymph node and spleen fragments	Medium with 20 per cent rabbit serum	Antibody synthesis measured by labelled amino acid incorporation, hemagglutination and quantitative precipitin. Addition of purines, pyrimidines, vitamins, coenzymes, lipids and carbohydrates did not effect antibody synthesis

TABLE II-A (Continued)

SECONDARY ANTIBODY RESPONSE BY FRAGMENT CULTURE IN VITRO. ANTIGEN ADMINISTERED IN VIVO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
Keston and Katchen (176)	Ovalbumin, bovine gamma-globulin and human gamma-globulin	Rabbit spleen, lymph node, lung and liver slices	Homologous serum or modified Krebs-ringer bicarbonate	Antibody synthesis shown by labelled amino acid incorporation. Only lymph node and spleen gave response.
Lazda and Starr (177)	Bovine gamma-globulin	Rabbit spleen fragments	Medium with 10 per cent rabbit serum	Antibody synthesis shown by labelled amino acid incorporation. Inhibition of antibody synthesis by actinomycin.
Thorbecke (149)	Bovine gamma-globulin and ovalbumin	Rabbit spleen and lymph node fragments	Medium with 10 per cent calf serum	Antibody synthesis shown by labelled amino acid incorporation.

TABLE II-B

SECONDARY ANTIBODY RESPONSE BY FRAGMENT CULTURE IN VITRO. ANTIGEN INCUBATED IN VITRO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
Michaelides (100) Michaelides and Coons (99)	Diphtheria toxoid and bovine serum albumin	Rabbit lymph node fragments	Medium with 25 per cent rabbit serum	Demonstration of antibody synthesis by hemagglutination
O'Brien et al (101)	Diphtheria toxoid and bovine serum albumin	Rabbit lymph node fragments	Medium with 25 per cent rabbit serum	Demonstration of antibody synthesis by hemagglutination
Stavitsky (178) Stavitsky and Wolf (179)	Diphtheria toxoid, bovine gammaglobulin and bovine serum albumin	Rabbit lymph node and spleen fragments	Medium with 20 per cent rabbit serum	Demonstration of antibody synthesis by labelled amino acid incorporation, hemagglutination and quantitative precipitation
Halliday and Garvey (102)	Bovine serum albumin and hemocyanin	Rabbit lymph node and spleen fragments	Medium with hydrocortisone	Antibody synthesis shown by hemagglutination. Hydrocortisone enhances antibody synthesis
Kritzman and Harper (180)	Human serum albumin	Rabbit spleen and lymph node fragments	Medium 199 with 20 per cent rabbit serum	Antibody synthesis shown by hemagglutination

TABLE II-B (Continued)

SECONDARY ANTIBODY RESPONSE BY FRAGMENT CULTURE IN VITRO. ANTIGEN INCUBATED IN VITRO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
O'Brien and Coons (181)	Diphtheria toxoid and bovine serum albumin	Rabbit lymph node fragments	Medium with 25 per cent rabbit serum	Antibody synthesis shown by hemagglutination
Stevens and McKenna (182)	Casein and bovine gamma-globulin	Rabbit spleen fragments	Modified Trowell's medium with 40 per cent rabbit serum	Antibody synthesis shown by hemagglutination. Inhibition of antibody synthesis by endotoxin
Ambrose and Coons (183)	Bovine serum albumin and diphtheria toxoid	Rabbit lymph node fragments	Eagle's medium with 25 per cent rabbit serum	Antibody synthesis shown by hemagglutination. Inhibition of antibody synthesis by chloramphenicol
Juhasz and Rose (104)	Rat serum albumin	Rabbit lymph node fragments	Eagle's medium with 20 per cent rabbit serum	Antibody synthesis shown by hemagglutination. Delay in appearance of antibody when serine was used

TABLE II-B (Continued)

SECONDARY ANTIBODY RESPONSE BY FRAGMENT CULTURE IN VITRO. ANTIGEN INCUBATED IN VITRO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
Juhasz and Richter (105)	Rat serum albumin and diphtheria toxoid	Rabbit lymph node fragments	Eagle's medium with 20 per cent rabbit serum or serine	Anti-diphtheria antibody was increased by the addition of hydrocortisone (not anti-rat albumin antibody) as measured by hemagglutination
Richter et al (106)	Rat serum albumin	Rabbit lymph node fragments	Eagle's medium with 20 per cent rabbit serum. Cultured between gel foam and gel film	Antibody synthesis shown by hemagglutination
Richards et al (184)	Bovine pancreatic ribonuclease	Rabbit lymph node fragments	Eagle's medium	Demonstration of antibody synthesis by labelled amino acid incorporation
Dresser (185)	Bovine gamma-globulin	Guinea pig lymph node fragments	Eagle's medium with fetal calf or guinea pig serum	Antibody synthesis measured by labelled amino acid incorporation. Higher antibody synthesis with fetal calf serum.

TABLE II-B (Continued)

SECONDARY ANTIBODY RESPONSE BY FRAGMENT CULTURE IN VITRO. ANTIGEN INCUBATED IN VITRO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
Tao (103)	Bovine serum albumin and bovine gamma-globulin	Rabbit lymph node fragments	Medium with 20 per cent rabbit serum	Non specific secondary immune response following phytohemagglutinin administration in vitro as shown by hemagglutination

TABLE III-A

PRIMARY ANTIBODY RESPONSE BY CELL SUSPENSION IN VITRO. ANTIGEN ADMINISTERED IN VIVO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
Roberts et al (186)	Sheep erythrocytes	Rat spleen and lymph node mince	100 per cent rat serum	Antibody synthesis measured by hemagglutination
Langevoort et al (187)	Bovine gamma-globulin	Rabbit spleen mince	Medium with per cent rabbit serum	Antibody synthesis shown by hemagglutination
Jerne and Nordin (188)	Sheep erythrocytes	Mouse spleen cell suspension	Medium in gel	Demonstration of plaque formation
Strander (189)	Sheep erythrocytes	Mouse spleen cell suspension	Medium in gel	Demonstration of plaque formation which could be inhibited by puromycin and actinomycin-D
Tannenberg (190)	Sheep erythrocytes	Mouse spleen cell suspension	Medium in gel	Demonstration of plaque formation

TABLE III-A (Continued)

PRIMARY ANTIBODY RESPONSE BY CELL SUSPENSION IN VITRO. ANTIGEN ADMINISTERED IN VIVO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
Massie and Frick (191)	Potassium Penicillin-G attached to hemocyanin	Mice spleen cell suspension	Medium in gel	Demonstration of plaque formation
Kern and Eisen (192)	Bovine gamma-globulin	Guinea pig lymph node cell suspension	Medium	Increase in the incorporation of phosphate by the cells. (Antibody synthesis not measured.)

TABLE III-B

PRIMARY ANTIBODY RESPONSE BY CELL SUSPENSION IN VITRO ANTIGEN INCUBATED IN VITRO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
Fishman (193, 194)	Bacteriophage T2	Rat lymph node and macrophage cell suspension	Millipore chambers implanted in peritoneal cavity	Antibody synthesis shown by phage inactivation
Friedman et al (195)	Bacteriophage T2	Rat lymph node and paritoneal exudate cell suspension	Trowell's medium	Antibody synthesis shown by complement fixation test
McKenna and Stevens (196)	Sterile mineral oil (in vivo) bovine gamma-globulin and ovalbumin (in vitro)	Rabbit peritoneal exudate cell suspension	Medium 199 with 25 per cent rabbit serum	Antibody synthesis shown by hemagglutination (titers up to 1:28)
Mishell and Dutton (107)	Sheep erythrocytes	Mice spleen cell suspension	Medium [illegible] in gel	Demonstration of plaque formation
Marbrook (197)	Sheep and horse erythrocytes	Mice spleen cell suspension	Medium [illegible] in gel	Demonstration of plaque formation

TABLE III-B (Continued)

PRIMARY ANTIBODY RESPONSE BY CELL SUSPENSION IN VITRO ANTIGEN INCUBATED IN VITRO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
Zaalberg et al (198)	Sheep erythrocytes	Mouse spleen cell suspension	Medium in gel	Demonstration of rosette and plaque formation
Bussard and Anderson (199)	Sheep erythrocytes	Mouse spleen cell suspension	Diffusion chambers implanted in peritoneal cavity	Demonstration of plaque formation
Mosier (200)	Sheep erythrocytes	Mouse spleen cell suspension	Medium in gel	Demonstration of plaque formation and its dependence on macrophages
Hege and Cole (201)	Sheep erythrocytes	Mouse spleen cell suspension	Medium in gel	Demonstration of plaque formation. Phytohemagglutinin enhances plaque formation. (X-irradiation and neonatal thymectomy has no effects).

TABLE III-B (Continued)

PRIMARY ANTIBODY RESPONSE BY CELL SUSPENSION IN VITRO ANTIGEN INCUBATED IN VITRO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
Robinson et al (202)	Sheep erythrocytes	Mouse spleen cell suspension	Medium in gel	Demonstration of plaque formation and its inhibition by colchicin, actinomycin-D and rabbit anti mouse globulin
Bussard and Lurie (203)	Sheep erythrocytes	Mouse peritoneal cell suspension	Medium in gel	Demonstration of plaque formation and its inhibition by puromycin
Beninelli and Wedderbern (204)	Sheep erythrocytes	Mouse peritoneal cell suspension	Medium in gel	Demonstration of plaque formation (Lymphocytes form more plaques than macrophages)
Bussard (205)	Sheep erythrocytes	Mouse peritoneal cell suspension	Medium in gel	Demonstration of plaque formation

TABLE IV-A

SECONDARY ANTIBODY RESPONSE BY CELL SUSPENSION IN VITRO. ANTIGEN ADMINISTERED IN VIVO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
Thorbecke et al (151)	Bovine gamma-globulin and ovalbumin	Rabbit spleen mince	Medium with 10 per cent rabbit or calf serum	Antibody synthesis measured by hemagglutination
Cremer (206)	Bovine serum albumin and hemocyanin	Rabbit spleen cell suspension	Medium with 15 per cent rabbit serum	Antibody synthesis shown by hemagglutination and ring test
Roberts et al (186)	Sheep erythrocytes	Rat spleen and lymph node mince	100 per cent rat serum	Antibody synthesis shown by hemagglutination
Fleischman (207)	Phage T2	Rabbit lymph node cill suspension	Medium	Demonstration of antibody synthesis by labelled amino acid incorporation
Dutton et al (208)	Heterologous serum proteins	Rabbit spleen cell suspension	Eagle's medium with 15 per cent rabbit serum	Demonstration of antibody synthesis by labelled amino acid incorporation

TABLE IV-A (Continued)

SECONDARY ANTIBODY RESPONSE BY CELL SUSPENSION IN VITRO. ANTIGEN ADMINISTERED IN VIVO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
Dutton et al (210) Dutton and Pearce (209)	Heterologous serum proteins	Rabbit spleen cell suspension	Eagle's medium with 15 per cent rabbit serum	Antibody synthesis measured by labelled amino acid incorporation
Smiley et al (110)	Bovine serum albumin	Rabbit spleen and lymph node cell suspension	Eagle's medium	Antibody synthesis measured by labelled amino acid incorporation
Steiner and Anker (112)	Bovine serum albumin	Rabbit spleen cell suspension	Medium with 50 per cent rabbit serum	Demonstration of antibody synthesis by direct precipitation and labelled amino acid incorporation
Vaughan et al (109)	Heterologous serum proteins	Rabbit spleen cell suspension	Eagle's medium with 15 per cent rabbit serum	Antibody synthesis shown by labelled amino acid incorporation
Smiley and Jasin (221)	Bovine serum albumin	Rabbit spleen and lymph node cell suspension	Eagle's medium with no rabbit serum	Antibody synthesis shown by labelled amino acid incorporation

TABLE IV-A (Continued)

SECONDARY ANTIBODY RESPONSE BY CELL SUSPENSION IN VITRO. ANTIGEN ADMINISTERED IN VIVO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
Dutton et al (212)	Heterologous serum proteins	Rabbit spleen cell suspension	Eagle's medium with 15 per cent rabbit serum	Antibody synthesis shown by labelled amino acid incorporation. Addition of high concentration of tritiated thymidine inhibited antibody synthesis
Sussdorf (213)	Sheep erythrocytes	Rabbit spleen sub-cellular suspension	Medium	Slight indication of hemolytic antibody produced by subcellular preperations
Krueger and Twedt (118)	Salmonella typhosa	Frog spleen cell suspension	Medium	Demonstration of synthesis by bacterial immobilization
Ainis (214)	Hemocyanin	Rabbit spleen cell suspension	Eagle's medium with 28 per cent rabbit serum	Antibody synthesis demonstrated by micro-precipitin test

TABLE IV-B

SECONDARY ANTIBODY RESPONSE BY CELL SUSPENSION IN VITRO. ANTIGEN INCUBATED IN VITRO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
Helmreich et al (114, 115)	Bovine gamma-globulin oval-bumin	Rabbit lymph node cell suspension	Medium	Antibody synthesis measured by labelled amino acid incorpor-ation
Scharff and Uhr (116)	Phage T2	Rabbit lymph node cell suspension	Eagle's medium with 20 per cent fetal serum	Demonstration of anti-body synthesis-labelled amino acid incorporation
Bussard and Huyny (215)	Sheep erythro-cytes	Rabbit lymph node cell suspension	Eagle's medium	Antibody synthesis shown by labelled amino acid incorporation
Popisil (216)	Brucella	Rabbit lymph node cell suspension	Medium	Antibody synthesis measured by labelled amino acid incorpora-tion (optimal pH 6.9-7.2).
Medzon and Vas (217)	Egg albumin and bovine serum	Rabbit spleen cell suspension	Medium 199	Synthesis measured by labelled amino acid incorporation. Inhib-ition of antibody syn-thesis by cytotoxicity of Newcastle disease virus.

TABLE IV-B (Continued)

SECONDARY ANTIBODY RESPONSE BY CELL SUSPENSION IN VITRO. ANTIGEN INCUBATED IN VITRO

Author(s)	Antigen Used	Species and Tissue	Culture Conditions	Observations
Capalbo et al (218)	Sheep erythrocytes	Rat and mouse spleen cell suspensions	Tyrode's solution. Diffusion chambers implanted in peritoneal cavity of x-irradiated recipients	Antibody synthesis shown by hemagglutination
Van der Meer and Koningsberger (219)	Phage X 174	Rat spleen cell homogenate	Medium	Antibody synthesis shown by phage neutralization assay
Richardson and Dutton (117)	Sheep erythrocytes	Rabbit spleen cell suspension	Medium in gel	Demonstration of plaque formation

CHAPTER 3

MATERIALS AND METHODS

3.1 Animals Used

Only adult, 5 to 7 pound outbred white New Zealand rabbits were used in this study. They were maintained in a well-ventillated, temperature-controlled animal room and were fed and watered at regular intervals of time.

3.2 Antigens Used

The antigens used were:

(1) Human serum albumin (HSA) (Hyland Laboratories, California, USA),

(2) Bovine serum albumin (BSA) (Pentex Inc., Kankakee, Ill. USA),

(3) Ovalbumin (OA) (Pentex),

(4) Horse serum albumin (HoSA) (Pentex),

(5) Dog serum albumin (DSA) (Pentex),

(6) Cat serum albumin (CSA) (Pentex),

(7) Sheep serum albumin (SSA) (Pentex),

(8) Bovine gamma-globulin (BGG) (Pentex),

(9) Human gamma-globulin (HGG) (Pentex),

(10) Rabbit serum albumin (RSA) (Pentex),

(11) Keyhole limpet hemocyanin (KLH) (Bio-Pacific Marine Corporation, Venice, California, USA),

(12) Salmonella O antigen (Salm O) (obtained from Dr. F. Daguillard, Division of Immunochemistry and Allergy, Royal Victoria Hospital, Montreal, Canada).

(13) Sheep erythrocytes (Srbc) (obtained from the Department of Microbiology, McGill University).

All the antigens were prepared in saline (0.85 per cent sodium chloride) or Medium 199 in the appropriate concentrations, filtered through seitz filter pads and stored at -10°C till used.

3.3 Phytohemagglutinin

Only phytohemagglutinin-M (PHA-M) (Difco Laboratories, Detroit, Mich., USA) was used in this study. The contents of a vial were dissolved in 5 ml of Medium 199. The PHA solutions were kept at -10°C.

3.4 Immunosuppresant Agents

Six mercaptopurine (6-MP) was obtained in a powder form from the Burroughs Welcome Laboratories. It was prepared in medium, in the appropriate concentration, prior to use. Not all the 6-MP was observed to dissolve in the medium.

3.5 Media Used

Medium 199 with bicarbonate and Hanks balanced salt solution (Hanks BSS) were obtained from Microbiological Associates, Bethesda, Md., USA. The pH of the medium, which was

checked periodically, was pH 7.2 ± 0.05. Except where otherwise stated, the medium was fortified with penicillin, streptomycin and normal rabbit serum (NRS) to yield final concentrations of 100 units per ml, 100 ug per ml and 15 per cent, respectively. This fortified medium is referred to as Med-PS-NRS in the text. The penicillin and streptomycin stock solutions were obtained from Microbiological Associates, Bethesda, Md., USA, while the normal rabbit serum was obtained from the Institute of Microbiology, University of Montreal.

3.6 Radioactive Compounds

The tritiated thymidine (Thymidine-H^3) and tyrosine -C^{14} were obtained from Schwarz Bio-Research Incorporated, Orangeburg, N.Y., USA.

3.7 Immunization Procedure

Rabbits were injected with the antigen via the intravenous route and/or the foot-pad or by the subcutaneous route according to the particular experimental procedure. For the intravenous administration, the antigen was dissolved in saline and Seitz-filtered prior to use. For the foot-pad injection, equal volumes of the antigen solution and Freund's complete adjuvant (Difco Laboratories, Detroit, Mich., USA) were mixed and the emulsion was prepared using two luer-lock syringes and a double-hub needle.

3.8 Induction of Immunological Tolerance

Neonatal rabbits were injected intraperitoneally at days 3 and 6 of age with 100 mg HSA (399, 400). They were left undisturbed with their uninjected littermates and all the rabbits were bled at 10 weeks of age. Several rabbits of each litter were then injected intravenously with an immunogenic dose of HSA (10 mg) and bled 8, 15 and 20 days later. Antibody titers were determined by the tanned cell hemagglutination technique (see below). The remaining animals of the litter, consisting of both control and "tolerant" rabbits, were sacrificed and the various lymphoid organs were excised. Cell suspensions and fragments were prepared from the various organs and these were tested for their immune reactivity by the fragment and cell culture techniques described below.

3.9 Fragment Culture Technique

The organ was sliced with two scalpels to yield fragments as uniform as possible, approximately 1-1.5 mm^3 in size. Eight fragments were distributed into each Leighton tube (Bellco Company, Vineland, N.J., USA) onto a gelfoam pad (Upjohn Company) via a Pasteur pipette and the fragments were covered with a strip of gelfilm (Upjohn Company) (106). One ml of Med-PS-NRS was added to each tube and the tubes were stoppered and placed in the horizontal position in a 37°C incubator. Where indicated, antigen

was added to the tubes at the commencement of culture and allowed to incubate with the fragments for two hours. The culture fluid containing the antigen was then withdrawn, traces of antigen eliminated by three consecutive washes of the tissue fragments with Med-PS-NRS, and the tubes were replenished with one ml of the culture medium. The medium was changed the next day and every third to fourth day thereafter and the supernatants were tested for antibody activity by the tanned cell technique (see below).

This technique of fragment culture has been found to be capable of maintaining antibody formation for periods up to 35-40 days (104, 105).

3.10 Cell Culture Technique

The technique of cell culture as used in this laboratory has been described in a previous communication (401). The rabbits were bled from the heart with a heparinized syringe and were then sacrificed by the intravenous administration of nembutal (50 mg per kg body wt.). The various organs were removed in rapid order and placed in Med-PS-NRS, the entire procedure taking no longer than several minutes. The organs removed were bone marrow (from the head and upper shaft of the tibia), popliteal lymph node, spleen, thymus, appendix and sacculus rotundus. The blood was diluted with 6 per cent dextran (mol. wt. 75,000) (Gentran, Baxter Laboratories, USA) in a ratio of 3:2 (blood:dextran). The mixture was introduced into sterile disposable plastic tubes (Falcon Plastics, Los Angeles, California, USA)

which were placed at an angle of 60° to the horizontal in a 37°C incubator and allowed to sediment for 40-60 minutes. The leucocyte-rich plasma-dextran layer was carefully decanted, diluted 10 fold with Medium 199 and centrifuged at 800 rpm for 10 minutes. The cells were resuspended in Medium 199 and washed once more. The organs other than the bone marrow were cut into small fragments and teased through a sterile wire mesh (50 mesh) by the application of slight pressure. The cells were collected into Med-PS-NRS. The bone marrow cell suspensions were prepared by flushing the bone marrow with several aliquots of normal rabbit serum into a sterile plastic tube. We have observed that the stability of the bone marrow cells is enhanced if NRS is used in place of Medium 199 containing heparin. The cell mass was gently shaken in the plastic tube and centrifuged at 500 rpm for 5 minutes. The fatty upper layer was decanted and the cell button was resuspended in Med-PS-NRS. The cell suspensions of all the organs were diluted in Med-PS-NRS to contain 10^6 lymphocytes per ml. The various cell suspensions were then all treated in a similar fashion. Four ml of the suspension were placed into disposable sterile plastic tubes (Falcon). The antigen was added, usually 0.1 to 0.25 ml, and the tubes were sealed and incubated at 37°C. Unless otherwise stated, the antigen was incubated with the cell suspensions for the duration of culture. Approximately 24 hours prior to the termination of culture, 2 uc tritiated thymidine (Specific activity 1.0 mc/Mm) was added to each tube. At

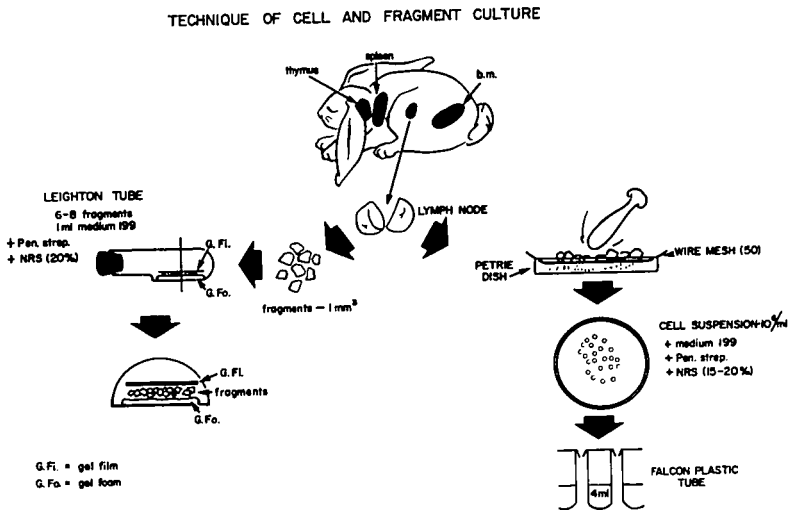


FIGURE 1 TECHNIQUE OF FRAGMENT AND CELL CULTURE

the end of culture the tubes were removed from the incubator and processed for their radioactive content by the method described below. The fragment and cell culture techniques are diagramatically presented in Figure 1.

3.11 Determination Of Radioactive Thymidine Incorporated by the Cell Suspensions

The tubes were centrifuged at 1000 rpm for 10-15 minutes, the supernatants were discarded and the cell buttons resuspended in two ml of five per cent trichloracetic acid. The tubes were centrifuged and washed once more in an identical fashion with trichloracetic acid. One half ml of Hyamine (Packard Instruments, USA) was then added to each tube and the tubes were permitted to digest for 24 hours at room temperature in the dark. The contents of the tubes were then transferred to scintillation counting vials using two washes of absolute ethanol (0.6 ml total). The vials were then incubated at 70-75°C for one hour, allowed to cool at room temperature, following which 15 ml of the scintillation solution (containing 400 gm naphthalene, 28 gm PPO, 1.2 gm POPOP, made up to 3.8 liters with dioxane) were added to each vial. The vials were analyzed for their radioactive content in a Model 4000 Packard liquid scintillation counter. The results are expressed as counts per minute. The results are also presented, where appropriate, as the specific thymidine incorporation, which is the ratio of thymidine incorporation in the presence of antigen to that incorporated in its absence. This procedure is essentially that described by Bain and Lowenstein (402).

3.12 Fractionation Of Normal Rabbit Bone Marrow Cells

A linear sucrose density gradient was prepared in a 15 ml sterile polypropylene tube using the Buchler Gradient Mixer equipped with a vibration type sterrer assembly. The proximal compartment was filled with a 15 per cent sucrose solution in water containing 20 per cent NRS. The distal compartment was filled with a 5 per cent sucrose solution made up in Hanks BSS containing 20 per cent NRS. The rate of entry of fluid from the distal to the proximal compartment was equal in volume to that dripping into the polypropylene tube from the proximal compartment, thus permitting for the establishment of a linear density gradient.

The bone marrow cells were washed several times with Medium 199 and prepared as a suspension in normal rabbit serum, diluted two fold, in a concentration of 200-300 x 10^6 cells per ml. One half to one ml of this cell suspension was layered onto the surface of the linear sucrose gradient and the tube was centrifuged at 100 x g for 3-5 minutes, following which the tube was punctured with a needle and the fractions collected in sterile plastic tubes (Figure 2). The cells were washed twice with Med-PS-NRS and were resuspended in this medium to give a final cell concentration of 10^6 cells per ml. The response of the bone marrow cells in each fraction to antigen was determined using the cell culture technique as outlined above.

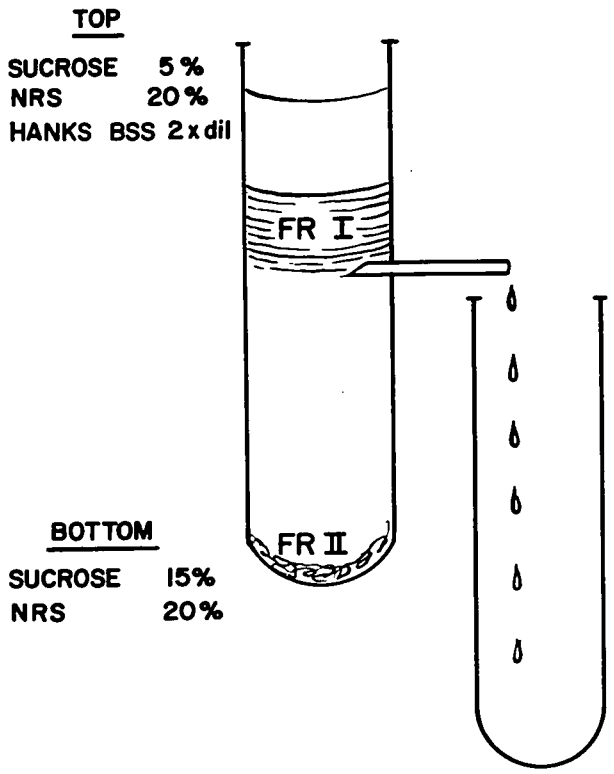


FIGURE 2 FRACTIONATION OF NORMAL RABBIT BONE MARROW BY CENTRIFUGATION IN A LINEAR SUCROSE DENSITY GRADIENT.

3.13 Radioautography

The radioautographic procedure followed for the analysis of the cell suspensions following incubation with the antigen was essentially that described by Kopriwa and Leblond (403). The cells were spread onto gelatin-coated glass slides (0.5 gm gelatin and 0.05 gm chrome alum, made up to 100 ml with distilled water) which were then air dried. The coating procedure was carried out in a photographic dark room. A safelight filter (Kodak, Wratten Series 2) was used with a 15 Watt lamp at a distance of 3 feet above the working area. Kodak NTB-2 Liquid Nuclear Track Emulsion was heated to 37°C in a water bath and kept at this temperature throughout the procedure. The slides were held vertically by the marked end and dipped into the emulsion. The excess emulsion on the back of the slide was wiped off with Kleenex tissue. The coated slides, maintained in a vertical position, were allowed to dry for 2-3 hours in complete darkness. During this period, the excess emulsion that drained was absorbed onto gauze. The slides were then stored in light-tight slide boxes containing a dessicating agent (Drierite pellets). The boxes were kept at 4°C for 2-4 days and then developed with Kodak D-19 developer for 3 minutes, followed by passage through a water rinse for one minute and Kodak fixer for 5 minutes. These solutions and the water used were maintained at room temperature. After washing, the slides were stained with Jenner's stain.

3.14 Fluorescent Antibody Technique

Human serum albumin, bovine gamma-globulin, bovine serum albumin and Keyhole limpet hemocyanin were conjugated to fluorescein isothiocyanate (FITC) (Dajac Laboratories, Philadelphia, Pa., USA) by the method of Clark and Shepard (404). The protein to be coupled was dissolved in 0.025 M bicarbonate buffer, pH 9.0, to a concentration of 10 mg per ml. Five ml of this solution were placed within a Visking cellophane sac (Visking Corporation, Lindsay, Ont.) and dialyzed for 12-15 hours against 10 volumes of a one per cent solution of FITC dissolved in the same buffer. The contents of the dialyzing sac were then dialyzed against phosphate buffer, pH 7.3, for several days, with daily changes of the buffer, until no coloured material could be discerned emanating from the Visking sac. The contents of the dialysis sac were then frozen in 2 ml aliquots and stored at -10°C until used. The FITC - protein conjugate was not absorbed with guinea pig liver powder or any other organ prior to use.

Rabbit antiserum to human serum albumin was precipitated with 40 per cent ammonium sulphate and the precipitate was washed with ammonium sulphate, dissolved in water, dialysed against distilled water for 24 hours at 4°C and lyophilized. This gamma-globulin fraction of the rabbit antiserum was then conjugated to FITC in the manner as described above.

All preparations were mounted in buffered glycerol and studied immediately with a Reichert "Zetopan" microscope equipped with an Osram HBO 200 watt super pressure mercury lamp with an emission curve with two peaks at 365 and 435 mu. An excitor filter and Wratten blocking filter 1/GG9 were interposed between the light source and the stage.

3.15 Tanned Cell Hemagglutination Technique

Boyden, in 1951, demonstrated that the treatment of red blood cells with low concentrations of tannic acid brings about a change in the properties of these cells, rendering them capable of absorbing protein molecules and of subsequently being agglutinated by the antibodies directed to the absorbed antigenic protein molecules (405). It has been suggested (406) that tannic acid alters the surface properties of the red cells, changing them from a hydrophilic to a hydrophobic state. Freund (407) assumed that tannic acid, like homologous antibody, brings about a change in the surface potential of the cells which, in the presence of certain electrolytes, results in their aggregation. Whatever the exact mechanism of the agglutination of the red cells by tannic acid, it seems not unlikely that the same factors are involved in the ability of the tannic acid to promote the absorption of proteins onto the red cells with their resultant agglutination by specific antibody. However, the exact mechanism by which tannic acid alters the red cell surface to facilitate absorption of protein is unknown.

The red cells were tanned by adding 6 ml of a 1:20,000 preparation of tannic acid, freshly prepared with phosphate buffered saline (PBS) (phosphate buffer, pH 7.2:saline-1:1) from a 1:100 stock solution of tannic acid, to 6 ml of a 2.5 per cent suspension of sheep red cells which were then incubated for 10 minutes at 37°C. The tube was centrifuged, the supernatant discarded and the tanned cells washed three times with 6 ml of PBS. The tanned cells were then incubated with 6 ml of the antigen solution, made up in PBS, for 15 minutes at 37°C following which the tube was centrifuged and the supernatant discarded. The cells were washed three times with normal rabbit serum diluted 100 fold in PBS and made up to a final cell concentration of 2.5 per cent. These cells are referred to as sensitized cells.

The hemagglutination test was performed in 13 x 100 mm lipped round bottom test tubes which were held in plastic frames containing 14 tubes to a row. The antiserum to be tested was diluted 10 fold with saline and doubling dilutions were prepared, using one ml volumes. One-tenth ml of the sensitized cells was added to each tube and the tubes were shaken well until the cells were evenly dispersed. The following controls were performed with each experiment (i) incubation of sensitized cells with diluent only and (ii) incubation of unsensitized cells with the antiserum. The tubes were allowed to stand at room temperature and usually read 16-20 hours later. In control tubes, the

cells settled to the bottom to form a compact button. This is considered to be a negative result. In the presence of antibodies, the red cells formed a gelatinous-like layer covering the entire bottom surface of the tube and this disposition of the sensitized cells constitutes a positive hemagglutination reaction. The titer of the antiserum is expressed as the maximum dilution of the antiserum capable of effecting agglutination of the sensitized erythrocytes.

3.15 Co-Precipitation Of Radioactively-Labelled Antibody

The technique followed was essentially that described by Haber et al (408). Tubes containing 4 ml of the cell suspension (10^6 cells per ml) were set up as described under Cell Culture Technique (Chapter 3.10). On day 2 of culture, the cell suspensions were centrifuged, the antigen (HSA) - containing supernatants were discarded and the cells resuspended in 6 ml of Med-PS-NRS. They were washed twice with this medium and resuspended in 4 ml of medium. One uc Tyrosine - C^{14} (specific activity 1 mc/Mm) was added to each tube and the tubes were placed in a 37°C incubator. After five days of culture, the tubes were centrifuged at 800 rpm for 10 minutes and the supernatants from all the tubes in the same series were pooled, dialyzed against distilled water for 48 hours at 4°C and lyophilized. The cells were washed once more with Med-PS-NRS, resuspended in 6 ml of this medium and a cell-free preparation obtained by subjecting the cells to ultrasonic

vibration (20,000 cycles/sec.) for 30 seconds (Ultrasonic Probe, Fisher Scientific Instruments). This preparation was also dialyzed against distilled water for 48 hours and lyophilized. Both supernatant and cell preparations were then dissolved in saline in one ml volumes which were divided into two equal aliquots. To one was added 0.5 ml rabbit anti-HSA antiserum and to the other was added 0.5 ml rabbit anti-BGG antiserum. The tubes were incubated in a 37°C water bath for one minute following which the optimal concentrations of the antigens, HSA and BGG, were added to the respective tubes. The optimal concentrations of the antigens were initially determined by quantitative precipitation tests to give the Dean and Webb antiserum constant antigen variable optimal proportions ratio. The tubes were shaken and kept in the water bath for two hours followed by incubation at 4°C overnight. The precipitates formed were washed twice with cold saline, dissolved in hyamine (0.5 ml) and analyzed for their radioactive content using the Packard scintillation counter (see Chapter 3.11).

3.17 Viability Studies

One tenth ml of a one per cent solution of trypan blue was added to one ml of the cell suspension. A count was performed immediately thereafter and after one hour of incubation at 37°C (409) in order to determine the proportion of dead cells.

3.18 Staining Procedures

The slides were stained with the Jenner-Giemsa stain. The smears were covered with Jenner's stain for six minutes, rinsed in buffered water, pH 6.8, and then covered with Giemsa stain for ten minutes. The stained slides were rinsed in distilled water, air dried, cover slipped and sealed with permount.

CHAPTER 4

EXPERIMENTAL PROCEDURES AND RESULTS

EXPERIMENT I

THE INDUCTION AND SPECIFICITY OF THE SECONDARY IMMUNE RESPONSE IN FRAGMENTSOF LYMPHOID TISSUES OF PREVIOUSLY IMMUNIZED RABBITS

Procedure

Rabbits were immunized with HSA by the foot-pad administration of 5 mg HSA emulsified in Freund's complete adjuvant twice at 7 day intervals. They were also give 15 mg HSA intravenously simultaneous with the foot-pad injections.

Circulating antibody titers were determined at regular intervals of time and the animals were sacrificed, by the intravenous administration of nembutal (50 mg per kg body weight) about 5 - 7 months after immunization, when the antibody titer had fallen to 10 - 20 per cent of that observed at peak antibody formation. The lymph nodes (popliteal), thymus and spleen were rapidly excised, placed in medium and cut into fragments and cultured as described in Chapter 3.9. The supernatants of the fragment cultures were analyzed for their antibody content by the tanned cell technique, as described in Chapter 3.14.

Results

As can be seen in Table V, the lymph node fragments responded with marked antibody formation following incubation with the antigen for 2 hours. The peak antibody titer was attained by day 10-13 and antibody released by the fragments could still be detected by day 34 of culture. Lymph node fragments which had not been initially incubated with the antigen either did not respond with the formation of detectable amounts of antibody or produced much lower levels of antibody. Results similar to those presented in Table V were obtained with lymph node fragments of 10 other immunized rabbits.

The specificity of the immune response initiated in vitro is documented in Table VI. Only red cells sensitized with HSA were agglutinated by specific antiserum and the culture supernatants. Furthermore, the agglutination of the antigen sensitized tanned cells by the culture supernatants could only be inhibited by the specific antigen.

The in vitro response with spleen fragments was at best equivocal, whereas fragments of thymus and appendix uniformly failed to respond with antibody formation (Table VII). This experiment was repeated three times with essentially similar results.

The effect of a known immunosuppressant, 6-mercaptopurine (6-MP), on the in vitro immune response is presented in Table VIII. The immune response was totally inhibited by 100 ug and 1000 ug of 6-MP while 10 ug of this compound had no obvious effect.

TABLE V

ANTIBODY TITERS IN SUPERNATANTS OF CULTURES OF LYMPH NODE FRAGMENTS OBTAINED FROM HYPERIMMUNIZED RABBITS, MAINTAINED IN MED. 199-NRS

Expt. No.	Antigen added (HSA)	Age, in days, of cultures when supernatants were tested for antibody content:							
		4	7	10	13	19	25	31	34
1	+	0	2,560	81,820‡	40,960	20,480	2,560	5,120	640
	+	0	5,120	40,960	40,960	20,480	2,560	1,280	320
	+	0	640	40,960	40,960	10,240	2,560	1,280	160
	-‡	40	640	2,560	1,280	640	160	80	80
	-	40	640	2,560	1,280	320	160	80	80
	-	40	640	2,560	1,280	640	40	20	20
2	+	0	10,240	10,240	2,560	2,560	640	80	160
	+	0	10,240	5,120	2,560	2,560	640	320	320
	+	0	20,480	10,240	5,120	10,240	1,280	640	640
	-	40	1,280	2,560	2,560	640	320	160	80
	-	20	1,280	2,560	2,560	640	160	160	80
3	+	0	2,560	1,280	640	1,280	640	320	320
	+	0	5,120	1,280	640	1,280	1,280	640	160
	+	0	5,120	2,560	1,280	1,280	1,280	640	160
	+	0	1,280	1,280	1,280	2,560	2,560	320	160
	-	160	320	640	160				
	-	320	640	320	80	80	20	20	N.D.*
	-	320	640	80	20	80	20	20	N.D.

* N.D. = not determined

‡ The antibody titer, as determined by the tanned cell technique, represents the reciprocal of the maximum dilution of the culture fluid capable of effecting agglutination of the antigen sensitized cells.

‡ Signifies no antigen added.

TABLE VI

THE SPECIFIC AGGLUTINATION OF SENSITIZED TANNED RED CELLS BY TISSUE CULTURE SUPERNATANTS

Antiserum or Supernatant tested	Antigen used to sensitize tanned cells	Inhibiting Antigen	Titer*
Rabbit Anti-HSA antiserum	HSA	-	800,000
Rabbit Anti-HSA antiserum	BGG	-	200
Rabbit Anti-HSA antiserum	KLH	-	20
Rabbit Anti-HSA antiserum	BSA	-	1,000
Tissue Culture Supernatant**	HSA	-	10,240
Tissue Culture Supernatant	KLH	-	10
Tissue Culture Supernatant	BGG	-	10
Tissue Culture Supernatant	HSA	HSA	10
Tissue Culture Supernatant	HSA	KLH	10,240
Tissue Culture Supernatant	HSA	BGG	5,120

* The titer represents the reciprocal of the maximum dilution capable of effecting agglutination of the antigen sensitized red cells.

** Obtained from cultures specifically stimulated with human serum albumin (HSA).

TABLE VII

ANTIBODY TITERS IN SUPERNATANTS OF CULTURES OF FRAGMENTS OF VARIOUS LYMPHOID TISSUES OBTAINED FROM IMMUNIZED RABBITS (FOOT-PAD), INCUBATED WITH ANTIGEN (HSA) IN VITRO.

Organ Cultured	Age, in days, of cultures when supernatants were tested for antibody content by the tanned cell hemagglutination technique.*					
	4	7	10	16	22	31
Lymph node (popliteal)	160	2,560	20,480	20,480	10,240	2,560
Spleen	0	0	40	0	0	0
Thymus	0	0	0	0	0	0
Appendix	0	0	0	0	0	0

* The antibody titer represents the maximum dilution of the antiserum capable of effecting agglutination of the sensitized red cells. Each value represents the mean of duplicate determinations. Values less than 10 are considered to be negative.

The supernatants of lymph node fragments incubated without antigen displayed a maximum antibody titer of 640 on day 10. Spleen, thymus and appendix fragments incubated without antigen produced no detectable amounts of antibody.

TABLE VIII

EFFECT OF 6 - MERCAPTOPURINE (6-MP) ON THE RESPONSE TO ANTIGEN OF FRAGMENTS OF LYMPH NODES OBTAINED FROM A HYPERIMMUNIZED RABBIT (HSA)

Materials incubated with the fragments	Antibody titers of supernatants of fragments cultured with HSA and/or 6-MP for the following periods of time (days)*			
	3 days	7 days	10 days	15 days
Control - no antigen	0	160	640	320
HSA - 1 mg	0	1,280	2,560	2,560
6-MP - 1000 ug	0	0	80	40
6-MP - 100 ug	0	0	160	0
6-MP - 10 ug	0	0	0	0
HSA(1 mg) + 6-MP(1000 ug)	0	0	0	0
HSA(1 mg) + 6-MP(100 ug)	0	0	0	0
HSA(1 mg) + 6-MP(10 ug)	0	640	2,560	2,560

* The titer is expressed as the maximum dilution capable of effecting the agglutination of sensitized tanned red cells. Each value represents the mean of duplicate determinations

EXPERIMENT II

DETERMINATION OF OPTIMAL CONDITIONS FOR THE INDUCTION OF BLASTOGENESIS AND INCORPORATION OF TRITIATED THYMIDINE BY LYMPHOID CELLS IN VITRO

Procedure

Experiments were performed with lymph node (popliteal), spleen and bone marrow cell suspensions derived from normal rabbits. The culture tubes were set up with four ml of Med-PS-NRS containing varying numbers of cells. The response to PHA was determined by adding 0.25 ml of PHA (1:10) to the appropriate tubes at the beginning of culture and radioactive thymidine at day two of culture (see Chapter 3.10). After three days of incubation, the tubes were centrifuged and the cell suspensions were analyzed for their incorporation of tritiated thymidine, as described in Chapter 3.11.

The optimal concentration of NRS was determined by incubating normal lymph node and spleen cells (4×10^6 per tube) in Med-PS containing varying concentrations of NRS. One quarter ml PHA (1:10) was added to the tubes and the response was determined following an incubation period of three days. The cell suspensions were analyzed for their incorporation of tritiated thymidine in the manner described above.

Results

As can be seen in Table IX, the lymph node, spleen and bone marrow cell suspensions all presented with optimal incorporation of tritiated thymidine when 4×10^6 cells were utilized per culture tube. The response with 8×10^6 cells was not very much enhanced over that obtained with 4×10^6 cells.

As can be seen in Table X, the lymph node and spleen cell suspensions (4×10^6 per tube) incubated with PHA responded with optimal blastogenesis and incorporation of tritiated thymidine when incubated in Med-PS containing fifteen per cent NRS.

No direct relationship was found to exist between the absolute number of cells incubated per culture tube and the maximum incorporation of tritiated thymidine by these cells. Similar results were obtained when varying concentrations of NRS were utilized to determine the concentration of NRS required for optimal blastogenesis in the presence of PHA.

In view of these results, all cultures described in the following experiments were performed on the basis of 4×10^6 cells per tube in the presence of Med-PS-NRS, using NRS in a concentration of 15 per cent except if otherwise stated.

TABLE IX

THE INCORPORATION OF TRITIATED THYMIDINE BY CELL SUSPENSIONS OF VARYING CONCENTRATION INCUBATED WITH PHA FOR THREE DAYS

Material incubated with cell suspensions	Number of cells incubated in 4 ml of medium	Incorporation of tritiated thymidine by the cell suspensions (counts per minute)*		
		Lymph node cells	Spleen cells	Bone marrow cells
Controls (No PHA added)	1 x 10^6 cells	120	572	5,765
	2 x 10^6 cells	300	699	9,920
	4 x 10^6 cells	1,060	1,688	22,775
	8 x 10^6 cells	1,720	1,897	24,358
	12 x 10^6 cells	2,200	2,335	N.D.**
PHA (1:10) - 0.25 ml	1 x 10^6 cells	6,798	4,187	12,868
	2 x 10^6 cells	15,030	18,359	26,600
	4 x 10^6 cells	98,057	48,864	82,628
	8 x 10^6 cells	112,560	54,370	91,475
	12 x 10^6 cells	79,058	60,114	N.D.

* Each value represents the mean of triplicate determinations

** N.D. = Not done

TABLE X

THE IN VITRO RESPONSE TO PHA OF CELL SUSPENSIONS (4×10^6 CELLS) INCUBATED FOR THREE DAYS WITH MEDIUM 199 CONTAINING VARYING CONCENTRATIONS OF NORMAL RABBIT SERUM (NRS)

Material incubated with cell suspensions	Concentration of NRS in the medium	Incorporation of tritiated thymidine by the cells incubated for three days (counts per minute)*	
		Lymph node cells	Spleen cells
Controls (No PHA added)	50 per cent	675	1,285
	20 per cent	582	2,667
	15 per cent	687	2,175
	10 per cent	438	1,941
PHA (1:10)- 0.25 ml	50 per cent	57,000	24,390
	20 per cent	49,271	27,777
	15 per cent	54,668	29,410
	10 per cent	47,050	33,586

* Each value represents the mean of triplicate determinations

EXPERIMENT III

THE IN VITRO BLASTOGENIC RESPONSE TO PROTEIN ANTIGENS OF LYMPHOID CELL SUSPENSIONS OBTAINED FROM PREVIOUSLY IMMUNIZED RABBITS

Procedure

Rabbits were immunized with HSA in the manner described above and sacrificed five to seven months later when their circulating antibody titers had diminished to ten to twenty per cent of peak titers. The lymph nodes (popliteal), spleen, thymus, appendix, sacculus rotundus and bone marrow were rapidly excised and placed in Med-PS-NRS. The cell suspensions were prepared as described in Chapter 3.10 and they were incubated for varying intervals of time with the immunizing (HSA), cross reacting (BSA) and unrelated (KLH and BGG) antigens. The incorporation of tritiated thymidine by the cell suspensions was determined according to the procedure detailed in Chapter 3.11.

For radioautographic analysis, the cell suspensions at the termination of culture were smeared onto gelatin-coated glass slides which were then dried, coated with the photographic emulsion , stored at 4°C and developed and stained in the manner described in Chapter 3.13.

Results

As presented in Tables XI and XII, the lymph node and spleen cell suspensions responded with marked blastogenesis

and tritiated thymidine incorporation when incubated with HSA in vitro. The maximum response was observed between days five and ten of culture. As little as one ug HSA was capable of stimulating a marked increase in blastogenesis and tritiated thymidine incorporation. A much lower response was observed when BSA, a cross-reacting antigen, was added to the culture tubes. The cell suspensions did not respond to the unrelated antigens (KLH and BGG).

Thymus, appendix and sacculus rotundus cell suspensions failed to respond to any of the antigens with which they were incubated in vitro (See Table XIII).

As can be seen in Table XIV the bone marrow cells responded minimally, if at all, to HSA on day three of culture, whereas these cells responded markedly to BGG and KLH, especially by days five to seven of culture. The significance of this latter result set the stage for the experiments reported upon below (Experiment V).

The results of the radioautographic analyses are presented in Figures 3-5. The majority of the labelled lymph node and spleen cells following incubation with antigen were distinguished as blasts and plasma-like cells (Figures 3-B and 4). There appeared to be a direct correlation between the uptake of tritiated thymidine by the cell suspensions as determined by scintillation counting and the number of labelled cells seen in radioautographs. Control specimens incubated in the absence of the antigen contained

mostly unlabelled cells, the majority of which were small lymphocytes (Figure 3-A). As can be seen in Figure 5, the bone marrow cell suspensions following incubation with the antigen contained few labelled cells, the majority of which were immature myeloid and erythroid cells. Radioautographic analysis of thymus, appendix and sacculus rotundas cell suspensions following incubation with the antigen failed to show any significant increase of labelling when compared with control cultures incubated in the absence of the antigen.

The entire experiment was repeated five times with essentially similar results.

TABLE XI

THE IN VITRO RESPONSE TO PROTEIN ANTIGENS OF LYMPH NODE CELLS OBTAINED FROM A RABBIT HYPERIMMUNIZED TO HSA FIVE MONTHS PRIOR TO SACRIFICE

Antigen incubated with cell suspensions	Uptake of tritiated thymidine by the lymph node cells during incubation with antigens for the following periods of time (counts per minute)*				
	3 days	5 days	7 days	10 days	15 days
Control	594	1,200	3,250	314	135
HSA - 25 mg	8,450	19,150	12,416	9,750	620
HSA - 10 mg	15,800	40,800	19,300	13,852	781
HSA - 1 mg	8,340	27,950	16,373	6,300	863
HSA - 100 ug	4,788	17,700	9,520	7,555	374
HSA - 10 ug	4,450	18,500	13,200	1,330	400
HSA - 1 ug	2,400	12,700	12,104	1,490	129
BSA - 10 mg	895	1,950	5,172	692	161
BSA - 1 mg	608	2,058	3,790	963	227
KLH - 1 mg	502	920	3,200	288	53
BGG - 10 mg	428	1,620	2,753	189	76

* Each value represents the mean of triplicate determinations

TABLE XII

THE IN VITRO RESPONSE TO PROTEIN ANTIGENS OF SPLEEN CELLS OBTAINED FROM A RABBIT HYPERIMMUNIZED TO HSA FIVE MONTHS PRIOR TO SACRIFICE

Antigen incubated with cell suspensions	Uptake of tritiated thymidine by the spleen cells during incubation with antigens for the following periods of time (counts per minute)*				
	3 days	5 days	7 days	10 days	15 days
Control	1,100	1,150	1,650	1,356	510
HSA - 25 mg	33,900	32,400	10,471	12,572	985
HSA - 10 mg	38,750	40,955	29,650	37,452	2,175
HSA - 1 mg	24,325	19,450	37,450	20,500	1,270
HSA - 100 ug	15,314	10,972	19,900	7,100	700
HSA - 10 ug	9,850	9,000	10,300	6,850	420
HSA - 1 ug	2,755	6,122	4,090	5,000	500
BSA - 10 mg	6,570	4,300	3,050	2,675	625
BSA - 1 mg	4,000	5,475	750	4,580	800
BGG - 10 mg	1,350	900	1,300	1,000	482
KLH - 1 mg	910	1,200	825	1,200	310

* Each value represents the mean of triplicate determinations

TABLE XIII

THE IN VITRO RESPONSE TO PROTEIN ANTIGENS OF THYMUS, APPENDIX AND SACCULUS ROTUNDUS CELLS OBTAINED FROM A RABBIT HYPERIMMUNIZED TO HSA FIVE MONTHS PRIOR TO SACRIFICE

Cell suspensions prepared from following organ	Antigen incubated with cell suspensions	Uptake of tritiated thymidine by the thymus cells during incubation with antigens for the following periods of time (counts per minute)*				
		3 days	5 days	7 days	10 days	15 days
Thymus	Control	803	180	131	90	38
	HSA - 25 mg	650	75	98	110	25
	HSA - 10 mg	900	137	100	55	68
	HSA - 1 mg	575	105	70	22	51
	HSA - 100 ug	605	115	133	38	22
	HSA - 10 ug	720	80	58	70	15
	BSA - 10 mg	829	69	49	50	30
	KLH - 1 mg	375	125	80	30	48
	BGG - 10 mg	400	200	105	75	10
Appendix	Control	636	362	122	N.D.**	N.D.
	HSA - 25 mg	657	326	121	N.D.	N.D.
	HSA - 10 mg	507	148	72	N.D.	N.D.
	HSA - 1 mg	343	152	76	N.D.	N.D.
	KLH - 1 mg	610	217	71	N.D.	N.D.
Sacculus Rotundus	Control	235	142	108	N.D.	N.D.
	HSA - 25 mg	231	181	82	N.D.	N.D.
	HSA - 10 mg	178	160	48	N.D.	N.D.
	HSA - 1 mg	129	130	72	N.D.	N.D.

* Each value represents the mean of triplicate determinations

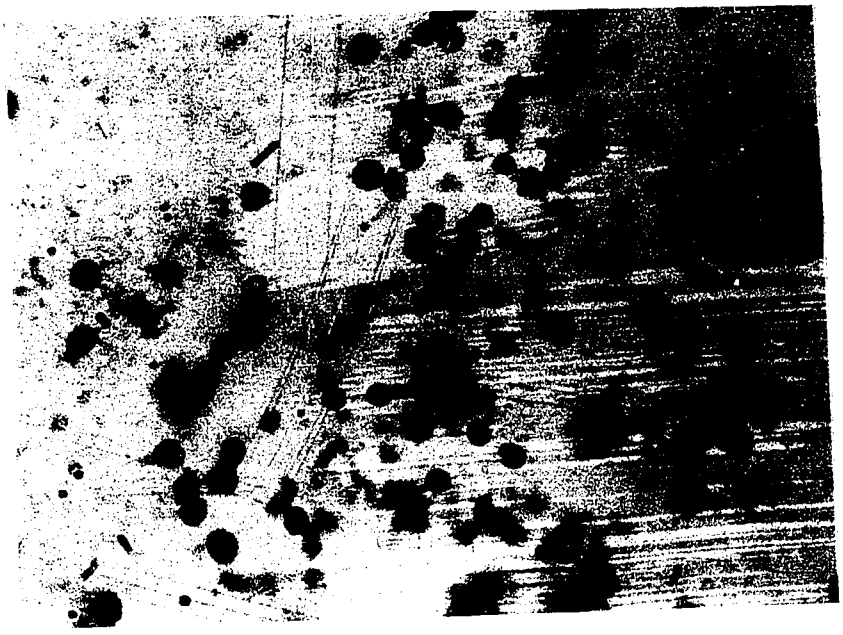
** N.D. = Not done

TABLE XIV

THE IN VITRO RESPONSE TO ANTIGENS OF BONE MARROW CELLS OF A RABBIT HYPERIMMUNIZED TO HSA FIVE MONTHS PRIOR TO SACRIFICE

Antigen incubated with cell suspensions	Tritium uptake by the bone marrow cells incubated in vitro with antigen for the following periods of time (counts per Minute)*				
	3 days	5 days	7 days	10 days	15 days
Control	36,005	4,683	3,465	1,296	444
HSA - 25 mg	33,500	3,900	2,300	1,350	350
HSA - 10 mg	37,685	3,808	2,975	1,049	520
HSA - 1 mg	47,440	5,182	3,204	1,050	580
KLH - 1 mg	50,200	15,020	10,700	3,600	1,200
BGG - 25 mg	44,100	10,800	8,942	2,900	760

* Each value represents the mean of triplicate determinations



(a)

(b)

FIGURE 3 RADIOAUTOGRAPH OF LYMPH NODE CELLS OF PREVIOUSLY IMMUNIZED RABBIT INCUBATED (a) WITHOUT HSA (b) WITH HSA FOR FIVE DAYS AT 37°C. 400 X MAGNIFICATION

FIGURE 4 RADIOAUTOGRAPH OF SPLEEN CELLS OF PREVIOUSLY IMMUNIZED RABBIT INCUBATED WITH HSA FOR FIVE DAYS AT 37°. 630 X MAGNIFICATION

FIGURE 4 RADIOAUTOGRAPH OF SPLEEN CELLS OF PREVIOUSLY IMMUNIZED RABBIT INCUBATED WITH HSA FOR FIVE DAYS AT 37°. 630 X MAGNIFICATION

FIGURE 5 RADIOAUTOGRAPH OF BONE MARROW CELLS OF PREVIOUSLY IMMUNIZED RABBIT INCUBATED WITH HSA FOR FIVE DAYS AT 37°C. 400 X MAGNIFICATION

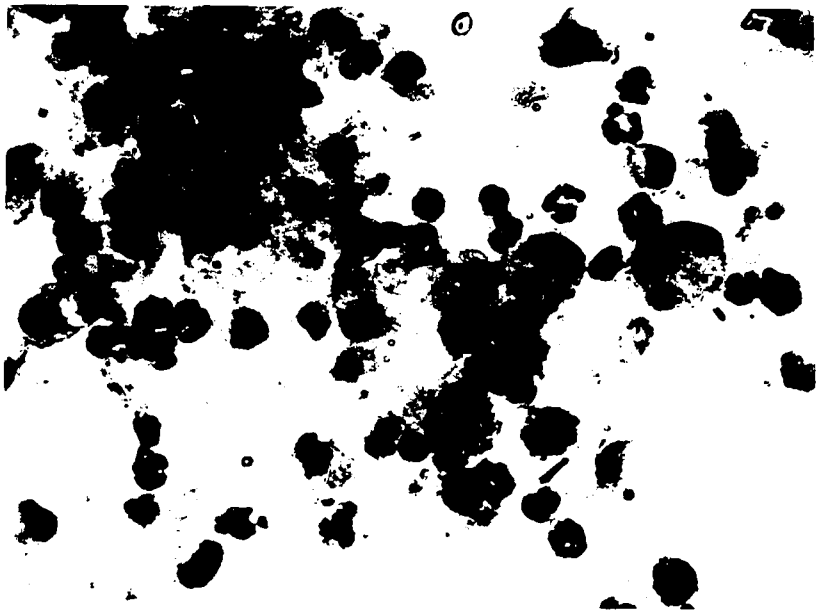


FIGURE 5 RADIOAUTOGRAPH OF BONE MARROW CELLS OF PREVIOUSLY IMMUNIZED RABBIT INCUBATED WITH HSA FOR FIVE DAYS AT 37°C.
400 X MAGNIFICATION

EXPERIMENT IV

RELATIONSHIP BETWEEN THE SECONDARY IMMUNE RESPONSE BY LYMPH NODE FRAGMENTS FROM PREVIOUSLY IMMUNIZED RABBITS AND THE BLASTOGENIC RESPONSE BY CELL SUSPENSIONS OBTAINED FROM THE LYMPH NODE OF THE SAME RABBIT

Procedure

Rabbits were immunized with HSA in the manner described previously and sacrificed five to seven months later. Fragment and cell culture studies were carried out with the lymph nodes (popliteal) in the manner described above (see Chapters 3.9 and 3.10). The supernatants from the fragment cultures were analyzed for their antibody content by the tanned cell technique as described in Chapter 3.15 and the cell suspensions were analyzed for their incorporation of tritiated thymidine as described in Chapter 3.11.

Results

As can be seen in Table XV the lymph node fragments responded with marked antibody formation following incubation with the antigen (1 mg HSA) for two hours. The peak antibody titer was attained by days ten to fourteen and antibody could still be detected by day twenty of culture.

The lymph node cell suspensions responded with marked blastogenesis and incorporation of tritiated thymidine upon incubation with HSA (1 mg) for the entire period of culture. The response was already initiated by day three of culture, attained

maximum levels by day seven to fourteen and could still be detected by day twenty when compared to control tubes. A definite relation was found to exist between the extent of antibody formation by the fragments and the incorporation of tritiated thymidine by the cell suspensions obtained from the lymph nodes of the same rabbit.

The entire experiment was repeated with the tissues of three other immune rabbits with essentially the same results.

TABLE XV

RELATIONSHIP BETWEEN ANTIBODY FORMATION BY LYMPH NODE FRAGMENTS FROM PREVIOUSLY IMMUNIZED RABBITS AND THE EXTENT OF RADIOACTIVE THYMIDINE INCORPORATION BY CELL SUSPENSIONS OBTAINED FROM THE SAME LYMPH NODE UPON INCUBATION WITH THE ANTIGEN (BSA) IN VITRO.

	Cells and supernatants analyzed after incubation for					
	3 days	7 days	10 days	14 days	17 days	20 days
Antibody titers* of fragments supernatants of						
Rabbit I						
Control	0	40	160	320	320	160
Plus Antigen (1 mg)	0	320	20,000	20,000	5,120	2,560
Rabbit II						
Control	0	40	80	20	0	0
Plus Antigen (1 mg)	0	640	2,560	320	320	80
Uptake of tritiated thymidine** by lymph node cells of						
Rabbit I						
Control	527	2,500	3,660	1,630	253	175
Plus Antigen (1 mg)	7,770	36,735	62,575	23,070	5,646	1,380
Rabbit II						
Control	335	895	1,600	1,100	600	52
Plus Antigen (1 mg)	3,880	42,314	39,975	8,845	3,200	430

* The titer is expressed as the maximum dilution capable of effecting agglutination of the sensitized red cells. Each value represents the mean of duplicate determinations. Titers less than 10 are considered to be negative.

** Expressed as counts per minute (per 4×10^6 cells incubated in 4 ml of medium). Each value represents the mean of triplicate determinations.

EXPERIMENT V

THE INCORPORATION OF TRITIATED THYMIDINE BY NORMAL RABBIT LYMPHOID CELL SUSPENSIONS INCUBATED WITH VARIOUS PROTEIN ANTIGENS

Procedure

The lymph node (popliteal), spleen, thymus, appendix, sacculus rotundus and bone marrow were obtained from normal unimmunized rabbits. The cell suspensions were prepared and incubated for varying intervals of time with various concentrations of several protein antigens (procedure as outlined in Chapter 3.10). The cell cultures were analyzed for their incorporation of tritiated thymidine in a manner described in Chapter 3.11.

For cell viability studies, the bone marrow cell suspensions at the termination of culture were analyzed in order to determine the proportion of dead cells (See Chapter 3.17).

The number of bone marrow cells required for optimal blastogenesis to protein antigen was determined by incubating various concentrations of these cells in Med-PS-NRS in the presence of HSA (25 mg).

Results

As presented in Table XVI the normal lymph node cells failed to respond to any of the antigens in vitro. Similar results were obtained with normal spleen cells (Table XVII), and normal thymus, appendix and sacculus rotundus cells (Table XVIII).

On the other hand, as presented in Table XIX, the bone marrow cell suspensions obtained from normal rabbits responded with marked blastogenesis and incorporation of tritiated thymidine upon incubation with various concentrations of several protein antigens. The response was definitely observed by day three of culture, attained maximum levels by days five to ten and could still be observed by day fifteen when compared to control cultures. The bone marrow cells failed to respond when incubated with rabbit serum albumin (RSA). Table XX shows the marked incorporation of tritiated thymidine obtained with normal bone marrow cells incubated with various concentrations of other protein antigens for three days in vitro.

If the results are expressed as specific incorporation of tritiated thymidine (the ratio of that incorporated in the presence of antigen to that incorporated in the absence of antigen), the values are 2.5-8 for bone marrow cells but approximately 1 for lymph node, thymic, spleen, appendix and sacculus rotundus cells.

As can be seen in Table XXI, the number of bone marrow cells normally cultured per tube (4×10^6) were found to respond optimally upon exposure to antigen for three and five days. Doubling the number of cells per tube (8×10^6) resulted in a minor increase in tritiated thymidine uptake. This experiment was performed in order to ensure that the conditions employed in the former and all subsequent experiments were indeed optimal for the reaction under investigation.

The cell viability studies showed that the normal bone marrow cell suspensions cultured in the absence of the antigen contained a greater number of dead cells than those cultured in the presence of antigen. The control cultures showed 14 to 17 per cent dead cells whereas the antigen-stimulated cultures showed only 5 to 7 per cent dead cells when cultured for five days. The cell viability counts performed immediately following the addition of trypan blue to the cells or after one hour of incubation at 37°C showed no marked difference in the number of stained dead cells.

TABLE XVI

INCORPORATION OF TRITIATED THYMIDINE BY NORMAL RABBIT LYMPH NODE CELL SUSPENSIONS INCUBATED WITH PROTEIN ANTIGENS IN VITRO

Antigen incubated with cell suspensions	Uptake of tritiated thymidine by the cells following incubation for the following periods of time (counts per minute)*				
	3 days	5 days	7 days	10 days	15 days
Control - no antigen	690	371	113	75	37
HSA - 50 mg	500	357	103	67	30
HSA - 25 mg	605	279	100	50	25
HSA - 10 mg	620	250	87	59	50
HSA - 1 mg	700	307	112	70	40
BSA - 25 mg	567	300	85	101	45
BSA - 10 mg	490	309	57	49	38

* Each value represents the mean of triplicate determinations

TABLE XVII

INCORPORATION OF TRITIATED THYMIDINE BY NORMAL RABBIT SPLEEN CELL SUSPENSIONS INCUBATED WITH PROTEIN ANTIGENS IN VITRO

Antigen incubated with cell suspensions	Uptake of tritiated thymidine by the cells following incubation for the following periods of time (counts per minute)*				
	3 days	5 days	7 days	10 days	15 days
Control - no antigen	959	1000	762	550	366
HSA - 50 mg	690	750	437	413	298
HSA - 25 mg	1000	803	720	528	300
HSA - 10 mg	875	906	400	320	205
HSA - 1 mg	1006	754	809	400	340
BSA - 25 mg	913	652	758	575	297
BSA - 10 mg	1040	1540	800	450	300

* Each value represents the mean of triplicate determinations

TABLE XVIII

INCORPORATION OF TRITIATED THYMIDINE BY NORMAL RABBIT THYMUS, APPENDIX AND SACCULUS ROTUNDUS CELL SUSPENSIONS INCUBATED WITH PROTEIN ANTIGENS IN VITRO

Cell suspensions prepared from following organ	Antigen incubated with cell suspensions	Uptake of tritiated thymidine by the cells following incubation for the following periods of time (counts per minute)*				
		3 days	5 days	7 days	10 days	15 days
Thymus	Control - no antigen	150	327	168	67	40
	BSA - 50 mg	120	275	100	58	38
	HSA - 25 mg	97	300	125	54	30
	HSA - 10 mg	149	228	162	28	46
	HSA - 1 mg	162	302	102	47	39
	BSA - 25 mg	107	52	77	20	58
	BSA - 10 mg	122	109	85	80	27
Appendix	Control - no antigen	572	176	102	N.D.**	N.D.
	HSA - 25 mg	546	115	100	N.D.	N.D.
	BSA - 25 mg	503	117	98	N.D.	N.D.
	KLH - 1 mg	176	217	76	N.D.	N.D.
Sacculus Rotundus	Control - no antigen	245	142	99	N.D.	N.D.
	HSA - 25 mg	127	106	87	N.D.	N.D.
	BSA - 25 mg	178	130	72	N.D.	N.D.

* Each value represents the mean of triplicate determinations

** N.D. = Not done

TABLE XIX

THE INCORPORATION OF TRITIATED THYMIDINE BY NORMAL RABBIT BONE MARROW CELLS FOLLOWING INCUBATION WITH VARIOUS PROTEIN ANTIGENS FOR VARYING PERIODS OF TIME

Antigen incubated with cell suspensions	Uptake of tritiated thymidine by the cells following incubation for the following periods of time (counts per minute)*						
	1 day	2 days	3 days	5 days	7 days	10 days	15 days
Control	194,192	126,602	32,905	2,296	1,349	267	155
RSA-50 mg	78,020	46,778	5,946	423	158	75	57
RSA-25 mg	105,263	91,342	30,204	1,505	1,039	113	114
RSA-10 mg	142,886	97,087	23,872	1,883	1,395	143	107
RSA- 1 mg	172,413	120,879	30,060	1,945	1,270	246	130
HSA-50 mg	142,881	143,892	92,282	5,715	2,134	1,095	217
HSA-25 mg	163,912	156,288	88,061	7,482	3,818	1,575	1,722
HSA-10 mg	173,934	141,221	65,040	5,653	2,083	847	345
HSA- 1 mg	145,833	129,546	31,653	2,731	1,418	495	301
BSA-50 mg	124,549	135,216	64,621	19,013	11,500	2,584	195
BSA-25 mg	163,978	140,845	97,563	19,380	4,991	1,814	350
BSA-10 mg	179,520	14,738	82,722	10,154	3,708	1,677	745
BSA- 1 mg	180,312	128,221	44,248	2,333	1,135	663	320
KLH- 2 mg	178,380	128,500	80,875	7,500	2,900	875	510
KLH- 1 mg	180,300	160,210	102,310	10,642	4,364	1,420	642
KLH- 0.5 mg	160,000	148,200	100,892	6,650	4,000	1,100	600

* Each value represents the mean of triplicate determinations

TABLE XX

THE INCORPORATION OF TRITIATED THYMIDINE BY NORMAL RABBIT BONE MARROW CELLS FOLLOWING INCUBATION WITH VARIOUS ANTIGENS FOR THREE DAYS.

Antigen incubated with cell suspensions	Uptake of tritiated thymidine by the cells following incubation for three days (counts per minute)*
Control - no antigen	25,800
DSA - 10 mg	45,700
1 mg	64,450
HoSA - 10 mg	35,270
SSA - 10 mg	26,212
CSA - 10 mg	42,700
1 mg	57,230
OA - 10 mg	40,402
HGG - 25 mg	45,000
10 mg	39,403
1 mg	35,609
BGG - 25 mg	50,200
10 mg	34,852
1 mg	26,208
Salm. O - 10 ug	39,345
1 ug	57,144
0.1 ug	48,875
0.01ug	30,503
Sheep rbc 0.2ml (10%)	45,555
0.1ml	42,379

* Each value represents the mean of triplicate determinations

TABLE XXI

THE INCORPORATION OF TRITIATED THYMIDINE BY NORMAL RABBIT BONE MARROW CELL SUSPENSIONS OF VARYING CONCENTRATION INCUBATED WITH HSA FOR THREE AND FIVE DAYS

Material incubated with cell suspensions	Number of cells incubated in 4 ml of medium	Incorporation of tritiated thymidine by the cell suspensions (counts per minute)*	
		3 days	5 days
Controls (No antigen added)	1×10^6 cells	5,765	1,556
	2×10^6 cells	9,923	4,250
	4×10^6 cells	14,771	8,546
	8×10^6 cells	16,358	10,005
HSA - 25 mg	1×10^6 cells	14,461	2,436
	2×10^6 cells	27,280	10,300
	4×10^6 cells	49,246	32,420
	8×10^6 cells	50,190	34,900

* Each value represents the mean of triplicate determinations

EXPERIMENT VI

SPECIFICITY OF RESPONSE OF NORMAL BONE MARROW CELLS TO ANTIGENS

EXPERIMENT VI-A

THE IN VITRO RESPONSE TO PROTEIN ANTIGENS OF BONE MARROW CELLS OBTAINED FROM RABBITS AT VARYING TIMES FOLLOWING THE ADMINISTRATION OF ANTIGEN

Procedure

Rabbits were given either a single intravenous injection of the antigen (HSA, BGG or KLH), in various concentrations, or a subcutaneous injection of the antigen or they were injected subcutaneously with the antigen in Freund's complete adjuvant. The animals were sacrificed at various intervals of time and their bone marrow cells were collected and cultured in the presence of the immunizing and other protein antigens according to the procedure outlined in Chapter 3.10. The incorporation of tritiated thymidine was determined in the manner described previously (See Chapter 3.11).

Results

The immediate effect of the intravenous administration of HSA on the in vitro response of bone marrow cells to various antigens is presented in Table XXII. The bone marrow cells

responded to HSA and other antigens in vitro when excised and cultured fifteen minutes following the administration of the antigen. However, the bone marrow cells obtained from the rabbits 24 hours after they had been injected with 100, 25 or 5 mg HSA intravenously did not respond to HSA in vitro and this response did not reappear for the duration of the experiment (10 days). The reactivity to HSA of the bone marrow cells obtained from rabbits injected with 1 mg HSA was still present by day ten but was absent by day fifteen. The reactivity of these in vivo "HSA primed" bone marrow cells to other antigens was comparable to that obtained with cells of normal, uninjected rabbits at all times. However, the response of these cells to BSA (a cross-reacting antigen) was somewhat lower than that observed with the other unrelated antigens.

The effect of the subcutaneous administration of HSA alone or in Freund's adjuvant on the in vitro response of the bone marrow cells to various antigens is presented in Tables XXIII and XXIV. In both cases the cells responded to HSA when cultured one or twenty four hours following the administration of 25 or 5 mg HSA, but this response was absent by day ten. The cells obtained from rabbits injected with 1 mg HSA responded to HSA in vitro on day 10 but not by day 15. The reactivity of bone marrow cells to other antigens was again not affected by the subcutaneous administration of HSA.

The effect of the intravenous administration of BGG or KLH on the in vitro response of the bone marrow cells in the presence of immunizing and other antigens is presented in Tables XXV and XXVI. The results observed in these experiments were essentially the same as those described above, in that bone marrow cells obtained from rabbits injected with BGG and KLH lost their capacity to respond in vitro when exposed to BGG and KLH, respectively. However, the response of the bone marrow cells to the non-immunizing antigens was not affected.

TABLE XXII

THE IN VITRO RESPONSE TO PROTEIN ANTIGENS OF BONE MARROW CELLS OBTAINED FROM RABBITS AT VARYING TIMES FOLLOWING THE INTRAVENOUS ADMINISTRATION OF HSA

Amount of Antigen Injected (HSA)	Time of sacrifice following administration of the antigen	Uptake of tritiated thymidine by the bone marrow cells during incubation with the following antigens for five days (counts per minute)*						
		Control	HSA 25 mg	HSA 10 mg	HSA 1 mg	KLH 1 mg	BSA 25 mg	BGG 25 mg
100 mg	15 mins	4,000	11,750	4,200	4,000	13,800	12,500	7,480
100 mg	1 hour	3,800	7,800	6,900	3,500	22,300	18,500	9,715
100 mg	24 hours	4,900	4,600	3,600	3,900	16,000	7,500	10,390
100 mg	10 days	5,200	5,400	6,090	6,400	26,585	10,570	10,500
25 mg	15 mins	4,200	10,800	7,800	4,100	15,450	11,560	8,250
25 mg	1 hour	3,875	6,705	5,200	3,002	14,048	9,050	8,836
25 mg	24 hours	5,780	5,361	5,000	4,840	16,200	8,200	10,254
25 mg	10 days	3,920	3,460	3,700	3,440	17,857	8,135	9,750
5 mg	15 mins	3,800	9,250	4,575	3,498	18,892	12,750	8,360
5 mg	1 hour	3,540	10,320	3,650	3,000	14,975	9,250	10,900
5 mg	24 hours	4,874	4,675	4,400	3,480	20,650	11,240	10,800
5 mg	10 days	3,000	2,907	2,630	2,408	15,650	6,875	9,270
1 mg	15 mins	3,400	9,794	4,370	3,258	13,950	12,183	8,502
1 mg	1 hour	4,200	11,860	5,400	4,200	22,725	13,450	9,175
1 mg	24 hours	4,475	10,128	8,005	5,748	18,298	11,385	11,050
1 mg	10 days	3,875	5,000	3,465	3,423	18,716	7,330	8,280

* Each value represents the mean of triplicate determinations

TABLE XXIII

THE IN VITRO RESPONSE TO PROTEIN ANTIGENS OF BONE MARROW CELLS OBTAINED FROM RABBITS AT VARYING TIMES FOLLOWING THE SUBCUTANEOUS ADMINISTRATION OF HSA

Amount of Antigen Injected (HSA)	Time of sacrifice following administration of the antigen	Uptake of tritiated thymidine by the bone marrow cells during incubation with the following antigens for five days (counts per minute)*						
		Control	HSA 25 mg	HSA 10 mg	HSA 1 mg	KLH 1 mg	BSA 25 mg	BGG 25 mg
25 mg	1 hour	3,650	14,490	8,250	3,520	22,352	16,667	9,950
25 mg	24 hours	4,886	9,979	8,000	4,200	15,506	13,600	9,954
25 mg	10 days	3,989	2,885	3,572	4,000	17,454	6,548	8,150
5 mg	1 hour	3,130	15,940	6,200	3,120	16,420	15,690	8,150
5 mg	24 hours	3,546	6,338	5,430	3,224	15,277	10,180	9,780
5 mg	10 days	4,486	3,985	4,270	4,000	14,562	7,354	7,924
1 mg	1 hour	3,260	14,446	5,470	4,080	14,771	13,360	10,847
1 mg	24 hours	5,847	10,395	5,656	4,868	14,458	13,600	10,556
1 mg	10 days	4,252	11,820	3,950	4,200	14,402	10,206	9,287
1 mg	15 days	3,376	5,250	2,120	3,259	10,482	8,251	8,188

* Each value represents the mean of triplicate determinations

TABLE XXIV

THE IN VITRO RESPONSE TO PROTEIN ANTIGENS OF BONE MARROW CELLS OBTAINED FROM RABBITS AT VARYING TIMES FOLLOWING THE SUBCUTANEOUS ADMINISTRATION OF HSA IN FREUND'S ADJUVANT

Amount of Antigen Injected (HSA)	Time of sacrifice following administration of the antigen	Uptake of tritiated thymidine by the bone marrow cells during incubation with the following antigens for five days (counts per minute)*						
		Control	HSA 25 mg	HSA 10 mg	HSA 1 mg	KLH 1 mg	BSA 25 mg	BGG 25 mg
25 mg	1 hour	3,583	13,879	7,604	4,405	14,692	12,634	10,329
25 mg	24 hours	5,400	12,860	6,764	4,909	15,306	14,562	10,336
25 mg	10 days	4,826	3,400	3,637	4,250	16,875	9,880	7,212
5 mg	1 hour	3,962	13,980	10,604	4,806	19,410	9,219	10,923
5 mg	24 hours	8,280	12,727	9,897	7,837	18,415	13,947	9,285
5 mg	10 days	7,826	4,780	7,078	7,230	17,415	13,947	9,208
5 mg	15 days	6,693	4,807	5,200	6,050	15,725	12,325	8,351
1 mg	1 hour	4,707	14,051	6,330	4,252	17,750	15,572	9,304
1 mg	24 hours	5,783	19,669	10,320	5,660	18,177	15,288	12,712
1 mg	10 days	4,729	8,250	5,236	4,000	13,502	10,876	7,625
1 mg	15 days	6,350	3,652	7,500	6,000	15,250	9,523	10,107

* Each value represent the mean of triplicate determinations

TABLE XXV

THE IN VITRO RESPONSE TO PROTEIN ANTIGENS OF BONE MARROW CELLS OBTAINED FROM RABBITS AT VARYING TIMES FOLLOWING THE INTRAVENOUS ADMINISTRATION OF BGG

Amount of Antigen Injected (BGG)	Time of sacrifice following administration of the antigen	Uptake of tritiated thymidine by the bone marrow cells during incubation with the following antigens for three days (counts per minute)*						
		Control	BGG 25 mg	BGG 10 mg	BGG 1 mg	KLH 1 mg	HSA 25 mg	BSA 25 mg
25 mg	15 mins	36,005	83,800	66,300	29,995	108,682	86,105	97,449
25 mg	4 hours	42,942	54,300	46,850	51,145	100,866	128,100	138,601
25 mg	24 hours	43,465	32,040	30,764	27,378	148,040	129,515	115,995
25 mg	2 days	44,352	18,300	32,608	36,709	129,287	72,256	68,876
25 mg	5 days	46,260	39,576	33,800	41,596	111,174	103,290	69,600
25 mg	10 days	28,680	20,595	27,630	13,957	48,306	64,490	78,259

* Each value represents the mean of triplicate determinations

TABLE XXVI

THE IN VITRO RESPONSE TO PROTEIN ANTIGENS OF BONE MARROW CELLS OBTAINED FROM RABBITS AT VARYING TIMES FOLLOWING THE INTRAVENOUS ADMINISTRATION OF KLH

Amount of Antigen Injected (KLH)	Time of sacrifice following administration of the antigen	Uptake of tritiated thymidine by the bone marrow cells during incubation with the following antigens for five days (counts per minute)*					
		Control	KLH 2 mg	KLH 1 mg	HSA 25 mg	BSA 25 mg	BGG 25 mg
10 mg	1 hour	9,325	20,708	23,200	24,607	28,529	14,000
10 mg	4 hours	11,250	7,500	9,750	30,746	39,000	15,900
10 mg	24 hours	8,500	6,775	8,320	19,860	33,258	13,590
2 mg	1 hour	6,528	18,256	21,900	17,308	22,878	10,500
2 mg	4 hours	5,650	4,350	4,520	40,240	14,848	9,875
2 mg	24 hours	6,500	4,275	5,600	15,775	18,229	9,408

* Each value represents the mean of triplicate determinations

EXPERIMENT VI-B

THE IN VITRO RESPONSE TO PROTEIN ANTIGENS OF BONE MARROW LYMPH NODE AND SPLEEN CELLS OBTAINED FROM RABBITS MADE TOLERANT TO HSA

Procedure

Neonatal rabbits were injected intraperitoneally on days three and six of age with 100 mg HSA. They were left undisturbed with their uninjected littermates until 10 weeks of age. Their state of immunologic tolerance to HSA was checked as described in Chapter 3.8. A group of littermates (Two "tolerant" and one uninjected) were sacrificed and their bone marrow, lymph nodes (popliteal) and spleen were excised. Cultures were set up with the lymph node fragments to study their capacity to synthesize antibody in vitro (see Chapter 3.9).

Cell suspensions of the bone marrow, lymph node and spleen of these same rabbits were prepared and cultured in the presence of HSA and other antigens for three days. At the conclusion of the culture the incorporation of tritiated thymidine was determined as described previously (see Chapters 3.10 and 3.11).

Results

The lymph node fragments failed to synthesize and/or release enough antibody which could be detected by the tanned cell hemagglutination technique (see Chapter 3.15).

None of the rabbits which had been injected with the antigen (HSA) on days three and six of age produced any antibody upon reinjection with the antigen at 10 weeks of age. On the other hand, littermates which had not been exposed to the antigen in the immediate neonatal period responded with antibody formation (hemagglutination titers of 640 - 5, 120) following administration of the antigen at 10 weeks of age. One may therefore assume that this regime of antigen administration in the neonatal period did indeed induce a state of immunological tolerance.

The lymph node and spleen cell suspensions did not respond to the immunising antigen. The bone marrow cells also failed to respond to HSA but they responded markedly to BGG and KLH (Table XXVII).

The response of the "tolerant" bone marrow cells to BSA was found to be somewhat depressed as compared to the response obtained with the normal bone marrow cells. The result can be attributed to the cross-reactivity exhibited by HSA and BSA.

The lymph node and spleen cells of normal littermates failed to respond to HSA in vitro. However, the bone marrow cells from these animals responded to all the antigens used in the in vitro culture.

The entire experiment was repeated with the fragments and cells of 2 tolerant and 2 control rabbits with essentially similar results.

TABLE XXVII

THE IN VITRO RESPONSE TO PROTEIN ANTIGENS OF BONE MARROW, LYMPH NODE AND SPLEEN CELLS OBTAINED FROM RABBITS MADE TOLERANT TO HSA

Cell suspensions prepared from following organs	Antigen incubated with cell suspensions	Uptake of tritiated thymidine by the bone marrow cells during incubation with antigens for three days (counts per minute)*		
		A. Tolerant Rabbit	B. Tolerant Rabbit	C. Normal Rabbit
Bone marrow	Control	58,415	52,000	43,910
Bone marrow	HSA 50 mg	39,873	51,200	89,185
Bone marrow	HSA 25 mg	50,825	46,028	120,748
Bone marrow	HSA 10 mg	55,275	49,440	101,455
Bone marrow	HSA 1 mg	57,321	42,015	41,270
Bone marrow	KLH 1 mg	104,830	120,575	129,900
Bone marrow	BSA 25 mg	70,080	59,175	87,300
Bone marrow	BSA 10 mg	70,000	53,475	80,590
Bone marrow	BGG 25 mg	80,840	89,948	58,780
Lymph node	Control	2,148	1,366	657
Lymph node	HSA 25 mg	1,357	1, 052	540
Lymph node	HSA 10 mg	1,704	1, 035	335
Lymph node	HSA 1 mg	1,311	1,311	515
Spleen	Control	1,755	1,235	1,198
Spleen	HSA 25 mg	1,481	1,140	1,048
Spleen	HSA 10 mg	1,359	849	1,125
Spleen	HSA 1 mg	1,146	1,215	942

* Each value represents the mean of triplicate determinations

EXPERIMENT VI-C

EFFECT OF 6-MERCAPTOPURINE (6-MP) ON THE RESPONSE TO ANTIGENS OF BONE MARROW CELLS OF NORMAL RABBITS AND OF LYMPH NODE AND SPLEEN CELLS FROM PREVIOUSLY IMMUNIZED RABBITS

Procedure

Normal rabbit bone marrow cells were collected and cultured (See Chapter 3.10) in the presence of a known immunosuppressant, 6 Mercaptopurine (6-MP), and/or with protein antigens for five days in vitro.

The lymph node and spleen cells were obtained from rabbits previously immunized with HSA and cultured in vitro in the presence of 6-MP and/or HSA for five days. The uptake of tritiated thymidine was determined as described previously (See Chapters 3.10 and 3.11).

Results

As can be seen in Table XXVIII neither 100 ug nor 1,000 ug of 6-MP had any detectable effect on the reactivity of either normal bone marrow cells or lymph node and spleen cells obtained from previously immunized rabbits cultured in the absence of any antigen. A marked suppression of incorporation of tritiated thymidine, in comparison to control cultures, was observed when the normal rabbit bone marrow cells and lymphoid cells from a previously immunized animal were incubated in the presence of the antigen and 6-MP.

TABLE XXVIII

EFFECT OF 6 - MERCAPTOPURINE (6-MP) ON THE RESPONSE TO ANTIGEN(S) OF ORGAN CELL SUSPENSIONS OBTAINED FROM HYPERIMMUNIZED AND NORMAL RABBITS

Immune state of donor rabbit	Cells of organ tested	Incorporation of radioactive thymidine by cell suspensions incubated with the following for five days (counts per minute)*								
		Control	6MP (1000ug)	6MP (100ug)	HSA	HSA + 6MP (1000ug)	HSA + 6MP (100ug)	KLH	KLH + 6MP (1000ug)	KLH + 6MP (100ug)
Hyperimmunized (HSA)	Lymph node	5,390	5,720	5,853	18,218	3,520	7,923	N.D.**	N.D.	N.D.
Hyperimmunized (HSA)	Spleen	9,657	8,738	10,270	23,702	8,360	10,762	N.D.	N.D.	N.D.
Normal, non-immune	Bone marrow	10,005	10,076	10,801	N.D.	N.D.	N.D.	37,463	14,344	22,415

* Each value represents the mean of triplicate determinations

** N.D. - Not done

EXPERIMENT VII

THE RELATION OF THE ANTIGEN INDUCED BLASTOGENIC RESPONSE OF NORMAL BONE MARROW LYMPHOID CELLS TO ANTIBODY FORMATION IN VITRO

EXPERIMENT VII-A

DETECTION OF ANTIBODY WITHIN BONE MARROW LYMPHOID CELLS BY THE FLUORESCENT ANTIBODY TECHNIQUE

Procedure

Bone marrow cells were obtained from normal uninjected rabbits and cell culture experiments set up as described in Chapter 3.10. The antigen (HSA), in a concentration of 25 mg per tube, was incubated with the cells for the duration of the culture. The tubes were then spun at 600 rpm for 8-10 minutes, the supernatants were decanted, the cells resuspended in Hank's BSS and centrifuged again at 600 rpm for 8-10 minutes. The cells were washed two more times in Hank's BSS. The cells were finally resuspended in a small volume of Hank's BSS (0.2 ml) and smeared onto pre-cleaned glass slides, which were then air dried, fixed with absolute ethanol at 4°C for 2-3 minutes and stored in Coplin jars at 4°C. The fluorescent antibody staining technique (partly described in Chapter 3.14) consisted of overlaying the fixed slides with 0.3 ml of each of the fluorescein isothiocyanate conjugated antigens (HSA-FITC, BGG-FITC and KLH-FITC) for 30 minutes. For the "sandwich" technique, the slides were first incubated with HSA or BGG or KLH, (each made up to 50 mg per ml in

buffered saline) for 30 minutes followed by a 30 minute wash with buffered saline (pH 7.2) and incubation with anti-HSA-FITC or anti-BGG-FITC. The fluorescein conjugate was left undisturbed on the slide for 30 minutes after which the slides were washed with buffered saline for 30 minutes. The slides were then coverslipped and examined under a fluorescence microscope as described in Chapter 3.14.

Results

As can be seen in Table XXIX-A, fluorescence was only observed when the HSA-incubated bone marrow cells were stained with HSA-FITC. BGG-FITC and KLH-FITC produced no fluorescence. Furthermore, anti-HSA-FITC and anti-BGG-FITC were also unable to stain the cells on the slide. When the "sandwich" technique was employed (incubation of the slide with unconjugated antigen followed by incubation with conjugated antiserum) only the HSA and anti-HSA-FITC combination produced fluorescence, which was quite marked (Figure 6).

TABLE XXIX - A

DETECTION OF SPECIFIC ANTIBODY (ANTI-HSA) IN ANTIGEN (HSA) - STIMULATED NORMAL RABBIT BONE MARROW CELLS BY THE FLUORESCENT ANTIBODY TECHNIQUE

Fluorescein isothiocyanate (FITC) conjugated protein incubated with bone marrow smears	Detection of fluorescence
HSA - FITC	++ to +++
BGG - FITC	No fluorescence
KLH - FITC	No fluorescence
Anti-HSA - FITC	No fluorescence
Anti-BGG - FITC	No fluorescence
HSA, followed by Anti-HSA - FITC	++++
HSA, followed by Anti-BGG - FITC	No fluorescence

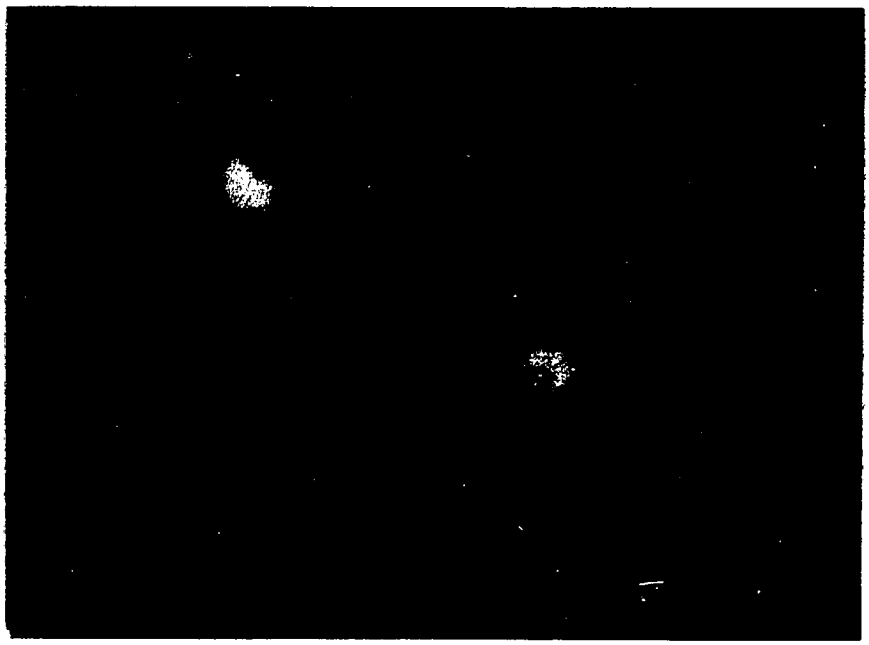


FIGURE 6 SPECIFIC FLUORESCENCE OBSERVED SUBSEQUENT TO INCUBATION OF NORMAL BONE MARROW CELLS WITH HSA FOR FIVE DAYS AT 37°C AND STAINED WITH HSA FOLLOWED BY ANTI-HSA-FITC.
630 X MAGNIFICATION

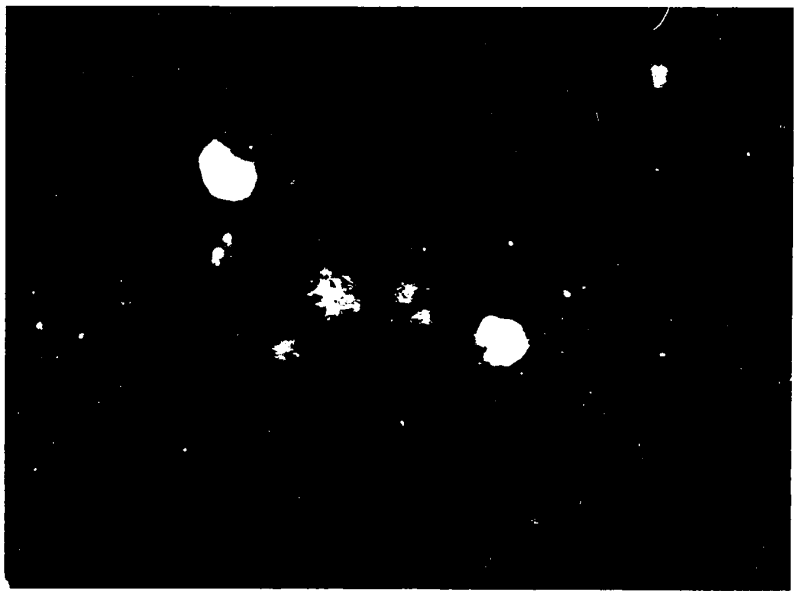


FIGURE 6 SPECIFIC FLUORESCENCE OBSERVED SUBSEQUENT TO INCUBATION OF NORMAL BONE MARROW CELLS WITH HSA FOR FIVE DAYS AT 37°C AND STAINED WITH HSA FOLLOWED BY ANTI-HSA-FITC. 630 X MAGNIFICATION

EXPERIMENT VII-B

DETECTION OF ANTIBODY SYNTHESIZED BY THE ANTIGEN-STIMULATED BONE MARROW CELLS BY THE CO-PRECIPITATION TECHNIQUE

Procedure

The procedure followed is that described in Chapter 3.16.

Results

As can be seen in Table XXIX-B, there is a suggestion that specific antibody was indeed synthesized by the bone marrow cells. Neither the supernatants nor sonicates of control suspensions nor supernatants of antigen-incubated suspensions appeared to contain antibody since the radioactivity detected in the precipitates brought down with HSA and anti-HSA was about equal to that brought down with BGG and anti-BGG.

On the other hand, the sonicates of normal bone marrow cells incubated with HSA yielded more precipitable radioactivity upon the addition of HSA and anti-HSA than that obtained following the addition of BGG and anti-BGG.

It can also be seen in Table XXIX-B that the cells incubated with HSA incorporated approximately twice the amount of Tyrosine-C^{14} as did bone marrow cells incubated in the absence of HSA.

This experiment was repeated a second time with essentially similar results.

TABLE XXIX - B

ANTIBODY FORMATION BY NORMAL BONE MARROW CELLS DURING INCUBATION WITH HUMAN SERUM ALBUMIN AND TYROSINE-C^{14} FOR FIVE DAYS

Cells incubated with the following	Preparation analyzed	Material added to preparation	Radioactivity detected in immune precipitate (counts per minute)
Control - no antigen	Cell supernatant (24×10^6 cells)	HSA + anti-HSA	632
		BGG + anti-BGG	620
	Cell sonicate (24×10^6 cells)	HSA + anti-HSA	1,208
		BGG + anti-BGG	1,000
	Total cell mass (4×10^6 cells)	Nil	20,000
HSA - 25 mg, for initial 48 hours of culture	Cell supernatant (24×10^6 cells)	HSA + anti-HSA	1,020
		BGG + anti-BGG	1,175
	Cell sonicate (24×10^6 cells)	HSA + anti-HSA	3,506
		BGG + anti-BGG	2,005
	Total cell mass (4×10^6 cells)	Nil	39,800

EXPERIMENT VIII

RESPONSIVENESS TO ANTIGENS OF THE LYMPHOCYTE-RICH FRACTION OF NORMAL RABBIT BONE MARROW CELLS

Procedure

Bone marrow cells were obtained from normal rabbits and a suspension prepared as described in Chapter 3.10. The marrow cells were layered onto the surface of the linear sucrose gradients prepared in plastic tubes. The tubes were centrifuged for 3 minutes at 100 x g, following which each tube was punctured (Figure 2) and the two fractions (Fractions I and II) were collected (see Chapter 3.12). The cell fractions were washed twice with Med-PS-NRS and resuspended in this medium. The response of the whole bone marrow cell suspension, Fraction I and Fraction II to antigens was determined using the cell culture technique as described previously (see Chapters 3.10 and 3.11).

For radioautographic analysis, the cell suspensions at the termination of culture were smeared onto gelatin-coated glass slides which were then air dried, coated with the photographic emulsion, stored at 4°C and developed and stained in the manner described in Chapter 3.13.

Results

Two fractions of bone marrow cells were collected following centrifugation in the sucrose density gradient, Fractions

I and II (See Figure 2). Fraction I consisted of cells suspended in a 3 ml zone in the upper third of the gradient whereas Fraction II consisted of cells which had sedimented into the lower third of the gradient during the centrifugation.

The cell content of the whole bone marrow suspension, Fraction I and Fraction II are illustrated in Figures 7, 8 and 9 respectively. Sixty to eighty per cent of the nucleated cells in Fraction I were lymphoid cells of various sizes, the remainder being mainly late erythroblasts, mature granulocytes and blast-like cells. Mature erythrocytes were also present in large numbers (Figure 8). On the other hand, Fraction II was composed mainly of mature granulocytes, granulocyte precursors, erythroblasts, undifferentiated blast-like cells and a few monocytoid cells and macrophage-like cells. Few mature lymphocytes were seen (Figure 9).

As can be seen in Table XXX, after 5 days of incubation with antigen, the uptake of tritiated thymidine by the whole bone marrow was 3-4 times as great as that obtained in the absence of antigen (HSA and KLH). The uptake of tritiated thymidine by Fraction I cells in the presence of antigen exceeded that of whole bone marrow whereas the incorporation of thymidine by fraction II cells was considerably less than that of whole bone marrow. If the results are presented in terms of specific thymidine incorporation (the ratio of thymidine incorporated in the presence of antigen to that incorporated in the absence of

antigen), the values are 3-4 for whole bone marrow, 11-20 for Fraction I and only 1.5-2 for Fraction II (Table XXX).

Whole bone marrow cell suspensions incubated for three hours in 15 per cent sucrose incorporated radioactive thymidine to the same degree as bone marrow cells not exposed to sucrose prior to incubation with the antigen in sucrose-free medium for five days.

By radioautographic analysis, the majority of the labelled cells following incubation with antigen were distinguished as blasts or blast-like cells (Figure 10). The other labelled cells consisted of granulocyte precursors, erythroblasts and a small number of mature granulocytes and macrophage-like cells. There appeared to be a direct correlation between the uptake of tritiated thymidine by the cell suspensions as determined by scintiallation counting and the number of labelled blast cells seen in radioautographs. Control specimens contained few labelled cells, the majority of which were immature myeloid and erythroid cells. These cell suspensions also incorporated very little radioactive thymidine.

The entire experiment was repeated three times with similar results.

TABLE XXX

THE RESPONSE OF WHOLE NORMAL RABBIT BONE MARROW AND BONE MARROW FRACTIONS TO PROTEIN ANTIGENS INCUBATED IN VITRO FOR FIVE DAYS

Antigen added	Uptake of tritiated thymidine by the cell suspensions (counts per minute)*								
	Whole bone marrow			Fraction I			Fraction II		
	A	B	C	A	B	C	A	B	C
Control - no antigen	3,555	6,451	4,620	1,000	241	550	2,000	3,400	2,800
HSA - 25 mg	9,285	12,490	12,050	11,890	10,480	10,800	4,205	5,224	4,037
KLH - 1 mg	12,650	18,755	15,650	20,605	23,060	18,420	3,666	8,575	4,600

* Each value represents the mean of triplicate determinations

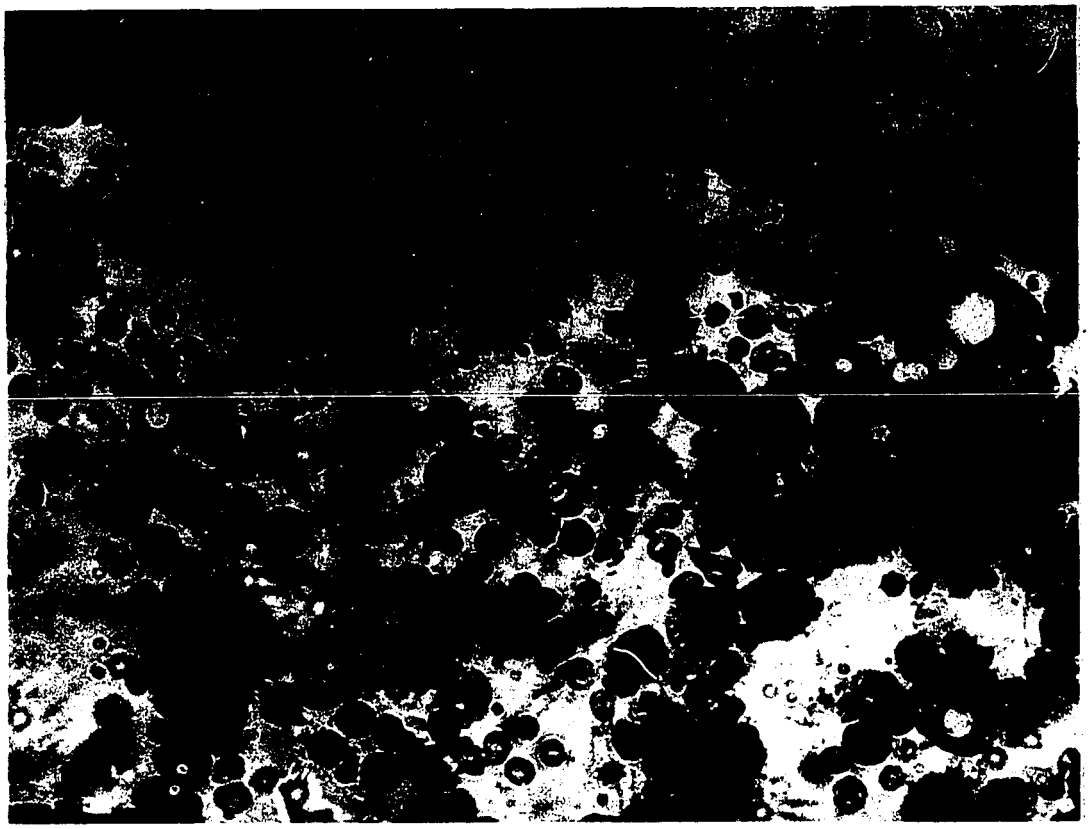


FIGURE 7 UNFRACTIONATED NORMAL RABBIT BONE MARROW.
400 X MAGNIFICATION

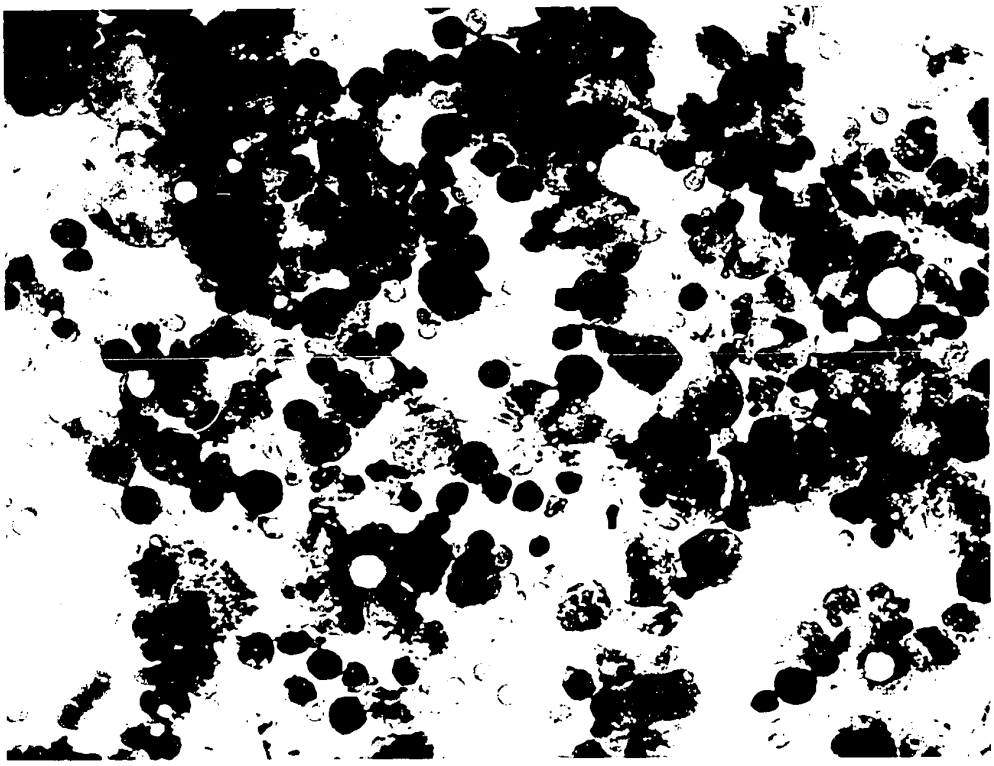


FIGURE 7 UNFRACTIONATED NORMAL RABBIT BONE MARROW.
400 X MAGNIFICATION

FIGURE 8 RABBIT BONE MARROW - FRACTION I
400 X MAGNIFICATION

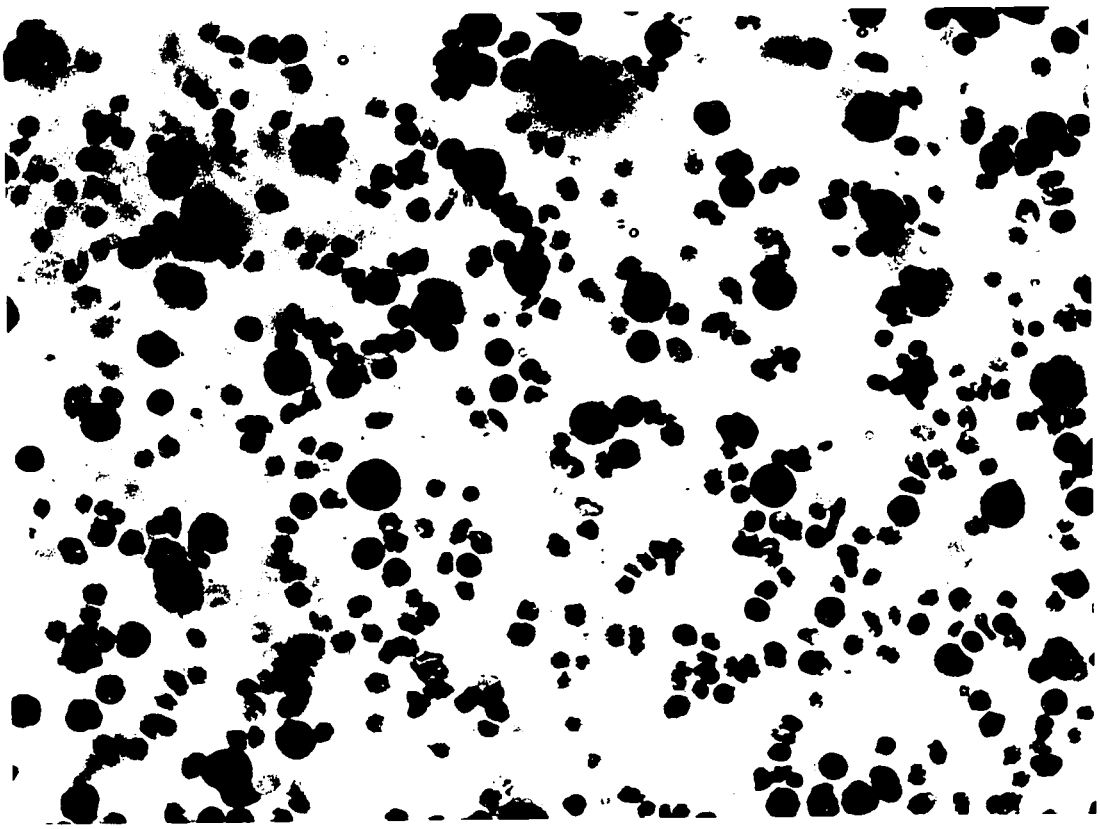


FIGURE 8 RABBIT BONE MARROW - FRACTION I
400 X MAGNIFICATION

FIGURE 9 RABBIT BONE MARROW - FRACTION II
400 X MAGNIFICATION

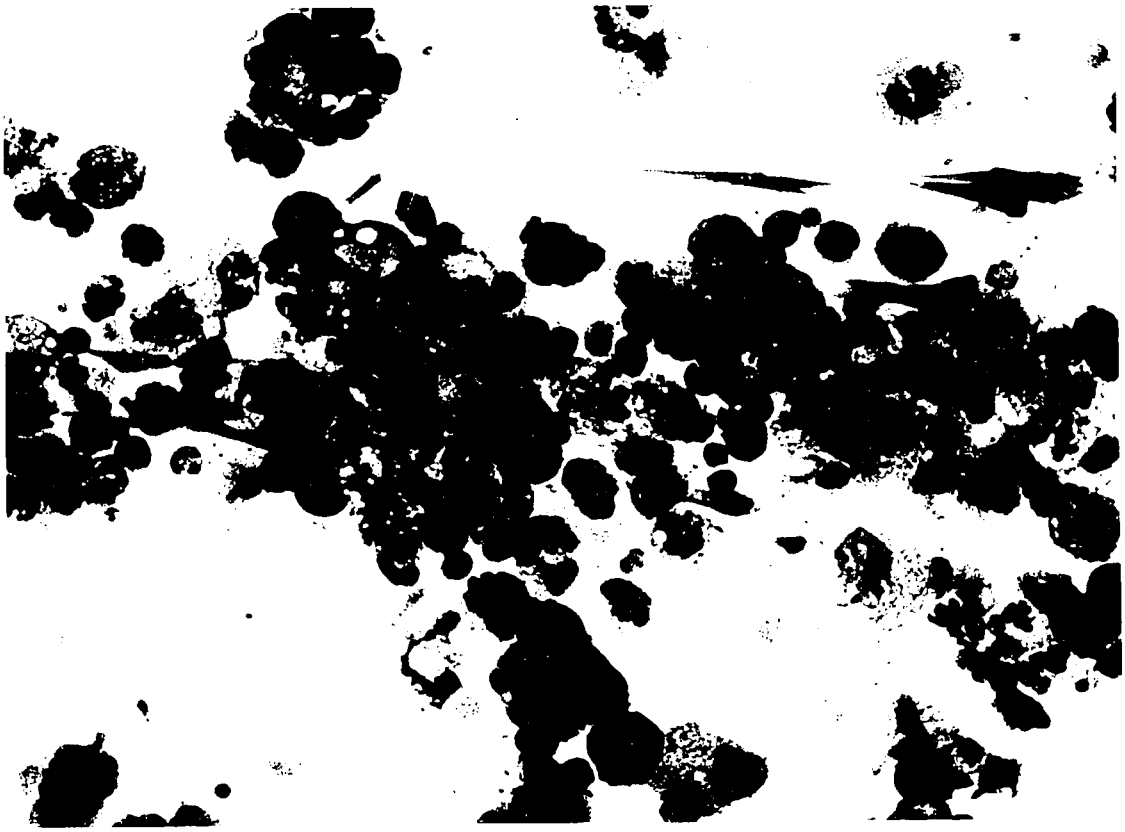


FIGURE 9 RABBIT BONE MARROW - FRACTION II
400 X MAGNIFICATION

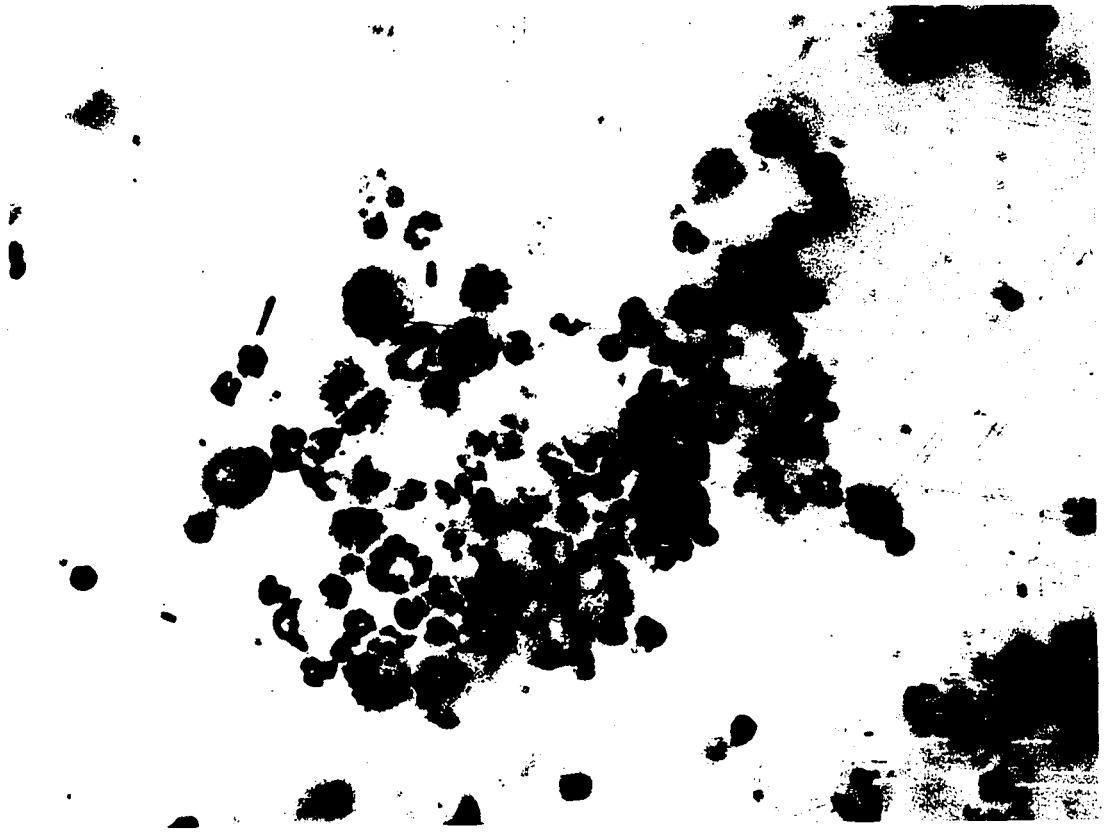


FIGURE 10 RADIOAUTOGRAPH OF WHOLE BONE MARROW CELLS INCUBATED WITH HSA FOR FIVE DAYS AT 37°C.
400 X MAGNIFICATION

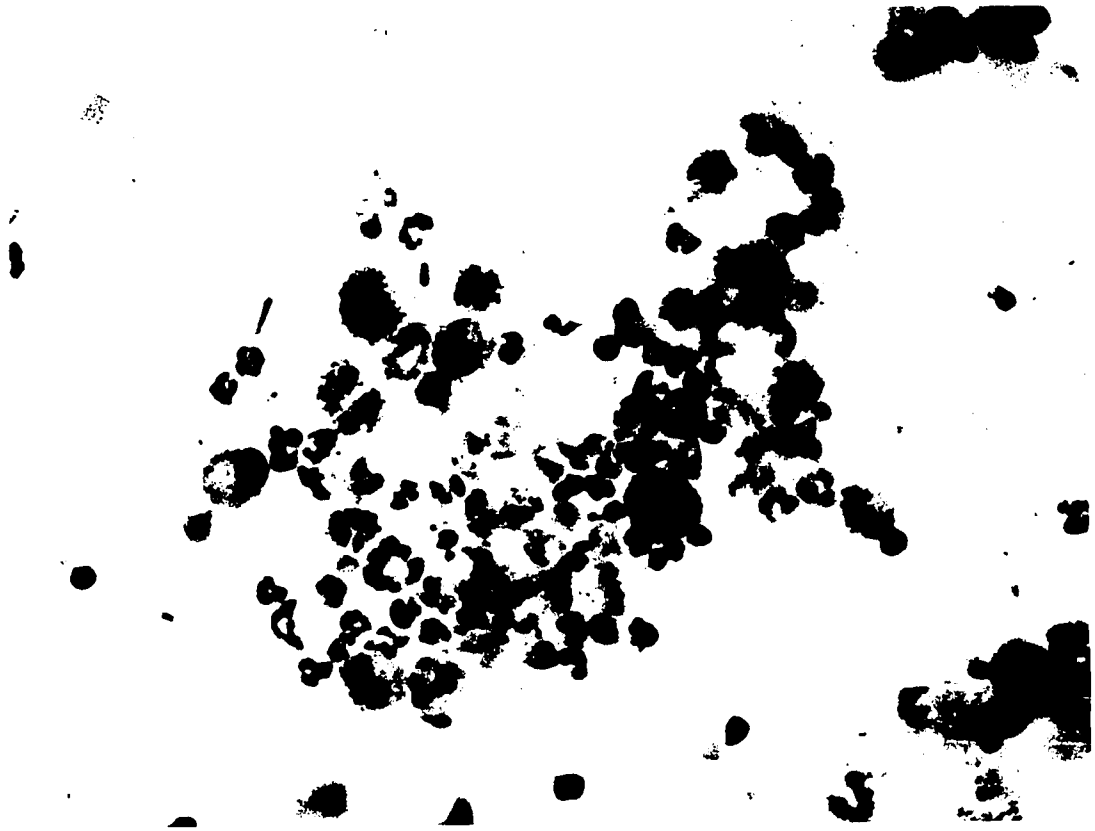


FIGURE 10 RADIOAUTOGRAPH OF WHOLE BONE MARROW CELLS INCUBATED WITH HSA FOR FIVE DAYS AT 37°C.
400 X MAGNIFICATION

EXPERIMENT IX

THE RESPONSE OF NORMAL RABBIT LYMPHOID CELL SUSPENSIONS TO PHYTOHEMAGGLUTININ IN VITRO

Procedure

Rabbits were bled from the heart with a heparinized sterile syringe in order to obtain peripheral white cells, following which they were sacrificed and the various lymphoid organs removed (bone marrow, popliteal lymph nodes, spleen, thymus, appendix and sacculus rotundus). The cell suspensions were prepared, 0.25 ml of PHA solutions of varying concentrations was added either at the commencement of culture or three days prior to the termination of culture (less for tubes removed after only one or two days in culture) and the incorporation of tritiated thymidine by the cell suspensions was determined in the manner described previously (see Chapters 3.10 and 3.11).

Results

As can be seen in Tables XXXI to XXXVII the response to PHA of the lymphoid cells obtained from the various organs varied markedly according to a number of criteria: the time of maximum stimulation, the time for maximum specific incorporation of tritiated thymidine, the magnitude of response, the duration of responsiveness and the optimal concentration of PHA. Table

XXXVIII and Figure 11 summarizes these results in a manner which permits rapid comparison of the responses to PHA of the different populations of lymphoid cells.

TABLE XXXI

BLASTOGENIC TRANSFORMATION OF NORMAL RABBIT LYMPH NODE CELLS BY PHYTOHEMAGGLUTININ (PHA) IN VITRO

Conc. of PHA added (0.25 ml)	Uptake of tritiated thymidine by normal rabbit lymph node cells incubated with PHA for the following periods of time. (counts per minute)*					
	1 day	2 days	3 days	5 days	7 days	10 days
Control	5,600	1,100	450	371	113	98
1:5	16,000	103,200	59,850	14,200	13,134	280
1:10	15,600	76,600	38,750	9,800	3,527	175
1:20	14,000	65,200	27,450	N.D.**	N.D.	N.D.
1:40	12,500	56,650	15,150	N.D.	N.D.	N.D.
1:80	10,600	45,400	5,850	N.D.	N.D.	N.D.
1:160	9,200	25,650	1,700	N.D.	N.D.	N.D.
1:320	9,050	10,000	1,200	N.D.	N.D.	N.D.
1:640	6,000	3,000	725	426	200	N.D.
1:1280	4,800	1,500	675	N.D.	N.D.	N.D.

* Each value represents the mean of triplicate determinations

** ND = Not done

TABLE XXXII

BLASTOGENIC TRANSFORMATION OF NORMAL RABBIT SPLEEN CELLS BY PHYTOHEMAGGLUTININ (PHA) IN VITRO

Conc. of PHA added (0.25 ml)	Uptake of tritiated thymidine by normal rabbit spleen cells incubated with PHA for the following periods of time. (counts per minute)*					
	1 day	2 days	3 days	5 days	7 days	10 days
Control	3,500	2,800	2,580	1,500	1,200	441
1:5	3,900	115,100	80,450	46,700	N.D.**	N.D.
1:10	4,800	120,500	87,800	44,700	2,500	860
1:20	5,600	134,100	109,000	55,000	2,250	1,250
1:40	5,700	130,700	117,000	53,400	N.D.	N.D.
1:80	6,150	77,000	49,600	24,200	N.D.	N.D.
1:160	3,000	19,000	14,500	7,500	N.D.	N.D.
1:320	2,120	5,000	3,700	2,000	1,500	663
1:640	1,200	2,060	2,580	N.D.	N.D.	N.D.
1:1280	598	950	2,000	N.D.	N.D.	N.D.

* Each value represents the mean of triplicate determinations

** ND = Not done

TABLE XXXIII

BLASTOGENIC TRANSFORMATION OF NORMAL RABBIT THYMUS CELLS BY PHYTOHEMAGGLUTININ (PHA) IN VITRO

Conc. of PHA added (0.25 ml)	Uptake of tritiated thymidine by normal rabbit thymus cells incubated with PHA for the following periods of time. (counts per minute)*					
	1 day	2 days	3 days	5 days	7 days	10 days
Control	32,950	9,150	1,400	168	127	110
1:5	40,500	12,850	5,150	190	98	123
1:10	39,800	13,700	6,050	215	106	175
1:20	43,150	18,750	5,300	N.D.**	N.D.	N.D.
1:40	44,700	19,000	6,500	N.D.	N.D.	N.D.
1:80	47,800	19,700	4,400	N.D.	N.D.	N.D.
1:160	40,450	15,900	4,100	N.D.	N.D.	N.D.
1:320	37,700	12,121	3,900	225	140	N.D.
1:640	32,350	16,100	2,850	N.D.	N.D.	N.D.
1:1280	30,900	10,000	2,250	N.D.	N.D.	N.D.

* Each value represents the mean of triplicate determinations

** N.D. = Not Done

TABLE XXXIV

BLASTOGENIC TRANSFORMATION OF NORMAL RABBIT APPENDIX CELLS BY PHYTOHEMAGGLUTININ (PHA) IN VITRO

Conc. of PHA added (0.25 ml)	Uptake of tritiated thymidine by normal rabbit appendix cells incubated with PHA for the following periods of time. (counts per minute)*				
	1 day	2 days	3 days	5 days	7 days
Control	18,000	3,500	1,950	275	625
1:10	20,400	17,500	9,100	5,500	1,000
1:20	18,150	15,000	10,200	3,580	660
1:40	17,500	12,250	6,250	1,200	390
1:80	17,400	7,700	4,300	484	95
1:160	17,000	8,010	3,180	550	320

* Each value represents the mean of triplicate determinations

TABLE XXXV

BLASTOGENIC TRANSFORMATION OF NORMAL RABBIT SACCULUS ROTUNDUS CELLS BY PHYTOHEMAGGLUTININ (PHA) IN VITRO

Conc. of PHA added (0.25 ml)	Uptake of tritiated thymidine by normal rabbit sacculus rotundus cells incubated with PHA for the following periods of time. (counts per minute)*				
	1 day	2 days	3 days	5 days	7 days
Control	10,200	1,500	375	150	46
1:10	14,300	16,175	14,224	1,895	28
1:20	10,500	8,781	5,711	175	38
1:40	10,648	7,381	3,672	300	42
1:80	7,629	4,686	1,375	150	36
1:160	9,683	2,636	1,548	156	50

* Each value represents the mean of triplicate determinations

TABLE XXXVI

BLASTOGENIC TRANSFORMATION OF NORMAL RABBIT BONE MARROW CELLS BY PHYTOHEMAGGLUTININ (PHA) IN VITRO

Conc. of PHA added (0.25 ml)	Uptake of tritiated thymidine by normal rabbit bone marrow cells incubated with PHA for the following periods of time. (counts per minute)*					
	1 day	2 days	3 days	5 days	7 days	10 days
Control	127,650	99,100	32,050	2,500	1,210	1,112
1:5	116,950	121,750	78,600	30,000	8,100	N.D.**
1:10	127,400	110,600	123,400	29,154	5,700	1,197
1:20	124,100	126,500	84,450	16,300	4,600	1,204
1:40	118,350	122,100	67,100	7,400	2,950	N.D.
1:80	116,150	108,300	51,000	4,919	2,428	N.D.
1:160	114,950	115,450	44,200	4,030	2,500	1,288
1:320	122,700	10,800	40,600	2,836	2,550	N.D.
1:640	117,400	104,850	39,350	2,200	1,672	N.D.
1:1280	118,100	101,150	30,500	2,400	1,050	N.D.

* Each value represents the mean of triplicate determinations

** ND = Not done

TABLE XXXVII

BLASTOGENIC TRANSFORMATION OF NORMAL RABBIT PERIPHERAL LEUKOCYTES CELLS BY PHYTOHEMAGGLUTININ (PHA) IN VITRO

Conc. of PHA added (0.25 ml)	Uptake of tritiated thymidine by normal rabbit peripheral leukocytes incubated with PHA for the following periods of time. (counts per minute)*			
	1 day	3 days	5 days	7 days
Control	1,308	450	284	300
1:10	1,396	2,452	1,400	340
1:20	N.D.**	1,380	N.D.	N.D.
1:40	1,245	1,400	380	310
1:80	1,184	910	200	295
1:160	N.D.	714	N.D.	N.D.

* Each value represents the mean of triplicate determinations

** N.D. = Note done

TABLE XXXVIII

SPECIFIC INCORPORATION* OF TRITIATED THYMIDINE BY NORMAL RABBIT HEMOPOIETIC AND LYMPHOPOIETIC CELLS INCUBATED WITH PHYTOHEMAGGLUTININ (PHA)

Cells of organ incubated	Time for maximum incorporation of tritiated thymidine** (days)	Specific incorporation at time of maximum incorporation of tritiated thymidine	Time for maximum specific incorporation of tritiated thymidine (days)	Optimum PHA concentration	Maximum specific incorporation of tritiated thymidine
Bone marrow	3 days	4	5 days	1:5	12
Lymph node	2 days	93	3 days	1:5	133
Spleen	2 days	48	2 days	1:20	48
Thymus	1 day	1.4	3 days	1:40	4.5
Appendix	2 days	5	5 days	1:10	20
Sacculus rotundus	2 days	10.7	3 days	1:10	40
Peripheral blood	3 days	5.5	3 days	1:10	5.5

* Specific incorporation is defined as the ratio of uptake of tritiated thymidine in the presence of PHA to that taken up in the absence of PHA.

** Greatestuptake of tritiated thymidine attributable to PHA stimulation (control counts subtracted).

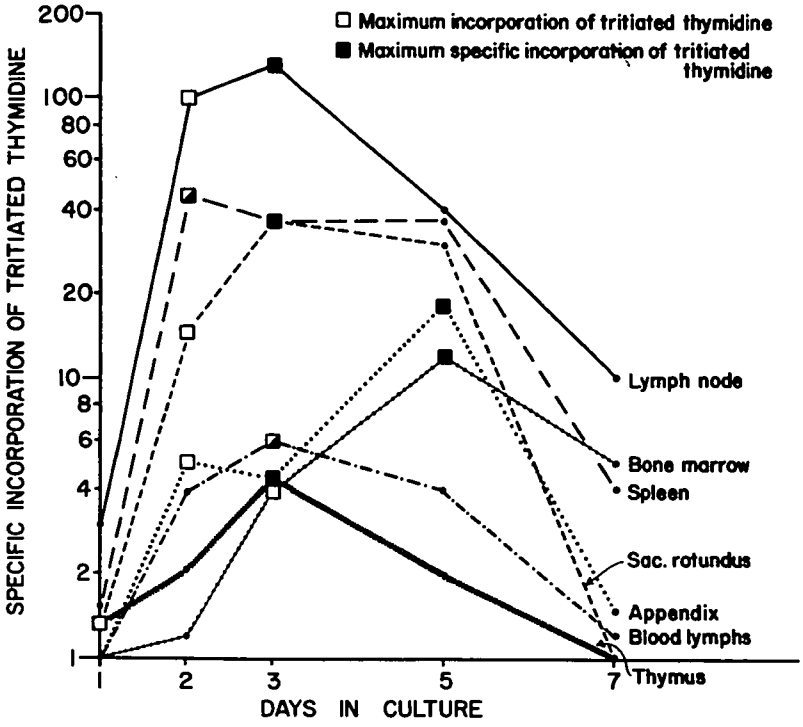


FIGURE 11 RESPONSE OF CELLS OF THE VARIOUS LYMPHOID ORGANS TO PHYTOHEMAGGLUTININ IN VITRO.

CHAPTER 5

DISCUSSION

The role which the various cells comprising the lymphopoietic system play in the induction and maintenance of the immune response has been, and still is, both an enigma and a puzzlement to immunologists. Undoubtedly, a major deterrent towards any detailed and protracted study of the role of the different lymphoid organs in the immune response has been the dearth of dependable in vitro systems required for such an investigation. Although the technique of fragment culture has been successfully employed since the early 1950's by a large body of investigators who have thus demonstrated the capacity of organ fragments to synthesize antibody in vitro, the extent of manipulation of the tissue is minimal and it is essentially a static system. More recently, it has been demonstrated that lymphoid cells can be maintained in a very viable state in the form of a suspension in culture and that their metabolic activities are unimpaired in spite of the loss of architectural organization. In comparing and contrasting the three systems available for study - the in vivo system, fragment culture and cell culture - it becomes obvious that each offers advantages and shortcomings to the investigator in terms of defining the

inter-relationship of the cells under study. The in vivo system is, of course, the most normal of the three and must provide the baseline in the definition of the antigenicity of a foreign material injected into the animal. However, it is also the least accessible system and sacrificing the animals and examining the tissues microscopically presents only the situation prevalent in the animal just prior to death. Furthermore, this situation does not permit for an evaluation of the role of the cells in the different lymphoid organs in the immune response. The fragment culture systems permits the maintenance, to a degree, of the original architecture of the organ, at least insofar as the environment of the cells within the fragment is concerned. Of course, the blood supply is no longer available and oxygen and nutrients must diffuse through the fragments in order to become available to the cells. This system does permit a sampling of the tissue under investigation at varying intervals of time and, theoretically at least, would permit the establishment of a relationship between the formation and/or release of antibody and the morphological characteristics of the fragments and the cells within them. However, here as well, the relationship of cells of different organs cannot be easily determined although fragments of the different organs can be cultured together in the same tube. The third technique, the cell culture method, allows for the greatest degree of flexibility and manipulation but it is the most "abnormal" of the three

situations insofar as the cell environment is concerned. The normal architecture of the organ is completely subverted to permit the maximum manipulation of the cells. This technique permits the study of cell-cell interactions, especially between cells of different organs, and for random sampling. The proliferative capacity of the cells can also be investigated by incorporating radioactive thymidine into the culture medium followed by radioautographic analysis of the cells. One may, therefore, conclude that (a) the in vitro system is the most normal, but also the most rigid system, (b) that the cell culture technique provides for the greatest number of degrees of freedom but imposes the most abnormal environmental conditions and restrictions upon the cells and (c) that the fragment culture technique serves as a compromise model. It is obvious that the information desired cannot be gleaned from any single one of the experimental systems discussed above but that the experimental procedure must involve all three in order to confer validity to results obtained with any one of them independently.

The significance and mechanism of the blastogenic reaction by lymphocytes in vitro has been well documented in a number of review articles (291, 370, 410, 411) and has been discussed in the Historical Review (Chapter 2). On the basis of experimental results obtained to date, one may assume that the reaction is an immunologically specific one in that it can only

be induced by specific antigen or by homologous or genetically-dissimilar isologous lymphocytes. It cannot be induced by the incubation of lymphocytes from genetically identical, monozygotic twins. In the present study, the cell culture technique was utilized along with the fragment culture method in the initial experiments in order to establish, in our hands, the immune nature of the blastogenic reaction obtained with the cell cultures. It was observed that spleen and lymph node cells prepared from the organs of rabbits immunized 5 - 7 months previously and cultured in the presence of the specific antigen were stimulated to undergo blastogenesis and mitosis and to incorporate radioactive thymidine to a degree 10-20 times that obtained with control preparations. Furthermore, only lymph node and spleen cells of previously immunized rabbits were capable of responding in this manner in vitro, and the lymph node cell response could be correlated with the antibody response obtained with the fragment cultures of the same lymph node. These initial experiments demonstrated that a direct relationship exists between antibody formation and blastogenesis. It was also observed that bone marrow cells obtained from a previously immunized rabbit, unlike lymph node and spleen cells, failed to respond with blastogenesis and thymidine incorporation in vitro upon exposure to the specific immunizing antigen, but did respond markedly to other antigens to which the rabbit had not been

previously exposed. Further investigations were carried out with cells of normal, uninjected rabbits. Normal bone marrow cells were observed to undergo blastogenesis and mitosis when incubated with a large variety of protein antigens, a bacterial antigen, and sheep red blood cells, although the magnitude of the response was somewhat different with respect to the different antigens. None of the cell cultures prepared from the other lymphoid organs of the normal rabbit-spleen, lymph node, thymus, sacculus rotundus and appendix-reacted to any of the antigens in vitro. The normal bone marrow cells were also shown to be capable of synthesizing specific antibody in vitro. These results strongly suggested that the normal bone marrow response to antigen in vitro is an immune response or the initial cellular event during the induction of the immune response. It should be noted that, as might be anticipated, the bone marrow cells failed to respond in the presence of commercially-available rabbit serum albumin. This finding, coupled with the failure of bone marrow cells from previously immunized rabbitsto respond to the specific antigens in vitro, precludes the possibility of attributing the response of normal rabbit bone marrow cells to a mitogenically active chemical contaminant in the antigen preparation. The high concentration of antigen required to induce the normal bone marrow blastogenic response upon initial exposure to antigen (10-25 mg) compared to that required to initiate the response in lymph node and spleen

cells of previously immunized rabbits (1 mg), may be correlated to the much larger amount of antigen required to initiate a primary, as compared to a secondary, immune response in vivo (150). Further evidence in favour of the immunologic nature of the bone marrow response emerged from the experiments in which rabbits were injected with a protein antigen (HSA or BGG or KLH), sacrificed at various times thereafter, and had their bone marrow cells cultured in the presence of the antigen which had been injected in vivo as well as with other non-cross-reacting antigens. In each case, the bone marrow cells lost their capacity to respond, within one hour to several days, to the antigen with which the marrow donor animal had been injected whereas the response to the other, non-cross reacting antigens was marked and uniform throughout the duration of the experiment. The specific failure of the bone marrow cells to respond to the immunizing antigen in vitro was still manifest even after 5 - 7 months. The specificity of the bone marrow response was further enhanced by the fact that bone marrow cells from tolerant rabbits did not respond to the tolerance-inducing antigen, whereas they did to the other antigens. That a true state of immunologic tolerance was achieved in the rabbits is indicated by the fact that they did not respond with antibody formation following reinjection of the antigen at 10 weeks of age, whereas control littermates responded vigorously. Furthermore, the lymph node

and spleen fragments of the tolerant animals, unlike those of immune animals, did not respond in culture upon exposure to the specific antigen. The immunologic nature of the bone marrow blastogenic response was demonstrated by (a) the detection of antibody within the stimulated cells using the fluorescent antibody technique; (b) the increased radioactive content, relative to controls, of immune precipitates formed with the sonicates of the cell cultures, following the addition of specific antiserum and antigen and (c) the extent to which the blastogenic reaction could be inhibited by 6-mercaptopurine (6-MP), a known inhibitor of antibody formation in vivo and which was also shown to inhibit the antibody response of immune lymph node fragments in vitro. The same concentrations of 6-MP were used in both the cell culture and fragment culture experiments.

Chapman et al (282) have also reported the failure of bone marrow cells of immunized rabbits ("immune" bone marrow) to respond to the specific antigen in vitro. In fact, they reported that the "immune" bone marrow cell response was somewhat inhibited, relative to controls, by the specific antigen. Similar results were observed in the experiments reported upon here. However, Champman et al (282) did not test the reactivity of the bone marrow cells with respect to other, non-cross-reacting antigens in vitro.

A finding which needs to be elaborated upon is the loss of reactivity of the bone marrow cells to the antigen in vitro within a few hours to a few days following administration of the antigen in vivo. This loss of reactivity is a specific one since the marrow cells were still able to respond optimally to the other non-cross-reactive antigens. These findings suggest that potentially immunocompetent cells are released from the bone marrow following contact with the antigen and that the bone marrow as a whole then becomes immunologically-tolerant with respect to this specific antigen. This tolerance, if not permanent, has been found to persist for at least seven months following the intravenous administration of the antigen. The cells which may be released from the bone marrow probably correspond to the potential antibody-forming cell of Mitchell and Miller (447) or to the "effector" cell of Claman et al (52) although the results definitely demonstrate that some bone marrow cells can actually form antibody. However, it may be that this antibody is a "primordial-type" antibody and is not released by the cell until it undergoes further maturation probably in the thymus, as the results of Mitchell and Miller would suggest (447). This interpretation of our results would also permit for an inexhaustable supply of potentially-immunocompetent cells in the bone marrow, since only a small number would actually be released in response to a single

antigen to proliferate in and populate the thymus and the peripheral lymphoid tissue. The cells lost from the bone marrow could be easily replaced by division of other primitive, uncommitted cells. The results also imply that the clonal selection theory of Burnet may indeed be operative at least insofar as the bone marrow is concerned since the loss of reactivity of the bone marrow to an antigen is specific to the injected antigen. It is interesting to note that a definite relationship was found to exist between the quantity of the antigen injected into the rabbit and the time required for the bone marrow cells to lose their reactivity to the specific antigen in vitro. In retrospect, such a result would be anticipated since the rate of migration of potentially immunocompetent cells out of the marrow would be dependent upon the rate of entry of antigen molecules into the marrow which, in turn, would be a function of the quantity of antigen injected. The bone marrow reactivity to antigen was lost irrespective of the mode of injection of the antigen. The "latent period", that is the period of time which elapsed between the administration of the antigen and the loss of reactivity of the bone marrow to this antigen, lengthened as the amount of antigen injected decreased. Furthermore, the bone marrow did not lose responsiveness when the donor animal was injected with a "sub-antigenic" dose of the antigen (i.e. 100 ug of HSA), thus supporting the supposition that the initial cellular event in the immune response in vivo is the release of potentially immuno-

competent cells. The fate of these cells, as discussed later, is still uncertain. However, the findings of Micklem et al (412) that the bone marrow, spleen and thymus of irradiated mice were all recolonized predominantly or exclusively by descendants of injected isogeneic bone marrow cells would suggest that a similar picture might exist in the rabbit.

There can be little doubt, when examining the literature concerned with the cell source of antibody formation, of which the references cited represent only a very small sampling (104, 105, 280, 379, 413-428), that the emphasis has been on the role of the lymphocyte in the immune response. Certainly, in the face of overwhelming evidence, one cannot doubt that the lymphocyte plays a major role in conferring immunity, both cellular and humoral. Although the role of the thymus gland in immunity has captivated a large number of investigators as well (429-433), the bone marrow has received relatively scant attention. Undoubtedly, the complex nature and heterogeneity of the cells comprising the bone marrow has deterred many investigators.

It has been known, for a number of years, that the injection of isologous or even homologous bone marrow cells into lethally x-irradiated animals can prevent death and many of the symptoms of radiation illness (434-445). Gengozian et al (445) injected bone marrow and spleen cells of normal or previously immunized mice into lethally-irradiated mice. The presensitized,

but not the normal, bone marrow conferred antibody forming capacity to the recipient mice. In contrast, both normal and presensitized spleen cells transplanted into irradiated hosts were able to initiate an immune response to the test antigen. Claman et al (52) injected irradiated mice with syngeneic thymus and/or spleen and/or marrow cells. Normal spleen cells produced some discrete areas of antibody production in recipient spleens, whereas mice receiving both marrow and thymus cells produced more centers of hemolytic-activity in their spleens as compared to those receiving cells of either type alone, whether from normal or immune isogeneic animals. These authors suggested that one cell population contains cells capable of making antibody ("effector cells") but only in the presence of the other cell population ("auxiliary cells"). The data presented did not establish which cell suspension, thymus or bone marrow, contains either effector or auxiliary cells or how the cells interact. A further relationship between marrow and thymus cells was demonstrated by Harris and Ford (446) who showed that at least some bone marrow cells migrate through the thymus. Thus, it is possible that the immunocompetence of transferred thymus cells from immunized donors may be due to the presence of marrow-derived cells within the thymus as has been suggested by Davies et al (514). These latter in-

vestigators (514) demonstrated that spleen cells containing bone marrow-derived cells, transferred from immunized mice to recipient X-irradiated host mice, could produce circulating antibody and give rise to a large number of plaque-forming cells, following antigenic challenge in vivo. Thus, bone marrow-derived cells on their own are capable of reacting immunologically upon re-exposure to the specific antigen in vivo. Miller and Mitchell (53) presented further evidence in favour of a bone-marrow-thymus relationship in the induction of antibody formation. They injected thymus cells and sheep red cells into X-irradiated, syngeneic mice (primary host). One week later, the primary host was sacrificed and the spleen cells transferred to an X-irradiated syngeneic mouse (secondary host) which also received sheep red cells and syngeneic bone marrow cells obtained from a normal donor. The secondary hosts were sacrificed at intervals of time and the spleen cells were assayed for hemolysin formation to sheep red cells by the Jerne plaque technique (188). A significant hemolysin-forming cell response was obtained only if the primary host had been injected with syngeneic thymus cells and sheep red cells. No significant response occurred if bone marrow cells were not given to the second irradiated host or if the first host had been given bone marrow cells instead of thymus cells. This finding suggested that thymus cells had to first react with antigen before interaction with bone marrow cells could produce a significant response. The results did not establish whether the interaction between thymus cells and antigen was a specific one. In a subsequent article, Mitchell and Miller (447) elaborated on this latter point in that they demonstrated that the thymic lymphocytes transferred to the first host had to first

react with the specific antigen before their interaction with bone marrow cells in a second irradiated host could produce a significant hemolysin response. They found that the specific antigen (sheep red cells) had to be given to the first host, which had been irradiated and given thymus cells, in order to obtain a significant response following transfer of their spleen cells, isologous marrow cells from another donor and sheep red cells to the second irradiated host. The administration of a non-cross-reactive antigen, horse red cells, to the first host resulted in a failure of its spleen cells to confer responsiveness to sheep red cells in the second host following the transfer of the spleen cells and syngeneic marrow cells. Mitchell and Miller (447) also injected syngeneic or allogeneic thymus cells or thoracic duct cells into neonatally thymectomized mice inoculated with sheep eythrocytes, with a resultant increase in the number of hemolysin-forming cells in the spleens of these animals. The immediate precursor of the hemolysin-forming cell was identified as a cell originating in the thymectomized host mice and not from the thoracic duct or thymus cells which had been administered. They concluded, on the basis of this and previous experiments (53), that the thymus lymphocyte had first to react with the specific antigen before interaction with bone marrow cells could produce a significant hemolysin response, that the 19S hemolysin-forming cell is marrow derived and that the thymus or thoracic duct lymphocytes recognize antigen and interact with it in the same way that triggers off differentiation of the essentially-passive bone marrow derived

precursor cells to specific antibody-forming cells. Mitchell and Miller (447) rule out any direct immunologic role for the macrophage or an epithelial cell since smears of suspensions of bone marrow and thymus revealed few, if any, cells which could be unequivocally identified as an epithlial cell or macrophage. Saline cell-free thymus extracts failed to produce more hemolysin-forming cells than were observed in neonatally thymectomized controls, thus demonstrating that the activity of the syngeneic thymus cells is dependent upon intact and undamaged cells. Since the bone marrow of neonatally thymectomized rodents is normal in appearance and resembles that of more mature marrow (53, 448), it could give rise to the hemolysin-producing cell in the thymectomized neonate.

A number of other experimental findings suggest a specific interaction between thymus lymphocytes and antigen(s). Davies et al (451, 514) observed that thymus-graft derived cells responded briskly to sheep red cells with a burst of mitotic activity. However, neither Davies et al (352) nor Mitchell and Miller (447) could detect any antibody-forming cells following this interaction between the thymus cells and antigen. Davies et al (514) transferred spleen cells, containing chromosomally-marked thymus and bone marrow derived cells, from a host mouse which had been immunized with sheep red cells, into an irradiated secondary host mouse. This latter host had been rendered isoimmune prior to irradiation so as to kill or prevent the proliferation of cells derived from the bone

marrow graft. They found that although thymus - derived cells were mitotically - reactive to antigenic stimulation in vivo, no antibody could be detected in the animals, suggesting that the thymus-derived cells are not capable of antibody formation nor of giving rise to antibody-forming cells. These investigators (447, 514) suggest that thymus-derived cells which have reacted to antigen produce an RNA-antigen complex which is necessary to trigger off transformation or conversion of the bone marrow-derived precursor of the antibody forming cell. As in the experiments of Gengozian et al (445) and Claman et al (52), Mitchell and Miller (447) also observed that spleen cells injected with rat red cells into irradiated mice produced much more antibody than a similar number of thymic lymphocytes. However, spleen cells, especially in rodents, would be expected to be immunologically effective since they are a mixture of thymus-derived and bone-marrow derived cells (447). Furthermore, the rodent spleen possesses hematopoietic function.

Dukor et al (449) injected thymectomized, irradiated mice with chromosomally-marked bone marrow and thymus cells and found both bone-marrow-donor type and thymus-donor type cells dividing in the lymphoid tissues. The majority of the dividing cells were of marrow origin. These findings confirmed those of Mitchell and Miller (447) cited above.

In a totally in vitro system, Globerson and Auerbach (450) observed that the competence of adult mouse spleen to evoke a

graft-versus-host reaction in vitro was lost upon sublethal irradiation but could be restored when the spleen fragments were grown in the presence of thymic tissue for two to three days. When lethal doses of irradiation were used, reactivation of immune competence did not occur unless both thymus and bone marrow tissues were present. Here, the bone marrow appeared to supply the immunocompetent cells.

On the basis of the results presented above, it appears that the immediate precursor of the antibody-forming cell, the "effector cell" of Claman et al (52) is derived from the bone marrow which need not migrate through the thymus. One may then ask what the role of the thymus or thoracic-duct cell is in the conversion of precursor cell of antibody-forming cell and to which cell lineage the "antigen-reactive cells" and the "auxiliary cells" of Claman (52) belong - the thymus or the bone marrow.

The results of experiments presented in this thesis strongly suggest that the bone marrow contributed the cells to the "immunocompetent cell pool". This conclusion is based on our findings that only bone marrow cells from normal, unimmunized rabbits reacted with blastogenesis and mitosis in the presence of a number of protein antigens. Lymph node, spleen or thymus cells of normal rabbits did not react in vitro. Although the basal activity of the various cell populations was high, as represented by the tritiated thymidine incorporation in the control tubes, the specific tritium incorporation, that is, the ratio of tritium incorporation in the presence

of the antigen to that incorporated in the absence of the antigen, was 3-10 for normal bone marrow cells whereas it was approximately 1 for normal lymph node, spleen and thymus cells. The other experimental findings presented and discussed above only serve to corroborate this interpretation of the role of the bone marrow in the immune response.

A study was then initiated in order to identify the cell(s) in the bone marrow which reacts to antigen in vitro. Normal rabbit bone marrow was separated into two fractions by centrifugation in a linear sucrose density gradient. Fraction I, which was enriched with small and medium-sized lymphocytes, responded markedly in the presence of the antigens whereas Fraction II, which contained the majority of the immature cells of the erythroid and granulocyte series in addition to a relatively small number of lymphocytes, responded poorly. The results suggest that the responsive cells form a part of the lymphoid population of the bone marrow rather than some of the other cell types. While Fraction I contained, in addition to lymphocytes, some larger blast-like cells which could conceivably have participated in the response, these cells were actually present in larger numbers in the poorly responding Fraction II. Furthermore, the difference in magnitude of response between Fractions I and II cannot be attributed to effects exerted on the cells by the varying sucrose concentration along the gradient since incubation of cell suspensions from whole bone marrow for 3 hours in medium containing 15 per cent sucrose, which is the high-

est sucrose concentration employed in the present fractionation technique, did not impair the response upon exposure to antigen.

These results provide support for the concept of a functional heterogeneity of lymphocytes, despite the morphological similarity of small lymphocytes, in the various lymphoid organs. The question of functional heterogeneity has also been raised by the findings presented herein with respect to the response of lymphoid cells of the various organs to phytohemagglutin (PHA) in vitro. The lymphocytes of each of the lymphoid organs investigated-bone marrow, spleen, lymph node, thymus, appendix, sacculus rotundus and blood-possess a distinct "PHA reactive profile" which is capable of differentiating the lymphocyte populations of each of the organs investigated. No two cell preparations reacted in a similar way. The five criteria by which these cell populations could be differentiated are:

(1) The concentration of PHA required for optimal blastogenesis.

(2) The latent period of the blastogenic response.

(3) The time required for maximum blastogenesis.

(4) The time required for maximum specific incorporation of tritiated thymidine.

(5) The duration of the blastogenic response.

These data suggest the presence of functionally-distinct lymphocyte populations in each of the lymphoid organs in the animal body. Furthermore, evidence has been presented in the current literature strongly suggesting the presence of a multiplicity of lymphocyte

types within the various lymphoid organs. Meuwissen et al (452) observed a failure of response to phytohemagglutinin by lymphocytes from children with congential thymic dyplasia although the response to allogeneic cells was unimpaired. Sahiar and Schwartz (453) observed marked histological differences in the spleens and lymph nodes of rabbits treated with 6-mercaptopurine (6-MP) and/or antigen. There was failure of germinal center enlargement in the spleens of rabbits given 6-MP. In contrast, however, the lymph nodes of the 6-MP treated rabbits displayed marked proliferative activity, particularly in the germinal centers. The authors suggested that these results are probably due to intrinsic differences in susceptibility to 6-MP between germinal center lymphocytes of the spleen and lymph node, implying functionally-heterogeneous lymphoid populations. The heterogeneity of splenic mononuclear cells has been demonstrated by the work of Plotz and Talal (454), who were able to fractionate splenic cells by passage over glass bead columns.

The functional heterogeneity of the lymphocytes in the various lymphoid organs has been further demonstrated on the basis of several other criteria. These are (a) their central as against their peripheral location, (b) the presence of thymus dependent and thymus independent areas in the peripheral lymphoid organs, notably the spleen and lymph nodes and (c) the life-span of the lymphocyte, since it has been demonstrated that there exist both long-lived and short-lived lymphocytes within the same lymphoid organ.

It has been shown, experimentally, that the lymphoid tissues may be classified as central and peripheral tissues (455). A lymphoid organ qualifies as central if its extirpation prior to a critical period in development compromises the integrity of the remaining lymphoid tissues. The thymus, in several animal species, and the bursa of Fabricius in the chicken have central lymphoid function. While the immunologic functions of the thymus and the bursa of Fabricius are different, under experimental conditions, these two organs show similarities of development and lymphoepithelial morphology. Both are composed of follicles of lymphoid tissues with cortical and medullary organization (462), their development is independent of antigenic stimulation (463) and they possess very few antibody-forming cells (464). However, thymic and bursal cells differ in that bursal lymphocytes are somewhat larger, they contain a higher RNA to DNA ratio and they display an abundance of polyribosomes (457, 465).

It has been presumed that lymphoid cells migrate from these central organs to peripheral lymphoid tissues such as the spleen and lymph nodes where they facilitate morphologic and functional maturity (455). In the rabbit, it has been demonstrated that the appendix (456, 457) and the sacculus rotundus (458) appear to possess central lymphoid function and these organs can be compared with the bursa of Fabricius, both in morphology and function (459). Peterson et al (460) and Cooper et al (461) have suggested

that the appendix, tonsils and other gut-associated lymphoid organs have a bursal role in man and rabbits. Cooper et al (461) removed the sacculus rotundus, appendix and Peyer's patches, which they refer to as the bursal homologue in man, from neonatal rabbits and observed that the humoral antibody response of these rabbits at a later date was much depressed compared to that of sham-operated rabbits. Their capacity to reject skin homografts was not impaired.

On the basis of the findings presented above it would appear that the bursa of Fabricius is concerned mainly with the establishment of the conditions leading to humoral antibody formation (457, 466) whereas the thymus gland is mainly, but not altogether, concerned with the establishment of a state of cellular immunity (429-433, 466-468). Cain et al (469) observed that thymus and spleen cells, obtained from ten week old Arbor White Rock chickens, demonstrate graft-versus-host activity, a cell-mediated immune reaction, when injected into fourteen day old Ghostly White Leghorn embryos, as determined by the extent of splenomegaly produced in the embryos by the transferred cells (470). Bursal cells injected into the embryos manifested no such reactivity. It has also been demonstrated that the thymus contributes towards full development of humoral immunity (466, 471-473) since thymectomy of young animals has been shown to decrease their humoral immune responsiveness to a number of well defined antigens in later life (466, 469, 471-475). Fichtelius et al (476) have questioned

whether this action of the thymus is a direct result of the absence of thymic function or an indirect result of depression of bursal differentiation as a consequence of the lack of vigorous thymic-dependent defense mechanisms.

Thymectomy at birth or soon afterwards is accompanied by incomplete development of the lymphoid tissue, lymphopenia and a generalized impairment of immunologic function (477, 478). In the adult animal, it has been demonstrated that thymectomy results in a relatively slow but progressive decrease in the number of small lymphocytes in the lymph nodes, blood and thoracic duct lymph (478-480) and a gradual reduction of the immunologic capabilities, both cellular and humoral, of the host (481-483). It has been shown that subjecting adult mice to both thymectomy and irradation results in a rapid and irreversible lymphopenia and in the reduction of the animals to the status of immunological cripples (484, 485).

The immunological unresponsivness induced in mice following neonatal thymectomy can be corrected by the injection of adult syngeneic spleen cells (486), neonatal spleen or lymph node cells (487) or thoracic duct cells (488) obtained from normal, adult mice. It can also be alleviated by the grafting of thymic tissue in a millipore diffusion chamber, which would prevent the emigration of thymic cells from the graft (489), thus lending further support to the theory that the thymus may function as an endocrine organ (42, 98, 181, 257). These results suggest that the immunological deficiency in thymectomized mice is related solely to a lack of circulating

lymphocytes. They also exclude the possibility that the unresponsiveness is the result of a defect in cellular "processing" of antigen or of an unfavourable environment for the proliferation of immunologically competent cells.

In 1962, Waksman et al (490) described areas of lymphocyte depletion in tissues of neonatally-thymectomized rats. These areas, which have since come to be regarded as the "thymus dependent areas", comprise the periarteriolar sheaths of the splenic white pulp and the diffuse lymphocyte fields, constituting the sub-flollicular and inter-follicular areas, of the lymph nodes and Peyer's patches. Similar areas of lymphocyte depletion were described by Parrott et al (491) in neonatally-thymectomized mice on the basis of the localization of radioactively-labelled spleen cells and thymocytes. Similar observation as to the presence of thymus-dependent lymphocytes have been made by Turk (492), de Sousa and Parrott (493) and Parrott and de Sousa (494). On the basis of results of experiments using radioactively-labelled lymphocytes and chromosome-markers, it has been demonstrated that thymus cells emigrate from the thymus to the lymph nodes in the guinea pig (495, 496), the mouse (497) and the rat (498). The experiments of Goldschneider and McGregor (499) and Parrott and de Sousa (500), using radio-actively labelled spleen and thymus cells injected into thymectomized animals, and those of Weissman (501), who injected labelled thymus cells into normal animals, have corroborated the presence of thymus-dependent areas in the lymphoid tissues of the

rat. Goldschneider and McGregor (499) have demonstrated that the thymus - dependent areas in lymphoid tissue are occupied by cells of the circulatory lymphocyte pool since the injection of isogeneic small lymphocytes into the neonatally-thymectomized rat resulted in their colonizing the thymus-dependent areas of the lymphoid tissue. Furthermore, the injected lymphocytes did not localize elsewhere. The small lymphocyte passed from the blood to lymphoid tissue along a route which included the marginal sinus in the splenic white pulp and post-capillary vessels in the cortex of lymph nodes and Peyer's patches. They concluded that neither the ability of small lymphocytes to populate lymphoid tissue nor the ability to traverse post-capillary venules are thymus-dependent functions (494). It had previously been shown (502) that rat small lymphocytes traverse from the blood into the interfollicular areas of the lymph nodes through the walls of the post-capillary venules. More specifically, the lymphocytes pass through the cytoplasm of the endothelial cells which line the walls of the vessels unlike the other leukocytes which penetrate the vessel wall at the endothelial cell junctions.

It has been shown by Everett et al (503, 504), in experiments using rats infused with tritiated thymidine, that lymphocytes in the rat have a various life span. There exists a short-lived population of lymphocytes mainly located in the thymus and bone marrow (504, 505) and a predominantly long-lived population of lymphocytes found in the spleen, lymph nodes and thoracic duct lymph (503, 504). The lymphoid cells in the germinal centers in the spleen

are also short-lived (506, 507). Fliedner et al (507) showed that, after a single injection of tritiated thymidine, 30-40 percent of the cells in the germinal centers in the rat spleen were labelled within one hour. However, these cells remained labelled for only 24-36 hours due to the rapid mitotic activity of these cells. The mean grain count in radioautographs of these cells diminished with a half-life of 13.4 hours. Cottier et al (508) presented evidence in favor of the independent proliferation of long-lived and short-lived lymphocytes. Extracorporeal irradiation of the blood of the calf completely depleted the animal of recirculating lymphocytes. Such depletion, however, failed to diminish the extent of proliferative activity of germinal centers although the surrounding cuffs of small lymphocytes were seriously reduced. Lymphopenia was found to persist for many months despite hypertrophy of the germinal centers. Radiation - induced chromosome aberrations have been used as markers to identify long-lived small lymphocytes capable of surviving for long periods in vivo without dividing (508). These cells could be identified by their chromosome alteration following stimulation with phytohemagglutinin in vitro. Nordman et al (509) estimated that the life span of the long-lived circulating human lymphocyte to be approximately 550 days. Weber and Nowell (175) cultured circulating lymphocytes from irradiated monkeys in the presence of phytohemagglutinin and observed cells with abnormal chromosomes in the circulation up to 8 months after irradiation.

In summary, it has been demonstrated that normal rabbit bone marrow cells are capable of reacting upon initial exposure to protein antigens in vitro with blastogenesis and mitosis and radioactive thymidine incorporation. On the basis of experimental results obtained, this response has been designated as a primary immune response or as the initial cellular event in the sequence of events leading up to antibody formation in the primary response. Furthermore, this response of the normal bone marrow cells to protein antigens in vitro appears to be localized to its lymphocyte population. It is anticipated that a more precise identification of the responsive cells will be achieved by further studies using the more highly purified lymphocyte fractions which may be obtained by differential density gradient centrifugation. Although it has been demonstrated that stimulated bone marrow cells contain specific antibodies, it has not yet been determined whether these antibodies are identical to the humoral antibodies detected in a classical immune response initiated and sustained in vivo. Experiments are currently in progress to attempt to elucidate further the nature of the in vitro bone marrow lymphocyte response and to relate it to the two main distinguishable immune responses - the humoral and cellular immune responses.

CHAPTER 6

SUMMARY

Fragments of lymph nodes and spleens of previously immunized rabbits were shown to be capable of synthesizing and releasing antibody when exposed to antigen and maintained in culture in vitro. Fragments of thymus did not respond with antibody formation in vitro. Cell suspensions of immune lymph node and spleen were also capable of undergoing blastogenesis and mitosis and of incorporating tritiated thymidine when maintained in culture with antigen in vitro. Cell suspensions of immune thymus and bone marrow did not respond in vitro.

On the other hand, neither fragments nor cell suspensions prepared from lymph nodes, spleen and thymus of normal, unimmunized rabbits and maintained in culture at 37°C responded with antibody formation or blastogenesis. However, normal bone marrow cells maintained in culture with the antigen for five days responded with marked blastogenesis and tritiated thymidine uptake. The specificity of this in vitro response was demonstrated by the fact that following the administration of a protein antigen in vivo the bone marrow cells lost their capacity to react to that particular protein antigen but not to other, non-cross-reacting antigens. Furthermore, bone marrow cells obtained from rabbits previously made tolerant to a protein antigen, HSA, failed to

respond to this antigen in vitro but did to other protein antigens. It also demonstrated that normal bone marrow cells incubated with antigen are capable of forming antibody which could be detected by the fluorescent antibody technique. However, the results of coprecipitation experiments suggest that this antibody is "cell bound" in nature since none, or very little, appeared to be secreted by the cells into the medium.

Centrifugation of the whole bone marrow cell suspension in a linear sucrose density gradient yielded two fractions, designated Fractions I and II. Fraction I, which was enriched with small and medium size lymphocytes, responded to a degree 5-10 times greater than that of the original whole bone marrow when exposed to the antigen in vitro. Fraction II, which contained most of the non-lymphoid cells, responded poorly.

It was also demonstrated that lymphoid cells obtained from the various lymphoid organs - lymph node, spleen, bone marrow, thymus, appendix, sacculus rotundus and peripheral blood - respond differently when incubated with phytohemagglutinin in vitro, thus demonstrating the presence of functionally distinct populations of lymphocytes in each of these organs.

It is concluded that the bone marrow lymphocyte, by its capacity to react with blastogenesis and mitosis and antibody formation upon initial exposure to the antigen, a property not possessed by lymphocytes of the other lymphoid organs, has a preemminent role in the sequence of cellular events leading to antibody formation in vivo.

CLAIMS OF ORIGINAL WORK

1. It has been demonstrated that lymph node and spleen cells obtained from previously immunized rabbits are stimulated to undergo blastogenesis and mitosis when exposed to the antigen and cultured in vitro at 37°C. Cells of the other lymphoid organs failed to respond upon incubation with antigen in vitro.

2. A correlation was found to exist between the capacity of the lymph node cells obtained from a previously - immunized rabbit to undergo blastogenesis and mitosis in response to antigen and the synthesis of antibody by fragment cultures of the same lymph node in vitro.

3. It has been demonstrated that bone marrow cells obtained from normal, unimmunized rabbits and cultured with various protein antigens in vitro are capable of undergoing blastogenesis and mitosis and of incorporating radioactively-labelled thymidine. This response was not observed with lymphoid cell suspensions obtained from the other lymphoid organs.

4. The specificity and immune nature of the blastogenic response of the normal bone marrow cells to antigens in vitro was demonstrated using the fluorescent antibody technique. Antibody could be specifically detected within the stimulated bone marrow cells.

5. The response of the whole bone marrow cell suspension to antigens in vitro was localized to the small and medium size lymphocyte populations of the bone marrow.

6. It was demonstrated that lymphocytes obtained from various lymphoid organs respond differently to phytohemagglutinin in vitro, suggesting that each lymphoid organ possesses a functionally - distinct population of lymphocytes.

BIBLIOGRAPHY

1. Marshall, A.H.E. and and White, R.G.
Brit. J. Exp. Path., 31: 157, 1950.

2. White, R.G.
in Mechanisms of Antibody Formation,
Czech. Acad. Sci., Prague, 1960.

3. Fagraeus, A.
Acta Med. Scand. Suppl. p. 204, 1948.

4. Wissler, R.W., Fitch, F.W., La Via, M.F. and Gunderson, C.H.
J. Cell. Comp. Physiol. 50: 265, 1957.

5. Ward, P.A., Johnson, A.G. and Abell, M.R.
J. Exp. Med., 109: 463, 1959.

6. Congdon, C.C. and Makinoden, T.
Amer. J. Path., 39: 697, 1961.

7. Thorbecke, G.J., Asofsky, R.M., Hochwald, G.M. and Siskind, G.W.
J. Exp. Med., 116: 295, 1962.

8. Gunderson, C.H., Juras, D., La Via, M.F. and Wissler, R.W.
J. A. M. A., 180: 1038, 1962.

9. Keuning, F.J., Meer, J. van der Nieuwenheis, P. and Ouderdijk, P.
Lab. Invest., 12: 156, 1963.

10. Langevoort, H.L.
Lab. Invest., 12: 106, 1963.

11. Bramms, W.G.
Acta Neerl, Morph., 4: 289, 1961.

12. Hanna, M.G.
A thesis, University of Tennesse, 1964.

13. Hanna, M.G.
Lab. Invest., 13: 95, 1964.

14. Craddock, C.G., Winkelstein, A.B., Matsuyuki, Y. and Lawrence, J.S.
J. Exp. Med., 125: 1149, 1967.

15. Ehrich, W.E. and Harris, T.N.
J. Exp. Med., 76: 335, 1942.

16. Hall, J.G. and Morris, B.
J. Exp. Physiol., 48: 235, 1963.

17. Hall, J.G. and Morris, B.
Lancet I: 107, 1964.

18. Smith, R.T.
Ciba Found. Symp. on Cellular aspects of immunity
p. 348, 1960. (Churchill, London)

19. Coons, A.H., Leduc, E.H. and Connolly, J.M.
J. Exp. Med., 102: 49, 1955.

20. Leduc, E.H., Coons, A.H. and Connolly, J.M.
J. Exp. Med., 102: 61, 1955.

21. White, R.G., Coons, A.H. and Connolly, J.M.
J. Exp. Med., 102: 73, 1955.

22. Keuning, F.J. and van der Slikke, L.B.
J. Lab. Clin. Med., 36: 167, 1950.

23. Nossal, G.J.V. and Makela, O.
J. Exp. Med., 115: 209, 1962.

24. Makela, O. and Nossal, G.J.V.
J. Exp. Med., 115: 231, 1962.

25. Ward, P.A., Johnson, A.G. and Abel, M.R.
Lab. Invest., 13: 180, 1963.

26. Cottier, M. Odartchenko, N., Keiser, G., Hess, M. and Stoner, R.D.
Ann. N.Y. Acad. Sci., 113: 612, 1964.

27. Sainte-Marie, G. and Coons, A.H.
J. Exp. Med., 119: 743, 1964

28. Hall, J.G., Morris, B., Moreno, G.D. and Bessis, M.C.
J. Exp. Med., 125: 91, 1967.

29. Cunningham, J.B.S. and Mercer, E.H.
J. Exp. Med., 124: 701, 1966.

30. Attardi, G., Cohen, M., Horibata, K. and Lennox, E.S.
J. Immunol., 92: 346, 1964.

31. Vazquez, J.J.
Lab. Invest., 10: 1110, 1961.

32. Deutsch, L.
Ann. Inst. Pasteur, 13: 689, 1899.

33. Chase, M. W.
Fed. Proc., 10: 404, 1951.

34. Chase, M. W.
in The Nature and Significance of the Antibody Response, Symp. Sect. Microbiol. N. Y. Acad. Med. (A. M. Pappenheimer, Jr., ed.), p. 156. Columbian Univ. Press, N. Y. 1952.

35. Landsteiner, K. and Chase, M. W.
Proc. Soc. Exp. Biol. N.Y., 49: 688, 1942.

36. Harris, J. and Harris, T. N.
Fed. Proc., 10: 409, 1951.

37. Harris, S. and Harris, T. N.
J. Exp. Med., 100: 269, 1954.

38. Harris, S. and Harris, T. N.
J. Immunol., 74: 318, 1954.

39. Harris, T. N., Harris, S. and Faber, M. P.
J. Immunol., 75: 112, 1955.

40. McGregor, D. D. and Gowans, J. L.
J. Exp. Med., 117: 303, 1963.

41. Gowans, J. L. and McGregor, D. D.
The origin of antibody forming cells. in Immunopathology, IIIrd Int. Symp., p. 89, 1963.

42. Trench, C. A. H., Watson, J. W. Walker, F. C., Gardner, P. S. and Green, C. A.
Immunol., 10: 187, 1966.

43. Naspitz, K., Singhal, S. K. and Richter, M.
Int. Arch. Allergy Immunol. 1968 (In Press).

44. Dixon, F. J., Roberts, J. C. and Weigle, W. O.
Fed. Proc., 16: 649, 1957.

45. Dixon, F. J., Weigle, W. O. and Roberts, J. C.
J. Immunol., 78: 56, 1957.

46. Konda, S.
Acta Haematol. Japan, 22: 656, 1959.

47. Perkins, E.M. Robins, M.A. and Makinodan, T.
J. Immunol., 86: 533, 1961.

48. Stoner, R.D. and Hale, W.M.
J. Immunol., 75: 203, 1955.

49. Rosenberg, L.T., Chandler, M.H., Gordon, A.S. and Fischel, E.E.
J. Immunol., 81: 136, 1958.

50. Taliaferro, W.H. and Taliaferro, L.G.
J. Infectious Diseases, 110: 165, 1962.

51. Doria, J., Goodman, J., Gengozian, N. and Congdon, C.C.
J. Immunol., 88: 20, 1962.

52. Claman, H.N., Chaperon, E.A. and Triplett, R.F.
Proc. Soc. Biol. N.Y., 122: 1165, 1966.

53. Miller, J.F.A.P. and Mitchell, G.F.
Nature, 216: 659, 1967.

54. Dixon, F.J. and Weigle, W.O.
J. Exp. Med., 105: 75, 1957.

55. Dixon, F.J. and Weigle, W.O.
Fed. Proc., 16: 411, 1957.

56. Trnka, Z.
Nature, 181: 55, 1958.

57. Harris, T.N., Harris, S. and Farber, M.B.
Proc. Soc. Exp. Biol. N.Y., 102: 495, 1959.

58. Harris, T.N., Harris, S. and Farber, M.B.
J. Immunol., 88: 199, 1962.

59. Nossal, G.J.V.
Immunol., 2: 137, 1959.

60. Dixon, F.J., Roberts, J.C. and Weigle, W.O.
J. Exp. Med., 105: 417, 1957.

61. Weigle, W.O. and Dixon, F.J.
J. Immunol., 82: 516, 1959.

62. Roux, W.
Z. Biol., 21: 411, 1885.

63. Jolly, J.
C.R. Soc. Biol. (Paris), 55: 1266, 1903.

64. Beebe, S.P. and Ewing, J.
Brit. Med. J., 2: 1559, 1906.

65. Harrison, R.G.
Proc. Soc. Exp. Biol. N.Y., 4: 140, 1907.

66. Burrows, M.T.
Anat. Rec., 6: 141, 1912.

67. Carrel, A.
J. Exp. Med., 17: 14, 1913.

68. Thomson, D.
Proc. Soc. Med., 7. Marcus Beck Lab Rep., 2: 21, 1914.

69. Carrel, A.
J. Exp. Med., 15: 516, 1912.

70. Ebeling, A.H.
J. Exp. Med., 17: 273, 1913.

71. Lewis, M.R. and Lewis, W.H.
Johns Hopk. Hosp. Bull., 22: 126, 1911.

72. Lewis, M.R. and Lewis, W.H.
Science, 39: 330, 1914.

73. Strangeways, T.S.P.
Proc. Roy. Soc., B 94: 137, 1922.

74. Fischer, A.
J. Exp. Med., 36: 393, 1922.

75. Champy, C.
C. R. Soc. Biol. Paris, 76: 31, 1914.

76. Cholopin, N.
Arch. Mikr., 96: 435, 1922.

77. Canti, R.G.
Arch. Exp. Zelltorsch, 6: 86, 1928.

78. Comandon, J., Levaditi, C. and Mutermilch, S.
C. R. Soc. Biol. Paris, 74: 464, 1913.

79. Carrel, A. and Baker, L.E.
J. Exp. Med., 44: 503, 1926.

80. Fischer, A.
J. Biochem., 43: 491, 1948.

81. Parker, R.C.
in Methods of Tissue Culture, 3rd ed.
(P.B. Hoeber Inc. N.Y.) 1960.

82. White, P.R.
Growth, 10: 231, 1946.

83. White, P.R.
J. Cell Comp. Physiol., 34: 221, 1949.

84. Waymouth, C.
Tex. Rep. Biol. Med., 13: 552, 1955.

85. Waymouth, C.
J. Cancer Inst., 17: 305, 1956.

86. Gey, G.O.
Amer. J. Cancer, 17: 752, 1933.

87. Medawar, P.B.
Nature, 148: 783, 1941.

88. Moscona, M. and Moscona, A.
J. Anat., 86: 287, 1952.

89. Rivaldini, L.M.
Nature, 173: 1134, 1954.

90. Dulbecco, R.
Proc. Nat. Acad. Sci. Wash., 38: 747, 1952.

91. Younger, J.
Proc. Soc. Exp. Biol. N.Y., 85: 202, 1954.

92. Pfeiffer, R. and Marx, Z.
Die Bildungsstatte der Choleraschutzsteffe. Z. Hyg.,
27: 272, 1898.

93. Ludke, M.
Berl. Klin. Wschr., 49: 1034, 1912.

94. Kucynski, M.A., Tannenbaum, E. and Werthermaun, A.
Virchow's Arch., 258: 687, 1925.

95. Meyer, K. and Lowenthal, H.
Z. Immun. Forsch., 54: 420, 1928.

96. Salle, A. J. and McOmie, W. A.
J. Immunol., 32: 157, 1937.

97. Askonas, B. A. and Humphrey, J. H.
Biochem. J., 68: 252, 1958.

98. Miller, J. F. A. P., Osoba, D. and Dukor, P.
Ann. N. Y. Acad. Sci., 124: 95, 1965.

99. Michaelides, M. C. and Coons, A. H.
J. Exp. Med., 117: 1035, 1963.

100. Michaelides, M. C.
Fed. Proc., 16: 426, 1957.

101. O'Brien, T. F. and Coons, A. H.
J. Exp. Med., 117: 1063, 1963.

102. Halliday, W. J. and Garvey, J. S.
J. Immunol., 93: 757, 1964.

103. Tao, T. W.
Science, 146: 247, 1964.

104. Jubasz, A. and Rose, B.
Can. J. Biochem., 43: 787, 1965.

105. Juhasz, A. and Richter, M.
Can. J. Biochem., 43: 803, 1965.

106. Richter, M., Juhasz, A., Drechsler, B. and Myers, J.
Nature, 210: 645, 1966.

107. Mishell, R. I. and Dutton, R. W.
Science, 153: 1004, 1966

108. McKenna, J. M. and Stevens, K. M.
J. Exp. Med., 111: 573, 1960.

109. Vaughan, J. H., Dutton, A. H., Dutton, R. W., George, M. and Marston, R. Q.
J. Immunol., 84: 258, 1960.

110. Smiley, J. D., Heard, J. G. and Ziff, M.
J. Exp. Med., 119: 881, 1964.

111. Stavitsky, A. B.
J. Immunol., 75: 214, 1955.

112. Steiner, D.F. and Anker, M.S.
Proc. Natl. Acad. Sci. U.S.A., 42: 580, 1956.

113. Svehag, S.E.
Science, 146: 659, 1964.

114. Helmreich, E., Kern, M. and Eisen, H.N.
J. Biol. Chem., 236: 464, 1961.

115. Helmreich, E., Kern, M. and Eisen, H.N.
J. Biol. Chem., 237: 1925, 1962.

116. Scharff, M.D. and Uhr, J.W.
Science, 148: 646, 1965.

117. Richardson, M., and Dutton, R.W.
Science, 146: 655, 1964.

118. Krueger, R.C. and Twedt, R.M.
J. Immunol., 90: 952, 1963.

119. Hulliger, L. and Sorkin, E.
Nature, 198: 299, 1963.

120. Hulliger, L. and Sorkin, E.
Immunol., 9: 391, 1965.

121. Nossal, G.J.V. and Lederberg, J.
Nature, 181: 1419, 1958.

122. Nossal, G.J.V.
Brit. J. Exp. Path., 39: 544, 1958.

123. Nossal, G.J.V.
Brit. J. Exp. Path., 40: 118, 1959.

124. Nossal, G.J.V.
Brit. J. Exp. Path., 40: 301, 1959.

125. Nossal, G.J.V.
Brit. J. Exp. Path., 41: 89, 1960.

126. Makela, O. and Nossal, G.J.V.
J. Immunol., 37: 447, 1961.

127. Makela, O. and Nossal, G.J.V.
J. Immunol., 87: 457, 1961.

128. Nossal, G.J.V. and Makela, O.
Elaboration of antibodies by single cells.
Ann. Rev. Microb., 16: 53, 1962.

129. Nossal, G.J.V. and Makela, O.
J. Immunol., 88: 604, 1962.

130. Attardi, G., Cohn, M., Horibata, K. and Lennox, E.S.
in Sym. on the biol. of cells modified by viruses or antigens. II On the analysis of antibody synthesis of the cellular level, Bact. Rev., 23: 213, 1959.

131. Jerne, N.K., Nordin, A.A., and Henry, C.
in Cell bound antibodies (B. Ames and H. Koprowski eds) Wister Inst. Press, p. 1094, 1963.

132. Ingraham, J.S.
Compt. Rend. Acd. Sci., 256: 5005, 1963.

133. Ingraham, J.S. and Bussard, A.
J. Exp. Med., 119: 667, 1963.

134. Friedman, H.
Fed. Proc., 24: 252, 1965.

135. Sterzl, J. and Mandel, L.
Folia Microb., 9: 173, 1964.

136. Rowley, D.A. and Fitch, F.W.
J. Exp. Med., 121: 671, 1964.

137. Bussard, A.E. and Binet, J.L.
Nature, 205: 675, 1965.

138. Sterzl, J. and Riha, I.
Nature, 208: 858, 1965.

139. Dresser, D.W. and Wortis, H.H.
Nature, 208: 859, 1965.

140. Weiler, E., Melletz, E.W. and Breuninger, P.E.
Proc. Natl. Acad. Sci. U.S.A., 54: 1310, 1965.

141. Berglund, K.
Nature, 204: 89, 1964.

142. Harris, T.N., Hummeler , K. and Harris, S.
J. Exp. Med., 123: 161, 1966.

143. Mountain, I.M.
J. Immunol., 74: 270, 1955.

144. Wolf, B. and Stavitsky, A.B.
J. Immunol., 81: 404, 1958.

145. Ambrose, C.T.
J. Exp. Med., 119: 1027, 1964.

146. Ambrose, C.T.
in Retention of Functional Differentiation in Cultured Cells (H. Koprowski ed.) Wister Inst. Press. Phil. p. 71, 1964.

147. Dresser, A.M.
Immunol., 9: 483, 1965.

148. Saunders, G.C. and King, D.W.
Science, 151: 1390, 1966.

149. Thorbecke, G.J. and Keuning, G.J.
J. Infect. Diseases, 98: 157, 1956.

150. Sterzl, J. and Jilek, M.
Nature, 216: 1233, 1967.

151. Thorbecke, G.J., Asofsky, R.M., Hochwald, G.M. and Siskind, G.W.
J. Exp. Med., 116: 295, 1962.

152. Dutton, R.W.
J. Exp. Med., 122: 759, 1965.

153. Holm, G., Perlmann, P. and Werner , B.
Nature, 203: 841, 1964.

154. Moller, E.
Science, 147: 873, 1965.

155. Patterson, R., Suszko, I.M. and Orr, M.F.
J. Immunol., 96: 139, 1966.

156. Eagle, H. and Piez, K.A.
J. Exp. Med., 116: 29, 1962.

157. Singhal, S.K. and Richter, M.
(Unpublished observations)

158. Moorhead, J.R., Connolly, J.J. and McFarland, W.
J. Immunol., 99: 413, 1967.

159. Stevens, K.M. and McKenna, J.M.
J. Exp. Med., 107: 537, 1958.

160. Mountain, I.M.
J. Immunol., 74: 278, 1955.

161. Kong, Y.M., and Johnson, A.G.
J. Immunol, 90: 672, 1963.

162. Schoenberg, M.D., Stabitsky, A.B., More, R.D. and Freeman, M.J.
J. Exp. Med., 121: 527, 1965.

163. La Via, M.F., Uris, S.A. and Ferguson, L.A.
J. Immunol., 84: 48, 1960.

164. Pernis, B., Cohen, M.W. and Thorbecke, G.J.
J. Immunol., 91: 541, 1963.

165. Grabar, P. and Carrazier, P.
in Cellular Aspects of Immunity (G.E.W. Wolstenholme and M. O'Conner eds.) Ciba Found Symp., Churchill London p. 198, 1960.

166. Berglund, S.E., Markey, P.A. and Mergenhagen, E.
Proc. Soc. Exp. Biol. Med., 126: 84, 1967.

167. Leibowitz, M.M.I. and Parks, J.J.
J. Exp. Med., 117: 197, 1963.

168. Tao, T.W. and Uhr, J.W.
Science, 151: 1096, 1966.

169. Globerson, A. and Auerbach, R.
Science, 149: 991, 1965.

170. Globerson, A. and Auerbach, R.
J. Exp. Med., 124: 1001, 1966.

171. Thorbecke, G.J. and Keuning, F.J.
J. Immunol., 70: 129, 1953.

172. McKenna, J.M. and Stevens, K.M.
J. Immunol., 78: 811, 1957.

173. Askonas, B.A., Humphrey, J.H. and Porter, R.R.
Biochem. J., 63: 412, 1956.

174. Ranney, H.M. and London, I.M.
Fed. Proc., 10: 562, 1951.

175. Weber, W.T. and Nowell, P.C.
J. Reticuloendothelial Soc., 2: 326, 1965.

176. Keston, A. S. and Katchen, B.
J. Immunol., 76: 253, 1956.

177. Lazda, V. and Starr, J. L.
J. Immunol., 95: 254, 1965.

178. Stavitsky, A. B.
Brit. J. Exp. Path., 39: 661, 1958.

179. Stavitsky, A. B. and Wolf, B.
Biochem. Biophys. Acta, 27: 4, 1958.

180. Kritzman, J. and Harper, A.
Proc. Soc. Exp. Biol. Med., 121: 489, 1966.

181. Osoba, D. and Miller, J. F. A. P.
Nature, 199: 653, 1963.

182. Stevens, K. M. and McKenna, J. M.
J. Exp. Med., 107: 537, 1958.

183. Ambrose, C. T. and Coons, A. H.
J. Exp. Med. 117: 1075, 1963.

184. Richards, F. F., Ambrose, C. T. and Haber, E.
J. Immunol., 96: 1013, 1966.

185. Dresser, A. M.
Immunol., 9: 483, 1965.

186. Roberts, S., Adams, E. and White, A.
J. Immunol., 62: 155, 1949.

187. Langevoort, H. L., Asofsky, R. M., Jacobson, E. B. de Vries, T. and Thorbecke, G. J.
J. Immunol., 90: 60, 1963.

188. Jerne, N. K. and Nordin, A. A.
Science, 140: 405, 1963.

189. Strander, H.
Immunol., 10: 45, 1965.

190. Tannenberg, W. J. K.
Nature, 214: 293, 1967.

191. Massie, F. S. and Frick, O. L.
J. Allergy, 40: 215, 1967.

192. Kern, M. and Eisen, H.N.
J. Exp. Med., 110: 207, 1959.

193. Fishman, M.
Nature, 183: 1200, 1959.

194. Fishman, M.
J. Exp. Med., 114: 837, 1961.

195. Friedman, H.P., Stavitsky, A.B. and Solomon, J.M.
Science, 149: 1106, 1965.

196. McKenna, J.M. and Stevens, K.M.
J. Exp. Med., 111: 573, 1960.

197. Marbrook, J.
Lancet, I: 1279, 1967.

198. Zalberg, O.B. van der Meul, V.A. and Van Twisk, J.M.
Nature, 210: 544, 1966.

199. Bussard, A.E. and Anderson, J.G.
Immunol., 11: 67, 1967.

200. Mosier, E.
Science, 158: 1573, 1967.

201. Hege, J.S., Cole, L.J.
J. Immunol., 99: 61, 1967.

202. Robinson, W.A., Marbrook, J. and Diene, E.
J. Exp. Med., 126: 347, 1967.

203. Bussard, A.E. and Lurie, M.
J. Exp. Med., 125: 873, 1967.

204. Bendinelli, M. and Wedderburn, N.
Nature, 215: 157, 1967.

205. Bussard, A.E.
Science, 153: 887, 1966.

206. Cremer, N.E.
J. Immunol., 90: 685, 1963.

207. Fleishman, J.B.
J. Immunol., 91: 163, 1963.

208. Dutton, R.W., Dutton, A.H. and Vaughan, J.H.
Nature, 182: 1317, 1958.

209. Dutton, R.W. and Pearce, J.D.
Immunol., 5: 414, 1962.

210. Dutton, R.W., Dutton, A.H. and Vaughan, J.H.
Biochem. J., 75: 230, 1960.

211. Smiley, J.D. and Jasin, H.E.
Immunol., 8: 49, 1965.

212. Dutton, R.W.
Nature, 192: 462, 1961.

213. Sussdorf, D.H.
Nature, 191: 770, 1961.

214. Ainis, M.
Nature, 194: 197, 1962.

215. Bussard, A. and Huyny, V.A.
Bioch. Biophy. Res. Comm., 3: 453, 1960.

216. Popisil, M.
Folia Microbiol., 8: 274, 1963.

217. Medzon, E.L. and Vas, S.I.
Can. J. Microb., 10: 535, 1964.

218. Capalbo, E.E. and Albright, J.F. and Bennett, W.E.
J. Immunol., 93: 243, 1964.

219. Van der Meer, J.H.H. and Koningsberger, V.V.
Biochem. Biophys. Acta. 103: 136, 1956.

220. Timofejewsky, A.D. and Benewetenskaja, S.W.
Arch. Exp. Zellforsch., 2: 31, 1925.

221. Timofejewsky, A.D. and Benewelenskaja, S.W.
Arch. Exp. Zellforsch., 4: 64, 1927.

222. Permain, G., Lycete, R.R. and Fitzgerald, P.H.
Lancet, I: 637, 1963.

223. Shrek, R.
Amer. Rev. Resp. Dis., 87: 734, 1963.

224. Hirschhorn, K., Kolodny, R.L., Hashem, N. and Bach, F.
Lancet, II: 305, 1963.

225. Marshall, W.H. and Roberts, K.B.
Lancet, I: 773, 1963.

226. Cowling, D.C., Quaglino, D. and Davidson, E.
Lancet, II: 1091, 1963.

227. Coulson, A.S. and Chalmers, D.C.
Immunol., 12: 417, 1967.

228. Hartog, M., Cline, M.J. and Grodsky, I.
Nature, 210: 314, 1966.

229. Caron, G.A. and Sarkany, I.
Nature, 210: 314, 1966.

230. Cump, D.W., Fekety, F.R. Jr., Urbanetti, J. and Nosenzo, G.
Amer. Rev. Resp. Dis. 95: 470, 1967.

231. Rouch, H.C.
Amer. Rev. Resp. Dis., 95: 220, 1967.

232. McFarland, W. and Heilman, D.H.
Amer. Rev. Resp. Dis., 93: 742, 1966.

233. Caron, G.A.
Int. Arch. Allergy, 30: 331, 1966.

234. Heilman, D.H. and McFarland, W.
Int. Arch. Allergy, 30: 58, 1966.

235. Heilman, D.H. and McFarland, W.
J. Immunol., 96: 988, 1966.

236. Fireman, P. Boesman, M. Harddad, Z.H. and Gitlin, D.
Science, 155: 337, 1967.

237. Hirschhorn, R. Hirschhorn, K. and Weissman, G.
Blood, 30: 84, 1967.

238. Vischer, T.L.
Lancet, II: 467, 1966.

239. Mayron, L.W. and Baram, P.
J. Immunol., 98: 1274, 1967.

240. Aspergren, N. and Rorsman, H.
J. Allergy, 35: 433, 1964.

241. Ricci, M., Passaleva, A., Ricca, M., Romagnani, S. and Bagnoni, E.
Lancet, II: 1031, 1966.

242. Lycette, R. R. and Pearmain, G. E.
Lancet, II: 386, 1963.

243. Elves, M. W., Roath, S. and Israeles, M. G. G.
Lancet, I: 806, 1963.

244. Elves, M. W., Roath, S., Taylor, G. and Israels, M. C. G.
Lancet, I: 1292, 1963.

245. Hirschhorn, K., Bach, F., Kolodney, R. L., Firschein, I. C. and Hashem, N.
Science, 142: 1185, 1963.

246. Ling, N. R. and Husband, E. M.
Lancet, I: 363, 1964.

247. Cowling, D. C. and Quaglino, D.
J. Path. Bact., 89: 63, 1965.

248. Girard, J. P., Rose, N. R. Kunz, M. L., Kobayashi, S. and Arbasman, C. E.
J. Allergy, 39: 65, 1967.

249. Wiener, S. and Brasch, J.
Med. J. Austr., I: 149, 1965.

250. Zitz, S. J., Van Arsdel, P. P. and McClure, D. K.
J. Allergy, 38: 321, 1966.

251. Richter, M. and Naspitz, C. K.
J. Allergy, 1968 (In Press)

252. Ricci, M., Passaleva, A. and Ricca, M.
Lancet II: 445, 1966.

253. Heitman, H. J.
Lancet II: 419, 1967.

254. Gotz, H. and Heitman, H. J.
Lancet I: 1380, 1967.

255. Holland, P. and Mauer, A. M.
Lancet, I: 1368, 1964.

256. Carron, G. A. and Sarkany, I.
Brit. J. Derm., 77: 556, 1965.

257. Feldman, F. and Globerson, A.
Ann. N. Y. Acad. Sci., 120: 182, 1964.

258. Ripps, C.S. and Hirschhorn, K.
Clin. Exp. Immunol., 2: 377, 1967.

259. Ripps, C.S. and Fellner, M.J.
Lancet II: 802, 1966.

260. Ripps, C.S. and Fellner, M.J. and Hirschhorn, K.
Lancet II: 95, 1965.

261. Gill, F.A.
J. Immunol., 98: 778, 1967.

262. Halpern, B., Ky, N.T., Amache, N., Lagrue, G. and Hazard, J.
La Presse Med., 75: 461, 1967.

263. Halpern, B., Ky, N.T. and Amache, N.
J. Allergy, 40: 168, 1967.

264. Woodliff, H.J. and Onseti, P.
Lancet II: 857, 1966.

265. Sarkany, I.
Lancet, I: 743, 1967.

266. Evans, D.A.
J. Allergy, 39: 399, 1967.

267. Hashem, N. and Barr, M.L.
Lancet II: 1029, 1963.

268. Fowler, I., Mossis, C.E. and Whitley, T.
New Engl. J. Med., 275: 1041, 1966.

268a. Hashem, N., Hirschhorn, K., Sedlis, E. and Holt, E.M. Jr.
Lancet II: 269, 1963.

269. Fjelde, A. and Kopecka, B.
Acta derm-venereal, 47: 168, 1967.

270. Pass, F., Larsen, W.G. and Lobitz, W.C.
Ann. Allergy, 24: 426, 1966.

271. Patrucco, A., Rothfield, N.F. and Hirschhorn, K.
Arthur. Rheumat. 10: 32, 1967.

272. Hirschhorn, K., Schereibman, R.R., Bach, F.H. and Siltzbach, L.E.
Lancet II: 842, 1964.

273. Cowling, D.C., Quaglino, D. and Bassett, P.K.M.
Brit. Med. J., I: 1481, 1964.

274. Aspegren, N. and Rorsman, M.
Int. Arch. Allergy, 24: 119, 1964.

275. Zweiman, B., Besdine, R.W. and Hildreth, E.A.
J. Immunol., 90: 672, 1966.

276. Zweiman, B., Besdine, R.W. and Hildreth, E.A.
Nature, 212: 422, 1966.

277. Mills, J.A.
J. Immunol., 97: 239, 1966.

278. Oppenheim, J.J., Wolstenecroft, R.A. and Gell, P.G.H.
Immunol., 12: 89, 1967.

279. Vischer, T.L., Stastny, P. and Ziff, M.
Nature, 213, 923, 1967.

280. Vischer, T.L., and Stastny, P.
Immunol., 12: 675, 1967.

281. Dutton, R.W. and Eady, J.D.
Immunol., 7: 40, 1964.

282. Chapman, N.D., Parkhouse, R.M.E. and Dutton, R.W.
Proc. Soc. Exp. Biol. Med., 117: 708, 1964.

283. Benezra, D., Gery, I. and Davies, A.M.
Proc. Soc. Exp. Biol. N.Y., 125, 1305, 1967.

284. Harris, G.
Nature, 211: 154, 1966.

285. Harris, G.
Immunol., 9: 529, 1965.

286. Zavaleta, A. and Stastny, P.
Clin. Exp. Immunol., 2: 543, 1967.

287. Marshall, W.H. and Roberts, K.B.
Quart. J. Exp. Physiol., 48: 146, 1963.

288. Mackinney, A.A., Stohlman, F. and Brecher, G.
Blood, 19: 349, 1962.

289. Hastings, J. Freedman, S., Rendon, O., Cooper, H.L. and Hirschhorn, K.
Nature, 192: 1214, 1961.

290. Tanaka, Y., Epstein, L.B., Brecher, G. and Stohlman, F.
Blood 22: 619, 1963.

291. Robins, J.H.
Experimentia, 20: 1, 1964.

292. Lindahl-Kiessling, K. and Book, J.A.
Lancet, II: 591, 1964.

293. Pisciotta, A.V., Westring, D.W., DePrey, C. and Walsh, B.
Nature, 215: 193, 1967.

294. Cooper, E.H.
Brit. J. Haemat., 8: 304, 1962.

295. Cooper, E.H., Barkhan, P. and Hale, A.J.
J. Haemat., 2: 101, 1963.

296. Lindahl-Kisessling, K. and Werner, B.
Lancet II: 862, 1964.

297. Rubio, C.A. and Zajicet, I.
Lancet I: 579, 1967

298. Winkelstein, A. and Craddock, C.G.
Blood, 29: 594, 1967.

299. McIntyre, O.R. and Segel, W.D.
Lancet I: 1265, 1966.

300. Bain, A.D. and Gould, I.K.
Brit. J. Exp. Path., 45: 530, 1964.

301. Lischner, H.W. and Punnett, H.H.
Lancet II: 640, 1966.

302. Wilson, J.A.
Lancet II: 52, 1966.

303. McIntyre, O.R. and Segel, W.D.
Lancet I: 1265, 1966.

304. Stroselli, E., Maccani, U. and Longe, A.
Hematologica, 50: 220, 1965.

305. Claman, H.N.
Proc. Soc. Exp. Biol. Med., 121: 236, 1966.

306. Oettgen, H.F., Silber, R., Miescher, P.A. and Hirschhorn, K.
Clin. Exp. Immunol., 1: 77, 1966.

307. Goldfarb, A.R. and Ullal, I.
Proc. Soc. Exp. Biol. Med., 119: 593, 1965.

308. Conen, P.E. and Erkman, B.
Lancet, I: 665, 1964.

309. Knight, S., Ling, N.R., Sell, S. and Oxnard, C.E.
Immunol., 9: 565, 1965.

310. Williams, T.W. and Ray, M.
Cytogenetics, 4: 365, 1965.

311. Elrod, L.M. and Schreck, R.
Exp. Cell. Res., 38: 418, 1965.

312. Metcalf, W.K.
Exp. Cell Res., 40: 490, 1965.

313. Johnson, L.I., Sullivan, P.A., Siegel, C.D., Chan, P.C. and Gordon, A.S.
Brit. J. Heamat., 13: 168, 1967.

315. Willard, H.G., Hoppe, I.B.H. and Nettesheim, P.
Proc. Soc. Exp. Biol. N.Y., 118: 993, 1965.

316. Macario, A.J.L.
Lancet, I: 823, 1966.

317. Sabesin, S.M.
Proc. Soc. Exp. Biol. Med., 122: 351, 1966.

318. Nichols, W.W. and Levan, M.
Blood, 20: 106, 1962.

319. Fikrig, S., Gordon, F. and Uhr. J.W.
Proc. Soc. Exp. Biol. Med., 122: 379, 1966.

320. Sell, S., Rowe, D. and Gell, P.G.H.
J. Exp. Med., 122: 823, 1965.

321. Rieke, W.O. and Schwartz, M.R.
Anat. Rec., 150: 383, 1964.

322. Rieke, W.O.
Science, 152: 535, 1966.

323. Metcalf, W.K. and Osmond, D.G.
Exp. Cell Res., 41: 669, 1966.

324. Foft, J.W. and Romero, P.A.
Lab. Invest., 13: 575, 1964.

325. Harris, G. and Littleton, R.J.
J. Exp. Med., 124: 621, 1966.

326. Schrek, R. and Batra, K.V.
Lancet, II: 444, 1966.

327. Weber, W.T.
Exp. Cell Res., 46; 464, 1967.

328. Schwartz, M.R. and Rieke, W.O.
Anat. Rec., 155: 493, 1966.

329. Weber, W.T.
J. Cell Physiol., 68: 117, 1966.

330. Schrek, R. and Donnelly, W.J.
Blood, 18: 561, 1967.

331. Bain, B., Vas, M.R. and Lowenstein, L.
Blood, 23: 108, 1964.

332. Hashem, N. and Carr, D.H.
Lancet, II: 1030, 1963.

333. Jones, A.L.
Transplantation, 4: 337, 1966.

334. Kasakura, S. and Lowenstein, L.
Nature, 208: 794, 1965.

335. Gordon, J. and McLean, L.D.
Nature, 208: 795, 1965.

336. Kasakura, S. and Lowenstein, L.
Transplantation, 5: 459, 1967.

337. Bach, F. and Hirschhorn, K.
Science, 143: 813, 1964.

338. Hashem, N.
Science, 150: 1460, 1965.

339. Hashem, N. and Rosen, F. S.
Lancet, I: 201, 1964.

340. Bain, B. and Lowenstein, L.
Science, 145: 1315, 1964.

341. Oppenheim, J. J., Whang, J. and Prei, E.
J. Exp. Med., 122: 651, 1965.

342. Moynihan, P. C., Jackson, J. F. and Hardy, J. D.
Lancet, I: 453, 1965.

343. Silvers, W. K., Wilson, D. B. and Palm, J.
Science, 155: 703, 1967.

344. Bach, F. H. and Voynow, N. K.
Science, 153: 545, 1966.

345. Main, R. K., Cole, L. J., Jones, M. J. and Haire, H. M.
J. Immunol., 98: 417, 1967.

346. Grasbeck, R., Nordman, C. T. and Chapelle, A. de la.
Lancet, II: 385, 1963.

347. Grasbeck, R., Nordman, C. T. and Chapelle, A. de la.
Acta Med. Scand. Suppl., 412: 39, 1964.

348. Holt, L. J., Ling, N. R. and Stanworth, D. R.
Immunochemistry, 3: 359, 1966.

349. Knight, S. and Ling, N. R.
Immunol., 12: 537, 1967.

350. Sell, S. and Gell, P. G. H.
J. Exp. Med., 122: 423, 1965.

351. Gell, P. G. H. and Sell, S.
J. Exp. Med., 122: 813, 1965.

352. Davies, A. J. S., Leuchars, E., Wallis, V., Marchant, R. and Elliot, E. V.
Transplantation, 5: 222, 1967.

353. Sell, S. and Gell, P. G. H.
J. Exp. Med., 122: 923, 1965.

354. Sell, S.
J. Exp. Med., 125: 289, 1967.

355. Sell, S.
J. Exp. Med., 125: 393, 1967.

356. Sell, S.
J. Immunol., 98: 786, 1967.

357. Oppenheim, J.J., Regentine, G.N. and Terry, W.D.
Fed. Proc., 26: 528, 1967.

358. Sabesin, S.M.
Science, 149: 1385, 1965.

359. Johnson, G.J. and Russel, P.S.
Nature, 208: 343, 1965.

360. Woodliff, H.J. and Onesti, P.
Lancet, II: 857, 1966.

361. Caron, G.A.
Brit. J. Heamat., 13: 68, 1967.

362. Pulvertoft, R.J.V. and Pulvertoft, I.
Lancet, II: 892, 1966.

363. Gill, F.A.
J. Immunol., 98: 778, 1967.

364. Farnes, P., Barker, P.E., Bjownhill, L.E. and Fanger, M.
Lancet, II: 1100, 1964.

365. Borjesson, J., Reisfield, R., Chessin, L.N., Welsh, P.D. and Douglas, S.D.
J. Exp. Med., 124: 859, 1966.

366. Chessin, L.N., Borjessen, J., Welsh, P., Douglas, S.C. and Cooper, H.L.
J. Exp. Med., 124: 873, 1966.

367. Hirschhorn, K., Schreibman, R.R., Verbe, S. and Gruskin, R.H.
Proc. Nat. Acad. Sc., 52: 1151, 1964.

368. Mazzei, D., Novi, C. and Bazzie, C.
Lancet, II: 803, 1966.

369. Stodolnik-Baranska, W.
Nature, 214: 102, 1967.

370. Bach, F.H. and Hirschhorn, K.
Seminars in Haemat., 2: 68, 1965.

371. Binet, J.L. and Mathe, C.
Nature, 193: 992, 1962.

372. Forbes, I.J.
Lancet, I: 198, 1965.

373. Ripps, C.S. and Hirschhorn, K.
Clin. Exp. Immunol., 2: 377, 1967.

374. Turner, K.J. and Forbes, I.J.
J. Immunol., 96: 926, 1966.

375. Schrek, R. and Stefani, S.
Nature, 200: 482, 1963.

376. Schrek, R. and Stefani, S.
J. Nat. Cancer Inst., 32: 507, 1964.

377. Stefani, S. and Oester, Y.T.
Transplantation, 5: 317, 1967.

378. Stefani, S.A.
Excerpta Medica, 89: 406, 1965.

379. Gowans, J.L. and McGregor, D.D.
Prog. All., 9: 1, 1965.

380. Parrot, D.M.V. and de Sausa, M.A.B.
Nature, 212: 1316, 1966.

381. Turk, J.L.
Brit. Med. Bull., 23: 3, 1967.

382. Scothorne, R.J.
Ann. N.Y. Acad. Sci., 64: 1024, 1957.

383. Uhr, J.W.
Physiol. Rev., 46: 359, 1966.

384. Dutton, R.W. and Page, G.M.
Immunol., 7: 665, 1964.

385. Naspitz, C.K., Eisen, A.H. and Richter, M.
Int. Arch. Allergy 1968 (In Press).

386. Oppenheim, J.J., Hersh, E.M. and Block, J.B.
in The biological effects of phytohemagglutinin. II (Robert Jones and Agnes Hunt Ortho. Hospital Owestry, England), 1966.

387. Heilman, D. and McFarland, W.
Fed. Proc., 26: 528, 1967.

388. Jones, A.L.
Transplantation, 4: 337, 1966.

389. Forbes, I.J.
Lancet, I: 198, 1965.

390. Caron, G.A.
in The biological effects of phytohemagglutinin II (The Robert Jones and Agnes Hunt Ortho. Hosp. Oswestry, England), 1966.

391. Agrell, I.P.S.
Exp. Cell Res., 42: 403, 1966.

392. Zech., L.
Exp. Cell Res., 44: 312, 1966.

393. Ioachim, H.L.
Nature, 210: 919, 1966.

394. Vassar, P.S. and Culling, C.F.A.
Nature, 202: 610, 1964.

395. Razavi, L.
Nature, 210: 444, 1966.

396. Conard, R.A.
Nature, 214: 709, 1967.

397. Rieke, W.O.
Lymphocyte work shop Washington, 1965.

398. Borgeson, J., Chessin, L.N. and Landy, M.
Int. Arch. Allergy, 31: 184, 1957.

399. Dietrich, F.M. and Weigle, W.O.
J. Exp. Med., 117: 621, 1963.

400. Smith, R.T. and Bridges, R.A.
J. Exp. Med., 108: 227, 1958.

401. Richter, M. and Naspitz, C.K.
Int. Arch. Allergy, 32: 288, 1967.

402. Bain, B. and Lowenstein, L.
in Effects of technical variations on the mixed leukocyte reaction. Series Haematd. 77, Histocompatibility testing, Munksgaard, Copenhagen, p. 217, 1965.

403. Kopriwa, B.M. and Leblond, C.P.
J. Histochem. Cytochem. 10: 269, 1962.

404. Clark, M.F. and Shepard, C.C.
Virology, 20: 629, 1963.

405. Boyden, S.V.
J. Exp. Med., 93: 107, 1951.

406. Reiner, L. and Fischer, O.Z.
Immunotatsfarschung, 61: 317, 1929.

407. Freund, J.
J. Immunol., 21: 127, 1931.

408. Haber, E., Page, L. and Richards, F.F.
Anal, Biochem., 12: 163, 1963.

409. Gorer, P.A. and O'Gorman, P.
Transplantation, Bull., 3: 142, 1956.

410. Robins, J.H.
Science, 146: 1684, 1964.

411. Naspitz, C.K. and Richter, M.
Prog. Allergy (In Press), 1968.

412. Micklem, M.S., Ford, C.E., Evans, E.P. and Gray, J.
Proc. Roy. Soc., Series, B., 165: 78, 1966.

413. O'Brien, T.F., Michaelides, M.C. and Coons, A.H.
J. Exp. Med., 117: 1053, 1963.

414. Singhal, S.K., Drechsler, B. and Richter, M.
Lancet, II: 567, 1967.

415. Cunningham, A.J., Smith, J.B. and Mercer, E.H.
J. Exp. Med., 124: 701, 1966.

416. McGregor, D.D., McCullagh, P.J. and Gowans, J.L.
Proc. Roy. Soc., Series B, 168: 229, 1967.

417. Ford, W.L. and Gowans, J.L.
Proc. Roy. Soc., Series B, 168: 244, 1967.

418. Gowans, J.L., McGregor, D.D. and Cowen, D.M.
Nature, Lond., 196: 651, 1962.

419. Gowans, J.L. and Uhr, J.W.
J. Exp. Med., 124: 1017, 1966.

420. Gowans, J.L.
Ann. N.Y. Acad. Sci., 99: 432, 1962.

421. Cochrane, C.G. and Dixon, F.J.
Antibody production by transferred cells.
Adv. Immunol., 2: 206, 1962.

422. Dutton, R.W.
In vitro studies of immunological responses of lymphoid cells. Adv. Immunol. 6: 253, 1966.

423. Stavitsky, A.B.
In vitro studies of the antibody response.
Adv. Immunol., 1: 211, 1961.

424. Nossal, G.J.V.
Antibody production in tissue culture in cells and tissues in culture (Willmer, Ed.) Academic Press, 3: 317, 1966.

425. Wilson, D.B. and Billingham, R.E.
Lymphocytes and transplantation immunity.
Adv. Immunol., 7: 189, 1967.

426. Nossal, G.J.V.
Mechanisms of antibody production. Ann. Rev. Med., 18: 81, 1967.

427. Stone, S.H.
Cellular commitments to immune responses
Ann. Rev. Microbiol, 21: 181, 1967.

428. Makinodan, T. and Albright, J.F.
Proliferative and differentiative manifestations of cellular immune potential, Prog. Allergy, 10: 1, 1966.

429. Miller, J.F.A.P., Marshall, A.H.E. and White, R.G.
The immunological significance of the thymus.
Adv. Immunol., 2: 111, 1962.

430. Wolstenholme, G.F.W.
The thymus: Experimental and clinical studies (Churchill, London, 1966).

431. Good, R.A. and Gabrielsen, A.E.
The thymus in immunobiology. Structure, function and role in disease, Harper and Row, 1964).

432. Cooper, M.D., Gabrielsen, A.D. and Good, R.A.
Role of the thymus and other central lymphoid tissues in immunological disease, Ann. Rev. Med., 18: 113, 1967.

433. Miller, J.F.A.P. and Osoba, D.
Physiol. Rev. 47: 437, 1967.

434. Jacobson, L.O., Marks, E.K., Gaston, E.O., Robson, M. and Zirkle, R.F.
Proc. Soc. Exp. Biol. Med., 70: 740, 1949.

435. Lorenz, E., Uphoff, D., Reid, T.R. and Shelton, E.
J. Nat. Cancer Inst., 12: 197, 1951.

436. Congdon, C.C.
Blood, 12: 746, 1957.

437. Trentin, J.J.
Proc. Soc. Exp. Biol. Med., 93: 98, 1956.

438. Van Putten, L.M., Van Bekkman, D.W., de Vries, M.J. and Balner, H.
Blood, 30: 749, 1967.

439. Morrison, J.H. and Toepfer, J.R.
Am. J. Physiol, 213: 923, 1967.

440. Mannick, J.A., Lochte, H.L., Ashley, C.A., Thomas, E.D. and Ferrebee, J.W.
Blood, 15: 255, 1960.

441. Congdon, C.C. and Urso, I.S.
Am. J. Path., 33: 745, 1957.

442. Strelin, G.S. and Schmidt, N.K.
Meditsinskaye Radiologiye, 7: 44, 1962.

443. Cole, L.J., Habermeyer, J.G. and Nowell, P.C.
Radiation Res., 7: 139, 1957.

444. Gengozian, N., Makinodan, T., Congdon, C.C. and Owen, R.D.
Proc. Nat'l. Acad. Sci., 44: 560, 1958.

445. Gengozian, N., Makinodan, T. and Shekarchi, I.C.
J. Immunol., 86: 113, 1961.

446. Harris, J.E. and Ford, E.E.
Nature, 201: 884, 1964.

447. Mitchell, G.F. and Miller, J.F.A.P.
Proc. Nat'l. Acad. Sci., 59: 296, 1968.

448. Bierring, F. and Grunnet, I.
Acte Anat., 59: 182, 1964.

449. Dukor, P., Miller, J.F.A.P., House, W. and Allman, V.
Transplantation, 3: 639, 1965.

450. Globerson, A., and Auerback, R.
J. Exp. Med., 126: 223, 1967.

451. Davies, A.J.S., Lenchors, E., Wallis, V. and Koller, P.C.
Transplantation, 4: 438, 1966.

452. Meuwissen, H.J., Bach, F.J., Hong, R. and Good, R.A.
Pediatrics, 72: 177, 1968.

453. Sahiar, K. and Schwartz, R.S.
Int. Arch. Allergy, 29: 52, 1966.

454. Plotz, P.H. and Talal, N.
J. Immunol., 99: 1236, 1967.

455. Cooper, M.D., Gabrielson, M.A. and Good, R.A.
Ann. Rev. Med., 18: 113, 1967.

456. Archer, O.K., Sutherland, D.E.R. and Good, R.A.
Nature, 205: 337, 1963.

457. Sutherland, D.E.R., Archer, O.K. and Good, R.A.
Proc. Soc. Exp. Biol. Med., 115: 673, 1968.

458. Cooper, M.D., Perry, D.Y., McKneelly, M.F., Gabrielson, A.E., Southerland, D.E.R. and Good, R.A.
Lancet, I: 1388, 1966.

459. Good, R.A. and Finstad, J.
in Germinal centers in immune responses. Cottier, H., Odartchenko, R., Schindler, R. and Congden, C.C., Eds. Spring-Varley, New York, Inc., Publishers, 1967.

460. Peterson, R.D.A., Cooper, M.D. and Good, R.A.
Am. J. Med., 38: 597, 1965.

461. Cooper, M.D., Perry, D.Y., Gabrielson, A.E., Sutherland, D.E.R., McKneally, M.F. and Good, R.A.
Int. Arch. Allergy, 33: 65, 1968.

462. Good, R.A., Gabrielson, A.E., Peterson, R.D.A. and Cooper, M.D.
Trans. and studies of the College of Physicians of Philadelphia, 33: 180, 1966.

463. Thorbecke, G.J., Gordon, H.A., Wostman, B., Wagner, M. and Reyniers, J.A.
J. Infect. Dis., 101: 237, 1957.

464. Dent, P.B. and Good, R.A.
Nature, 207: 491, 1965.

465. Clawsen, C.C., Cooper, M.D. and Good, R.A.
Lab. Invest., 16: 407, 1967.

466. Cooper, M.D., Peterson, R.D., South, M.A. and Good, R.A.
J. Exp. Med., 123: 75, 1966.

467. Jankovii, B.D. and Ipvaneski, M.
Int. Arch. Allergy, 23: 188, 1963.

468. Cooper, M.D., Peterson, R.D.A. and Good, R.A.
Nature, 205: 143, 1965.

469. Cain, W.A., Cooper, M.D. and Good, R.A.
Nature, 217: 87, 1968.

470. Siminsen, M.T.
Acta Path. et Microbiol. Scand., 40: 480, 1957.

471. Humphrey, J.H., Parrott, D.M.V. and Cast, J.
Immunol., 7: 419, 1964.

472. Pinnas, J.L. and Fitch, F.W.
Int. Arch. Allergy, 30: 217, 1966.

473. Miller, J.F.A.P.
Proc. Roy. Soc., Series B., 156: 415, 1962.

474. Good, R.A., Dalmasso, A.P., Martinez, C., Archer, O.K., Pierce, J.C., and Papermaster, B.W.
J. Exp. Med., 116: 733, 1962.

475. Jankovic, B.D., Waksman, B.H. and Arneson, B.G.
J. Exp. Med., 116: 159, 1962.

476. Fichteluis, K.E., Louvell, G. and Phillipson, L.
Acta Path. et Microbiol. Scand., 51: 81, 1961.

477. Miller, J.F.A.P.
in The thymus experimental and clinical studies.
Ciba Foundation Symphosium, Wolstenholme, G.E.W. and Porter, R. Eds. Little, Brown and Co., Boston, Publishers 1966.

478. Good, R.A., Gabrielson, A.E., Peterson, R.D.A., Finstad, J. and Cooper, M.D.
in The thymus: experimental and clinical studies. Ciba Foundation Symposium. Wolstenholme, G.E.W. and Porter, R., Editors, Little, Brown and Co., Boston, Publishers, 1966.

479. Metcalf, D.
Brit. J. Haematol., 6: 324, 1960.

480. Schooley, J.C. and Kelly, L.S.
in The thymus in immunobioloby, Good, R.A. and Gabrielson, A.E., Editors. Harper and Row, New York Publishers 1964.

481. Biening, F.
in Ciba Foundation Symposium on Haemopoiesis. Wolstenholm, G.E.W. and O'Connor, M., Editors J. and A. Churchill, Ltd., London, Publishers, 1960.

482. Taylor, R.B.
Nature, 208: 1334, 1965.

483. Metcalf, D.
Nature, 208: 1336, 1965.

484. Miller, J.F.A.P.
Nature, 185: 1318, 1962.

485. Miller, J.F.A.P., Doak, S.M.A. and Cross, A.M.
Proc. Soc. Exp. Biol. Med., 112: 785, 1963.

486. Dalmasso, A.P., Martinez, C., Sjodin, K. and Good, R.A.
J. Exp. Med., 118: 1089, 1963.

487. Parrott, D.M.J. and East, J.
in The thymus in immunology. Good, R.A. and Gabrielson, A.E., Editors, Harper and Row, New York, Publishers, 1964.

488. Miller, J.F.A.P., Mitchell, G.F. and Weiss, N.S.
Nature, 214: 992, 1967.

489. Miller, J.F.A.P.
Science, 144: 1599, 1964.

490. Waksman, B.H., Arneson, B.G. and Jankovic, B.D.
J. Exp. Med., 116: 187, 1962.

491. Parrott, D.M.V. de Sousa, M.A.B. and East, J.
J. Exp. Med., 123: 191, 1966.

492. Turk, J.L.
Brit. Med. Bull., 23: 3, 1967.

493. de Sousa, M.A.B. and Parrott, D.M.V.
in Germinal Centers in immune responses.
Cottier, H., Odartchenko, N., Sehindler, R. and
Congdon, C.C. Editors, Spring-Varley, New York Inc.,
Publishers, 1967.

494. Parrott, D.M.V. and de Sousa, M.A.B.
Nature, 212: 1316, 1966.

495. Nossal, G.J.V.
Ann. N.Y. Acad. Sci., 120: 171, 1964.

496. Murray, R.G. and Woods, P.A.
Anat. Record, 150: 113, 1964.

497. Harris, J.E., Ford, C.E., Barnes, D.W.H. and Evans, E.P.
Nature, 201: 886, 1964.

498. Weisman, I.
in Ciba Foundation Symposium. The thymus (quoted by
H.S. Kaplan). Wolstenholme, G.E.W., and Porter, R., eds.,
Little, Brown and Co., Boston, Publishers, 1966.

499. Goldschneider, I. and McGreger, D.D.
J. Exp. Med., 167, 155, 1967.

500. Parrott, D.M.V. and de Sousa, M.A.B.
Immunol., 13: 133, 1967.

501. Weissman, I.L.
J. Exp. Med., 126: 291, 1967.

502. Marchesi, V.T. and Gowens, J.L.
Proc. Roy. Soc., Series B, 159: 283, 1964.

503. Everett, N.B., Caffrey, R.W. and Reike, W.O.
Ann. N.Y. Acad. Sci., 113: 887, 1964.

504. Everett, N.B. and Tyler, R.W.
Int. Rev. Cytology, 22: 205, 1967.

505. Osmond, D.G.
in The lymphocyte in immunology and haemoporesis.
Yoffey, J.M., editor, Edward Arnold Ltd., London
Publishers, 1967.

506. Craddock, C. G., Winkelstein, A., Matsuyuki, Y. and Lawrence, J. B.
J. Exp. Med., 125: 1149, 1967.

507. Fliedner, T. M., Kesse, M., Cronkite, E. P. and Robertson, J. S.
Ann. N. Y. Acad. Sci., 113: 578, 1964.

508. Cottier, H., Cronkite, E. P., Jansen, C. R., Rai, K. R., Singer, S. and Sipe, C. R.
Blood, 24: 241, 1964.

509. Nordman, A., Sasaki, M. S., Ottoman, R. E. and Figenhurt, A. G.
Science, 147: 745, 1964.