Ph.D. EXPERIMENTAL MEDICINE

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THE ROLE OF THE BONE MARROW IN THE IMMUNE RESPONSE

ABSTRACT

Normal rabbit bone marrow cells, but not cells of the other normal lymphoid organs could transfer immunocompetence to lethally irradiated or tolerant allogeneic recipients. However, bone marrow cells of rabbits immunized 24 hours previously could not transfer immunocompetence with respect to the immunizing antigen. The bone marrow immunocompetent cell was identified as the antigen reactive cell (ARC) and not the antibody forming cell (AFC) by the use of allotypic markers. The ARC which were morphologically small lymphocytes, could be isolated by passage of the normal bone marrow cells through an antigen-sensitized glass bead column. Immunocompetence following irradiation correlated with the reappearance of the bone marrow ARC. Furthermore, the ARC activity could be initiated by incubation of the cells with the antigen in vitro.

The results suggest that rabbit bone marrow is the main source of ARC and that they migrate out of the bone marrow following immunization.

THE ROLE OF THE BONE MARROW

IN

THE IMMUNE RESPONSE

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ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

1. The bone marrow is the prime source of the immunocompetent antigen-reactive cell(s) in the unimmunized adult rabbit.

2. Normal rabbit bone marrow does not possess any antibody-forming cells.

3. Only viable bone marrow cells could transfer immunocompetence to irradiated recipients. Sonicates or heat killed cell preparations lost this capacity.

4. It was demonstrated that the antigen-reactive cell is irradiation sensitive whereas the antibody-forming cell is irradiation resistant.

5. The antigen reactive cell, not the potential antibody forming cell, is the "tolerant" cell in the immune tolerant state.

6. Clones of normal, unstimulated, antigen reactive cells are precommitted to react with only one antigen. These

clones can be isolated from a heterogenous population of antigen-reactive cells by means of specific immuno-adsorbents.

7. Following the intravenous administration of an antigen, the bone marrow is rendered specifically immunoincompetent with respect to the immunizing antigen. Evidence has been presented showing that, following immunization, the bone marrow antigen reactive cells are not rendered tolerant following in vivo interaction with the antigen but rather they migrate out of the bone marrow.

8. The clonal selection theory of Burnet has been confirmed by the present investigation. However, it would appear to relate only with respect to the antigen-reactive cell and not the antibody-forming cell, in the normal rabbit.

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CHAPTER I

BASIS FOR AND OBJECTIVES OF THE PRESENT INVESTIGATION

During the past few years progress has been made in identifying the types of cells involved in the immune response. It has been demonstrated that the cell(s) capable of synthesizing and/or storing humoral type antibodies are the cells belonging to the plasma cell series and that the cells mediating cellular immunity are the small lymphocytes. On the basis of recent investigation, it is now believed that more than one type of cell is required to participate in the events that preceed humoral antibody synthesis. These cells are referred to as the antigen reactive cell and the antibody forming cell. However, little is known about the early and initial cellular events following the in vivo administration of the antigen for the first time.

The basis for this study is the previous observation from this laboratory that normal rabbit bone marrow cells are the only lymphoid cells in the normal rabbit capable of undergoing blastogenesis and mitosis upon in

vitro incubation with various antigens. Evidence of an inconclusive nature has been presented suggesting the immune nature of this response.

The objective of this investigation is to provide information with regard to the following questions pertaining to the humoral primary immune response in the rabbit:

1. What is the organ source and morphology of the antigen reactive cell(s) in the rabbit?

2. What are the initial events that follow the initial in vivo contact of an antigen with the antigen reactive cell?

3. Is the lesion in the immune tolerant state at the level of the antigen reactive cell and/or the antibody forming cell?

4. Are antigen reactive cells precommitted to interact with only one antigen? If this is the case, can these cells be separated into specific clones following their interaction with the antigen?

5. Is immunocompetence dependent on the presence of antigen reactive cells in the body?

6. Can the events that follow the interaction of the antigen with the antigen reactive cell be initiated in vitro? If so, is that interaction specific?

CHAPTER II

DEFINITIONS AND ABBREVIATIONS

A. Definitions of terms used in the thesis

Stem Cell: Immature cell capable of giving rise to mature forms.

Differentiation: Acquisition of new properties by a non-dividing cell, i.e., qualitative changes.

Maturation: Intensification of pre-existing properties of the cell, i.e., quantitative changes in the same cell.

Proliferation: An increase in the number of the cells achieved by mitosis of precursor cells.

Central Lymphoid Organ: An organ the extirpation of which will result in depletion of lymphoid cells in another lymphoid organ.

Peripheral Lymphoid Organ: An organ that contains the fully differentiated lymphoid cells and the extirpation of which will not result in depletion of lymphoid cells in another lymphoid organ.

Immune Competent Animal: An animal which, though capable of an immunological response, is not yet indulging in one.

Immune Competent Cell: A cell capable of carrying out a specific aspect of an immune response.

Antigen Reactive Cell: A cell that recognizes an antigen and responds by differentiation and proliferation but does not form antibody.

Antibody Forming Cell: A cell that synthesizes and/or secretes specific antibody.

Primary Immune Response: Events that follow the first exposure to an antigen.

Secondary Immune Response: Events that follow the second exposure to the same antigen used to induce the primary immune response.

Normal Animal: An animal that has never been exposed to an antigen or any other form of treatment.

Primed Animal: An animal that has been in contact with an antigen for less than 48 hours.

Hyperimmune Animal: An animal that has been repeatedly exposed to the same antigen.

Normal, Primed, Immune or Hyperimmune Cells: Cells obtained from normal, primed, immune or hyperimmune animals, respectively.

Stimulated Cell: A cell obtained from a normal animal incubated with the antigen in vitro.

B. Abbreviation of words used in the thesis

Ab:	Antibody
Ag:	Antigen
Ig:	Immunoglobulin
DH:	Delayed hypersensitivity
GVHR:	Graft-versus-host reaction
MLR:	Mixed leucocyte reaction
ARC:	Antigen reactive cell(s)
AFC:	Antibody forming cell(s)
PFC:	Plaque forming cell(s)
HSA:	Human serum albumin
BGG:	Bovine gamma globulin
BSA:	Bovine serum albumin
SRBC:	Sheep red blood cells
SCS:	Sheep cell sonicate
HRBC:	Horse red blood cells
HCS:	Horse cell sonicate
RRBC:	Rat red blood cells
PHA:	Phytohemagglutinin

CHAPTER III

LITERATURE REVIEW

A. The immunocompetent cell: General considerations

The lymphocyte has been defined by morphological criteria as a small round cell ranging in size from 5 - 12 u present in the blood, lymph, lymphoid organs and body fluids characterized by basophilic nucleus and a thin rim of cytoplasm (1). The lymphocytes represent a highly mobile cell population vital to the defence of the mammalian organism. No longer, however, does it seem reasonable to write in terms of a single function of a lymphocyte since data are now accumulating in the literature showing that there is marked heterogeneity among lymphocytes, both structurally and functionally (2). Lymphocytes which appear to be morphologically identical under the microscope have various highly specialized functions in the different peripheral lymphoid organs and they are probably under the influence of separate regulatory mechanisms (2). Investigation

aimed at relating morphological structure with the function of the lymphoid apparatus in the ontogeny and phylogeny of these organs has led to the concept of the central lymphoid organs as specialized sites in which transformation of immunocompetent cells into antibody-forming cells or into cells which mediate cellular immunity takes place (3). The origin, fate and distribution, as well as the rate of proliferation and death of these cells is more understood after the use of isotopically-labelled cells. Also, progress has recently been achieved in the study of the complex interactions which take place between the different lymphoid cells (4, 5, 6). These findings permit a better understanding of the relationship of structure to function and clarify the sequence of cellular events in the induction of the primary immune response.

In this chapter, the role that the lymphoid system plays in the immune response is described, with particular emphasis placed on the heterogeneity of the lymphoid cells. Our present knowledge regarding the migration pathways of the different lymphoid cells during the immune response is outlined.

1. Lymphocyte Heterogeneity

a) The central versus the peripheral lymphoid system - The lymphoid system can be classified as a cellular compartment concerned primarily with differentiation (central compartment) and a second cellular compartment that includes the fully differentiated lymphoid cells (peripheral compartment) (7). One may also refer to a lymphoid organ as central or primary if its extirpation results in depletion of lymphoid cells in another lymphoid organ. In this context, as will be seen below, the thymus and the bursa of Fabricius constitute the central lymphoid organ system. The findings of Glick (8) and of Cooper et al (3, 7) have demonstrated that in the bird, the bursa of Fabricius is essential for the development of the lymphoid cells capable of antibody production. The studies of Miller et al (9) and of Good et al (10) have shown that the thymus, like the bursa, is a central lymphoid organ but that it is mainly engaged in cellular immunity. These central organs were shown by Auerbach (11) to represent tissues derived from epithelial embryonic structures in which precursors of lymphoid cells undergo active pro-

liferation and obtain the capacities and characteristics of the different populations of lymphoid cells. According to this view (10, 11), an undifferentiated lymphoid bone marrow stem cell entering the thymus comes directly under the influence of that organ, where it gives rise to a population of cells which can, after distribution to the periphery, become capable of expressing the thymus-dependent functions. On the other hand, lymphoid stem cells entering the bursa of birds or the gut-associated lymphoid tissues of certain mammals come under the influence of this environment and give rise into a population of lymphoid cells capable of producing antibodies in appropriate locations in the peripheral lymphoid tissues (3). Clinical (12), immunologic and phylogenetic (7, 13) studies have confirmed this two-component (central and peripheral) concept of the lymphoid system. The central lymphoid tissues can be expected to contain many lymphoid cells in the process of differentiation which do not exercise the functions attributable to the fully differentiated peripheral lymphoid cells. For example, the capacity of cells from the thymus and bursa to exercise graft-versus-host reactions (14, 15, 16) and to produce antibodies (17),

respectively, has been found to be deficient.

b) The thymus-dependent versus the gut-dependent lymphocytes - The distribution of lymphocytes in the peripheral lymphoid tissues has recently been classified into a thymus-dependent population of lymphocytes responsible for the expression of delayed hypersensitivity, allograft rejection and graft-versus-host reactions, and a gut-dependent lymphocyte population responsible for antibody production, germinal center and plasma cell formation (7).

Histological studies of lymph nodes and spleens following neonatal thymectomy or bursectomy have revealed the distribution of each type of lymphocyte. Thymus-dependent areas have been shown to be located in the deep cortical areas of the lymph nodes and in the splenic white pulp (7). The gut-dependent areas have been located in the extreme cortical parts, in the hilar areas and in the germinal centers of the lymphoid follicles of the lymph nodes (7). By in vivo labelling with ^{3}H-thymidine (18), the follicles in the gut-associated lymphoid tissues (appendix, Peyer's patches and Sacculus rotundus) have been shown to be composed

of cortical areas containing large blastoid cells with a high labelling index, and medullary areas consisting primarily of small dark cells with a low labelling index. In contract, the diametrically opposite situation pertains with respect to the follicles in the peripheral lymphoid tissues, i.e., lymph nodes and spleen.

In addition to thymus-dependent and gut-dependent populations of lymphoid cells, there appear to exist two other populations of lymphocytes involved directly in the immune reactions. These include hemopoietic stem cells, shown by Everett et al (19, 20) to be among the cells which have the morphological appearance of lymphocytes and fixed or free macrophage-like cells indistinguishable from lymphocytes by classical morphology (21, 22, 23). It is not yet certain whether this latter cell population is completely separable from thymus or gut dependent lymphocytes described above, or from monocytes which have the characteristics of sticking to glass and of engaging in the antigen processing step (see Chapter III, B-2).

c) Small, medium and large lymphocytes - Lymphocytes can be divided morphologically into small, medium and large cells (1). This classification does not add to our understanding of lymphocytes since cells which fall into the same morphological category do not necessarily have the same origin, fate or function. Although this classification is convenient, it is an arbitrary one since it has now been shown that the size of the cell viewed under the microscope varies according to the method of cell fixation (1). Also, it is not known what the relationship is between the large, medium and small lymphocytes described in the germinal centers of lymph nodes and those found in blood and lymph.

Lymphocytes of each class exhibit marked functional heterogeneity and they constantly change their size. The work of several investigators (24, 25, 26) has shown that the small lymphocytes in different organs do not respond to the same degree after the administration of anti-metabolites: Six mercaptopurine was shown to cause a profound fall in the number of small lymphocytes in the bone marrow of rats without affecting those in the lymph

nodes (26). Moreover, it has been shown that small lymphocytes do transform into large lymphocytes. Studies with thoracic duct lymph from the rat have shown that some of the large lymphocytes divide to form small lymphocytes in diffusion chambers (19) and after transfusion into syngeneic recipients (27). Small lymphocytes in culture, on the other hand, can transform into large blast cells when incubated with various mitogens (see Section III, A, 1f). These findings indicate that the small and large lymphocytes are not in a static balance with each other but rather that one may transform into the other. Therefore, the arbitrary division of lymphocytes according to conventional morphologic criteria into large, medium and small cells offers little advantage.

d) Short-lived versus long-lived lymphocytes - A class of small lymphocytes with a considerably shorter life span than the remainder has been described in the blood, lymph and lymph nodes of rats (28). By labelling the DNA of lymphocytes it was shown that the short-lived lymphocytes are located primarily in the thymus cortex, bone marrow and germinal centers (29). They are highly

sensitive to the lethal effects of irradiation and to the lytic action of cortisol (29). Long-lived small lymphocytes, on the other hand, make up 90% or more of the total cells in the thoracic duct lymph, blood and lymph nodes (26). They do not incorporate tritiated thymidine during incubation in vitro and have a life span in excess of 100 days (30). Ottsen (31) studied the decay of ^{32}P in the DNA of blood lymphocytes from human subjects. He identified two populations of cells: about 20% had a mean age of 2-3 days and the remainder a mean age of 100-200 days. Buckton and Pike (32) and Norman et al (33), using chromosomally marked lymphocytes in man, have suggested that the potential life span of the long-lived small lymphocytes may extend up to 10 years.

Craddock et al (29) have shown that both short and long-lived lymphocytes are required for the induction of the primary immune response. Short-lived lymphocytes are involved in the earliest phases of particle trapping, phagocytosis, antigen processing and for the interaction between the antigen and the phagocytic cells. Long-lived lymphocytes, on the other hand, are concerned with

the expression of the specific immune response and immunologic memory. They have been shown to be capable of responding to stimulation with phytohemagglutinin (PHA) and antigen in vitro (33, 34).

e) Lymphocytes involved in cellular and humoral immunity - Cellular immune reactions are mediated by specifically sensitized thymus-dependent lymphocytes. Turk labelled these cells with H^3 Thymidine (35), injected them into normal guinea pigs, and showed that relatively few of the cells which had infiltrated the site of the cellular immune reaction were labelled. He therefore speculated that very few of the infiltrating cells are specifically sensitized and that the reaction of the few sensitized cells with the antigen in tissues attracts a large number of unlabelled non-specific cells which infiltrate the area with subsequent tissue destruction (36). The bone marrow has recently been implicated to be the source of these non-specific cells (37, 38).

Based on the present state of our knowledge a general, partially hypothetical scheme for the role of lymphocytes

in cellular reactions is proposed. Following the injection of the antigen into a previously sensitized animal, sensitized circulating lymphocytes are attracted to the antigen depot and come in contact with the antigen. A few hours later, migration inhibitory factor (39), produced by these cells, diffuses out into the tissue, reacts with the macrophage, and in some way immobilizes them. The macrophages are thus prevented from leaving the site. Accumulation of these cells causes tissue engorgement and the newly formed macrophagic lysozomal enzymes may contribute to tissue injury; at the same time sensitization of lymphocytes is enhanced by improved antigen processing. The sensitized lymphocytes activated by antigen now undergo blastoid transformation and mitosis, leading to the formation of many more sensitized cells. Vigorous recruitment of unsensitized lymphocytes will lead to a logarithmic increase in cell numbers at the site of antigen deposition or in regional lymph nodes and to rapid elimination of the antigen (40).

The role of the lymphocyte in antigen recognition, antibody formation, and various humoral immune reactions will be discussed in Chapters B4 and D2.

f) The Differential Transformation of Lymphocytes Stimulated by Various Mitogenic Agents in Vitro - The peripheral lymphocytes of many animals may be cultured in vitro and may be stimulated to synthesize RNA, DNA and protein, to transform into immature blast cells, and to undergo mitosis upon stimulation by non-specific mitogenic agents such as PHA, pokeweed mitogen, streptolysin S or filtrates from cultures of staphylococcus aureus (41). Experiments using thoracic duct lymphocytes, purified lymphocyte preparations and histochemical staining clearly implicate the thymus-dependent small lymphocyte as the precursor of the blast cell in the in vitro response to non-specific mitogens (42).

In vitro mixtures of lymphoid cells from two genetically different individuals, but not of genetically identical individuals, are also capable of undergoing transformation (43). The mixed lymphocyte reaction (MLR) has been suggested as a histocompatibility test in vitro (44). The mechanisms of recognition and reaction of lymphocytes in the MLR are unknown. However, the specificity of the reaction implies a specific recognition mechanism whereby

the lymphocytes from one donor can identify and be stimulated by the lymphocytes from a genetically different donor.

Specific antigens may also stimulate lymphocyte transformation if added to cultures of peripheral lymphocytes obtained from sensitized animals and man (41). However, in the rabbit the bone marrow lymphocytes respond differently than those of other lymphoid organs and the immune rabbit differently from normals. For example, the work of Singhal and Richter (45, 46) has shown that normal rabbit bone marrow cells, but not lymphocytes obtained from the other rabbit lymphoid organs, respond in vitro with blastogenesis and mitosis if incubated with different antigens. On the other hand, bone marrow cells obtained from an immunized rabbit cannot be stimulated in vitro if incubated with the immunizing antigen but can respond to non-cross-reacting antigens. On the other hand, lymphocytes obtained from the spleen and lymph nodes of the immunized animal can respond in vitro to the immunizing antigen (45, 46).

It has also been demonstrated (47, 48) that lymphocytes of different lymphoid organs of the rabbit possess a distinct PHA-reactive profile, i.e., there are marked differences in the in vitro response to PHA as shown by the degree and time of maximum specific incorporation of tritiated thymidine and for the optimum PHA concentration required to elicit a maximum blastogenic response.

The above data unequivocally indicate the marked heterogeneity of the lymphocytes in the animal body and the absence of a clear-cut relationship between their morphological differences on the one hand, and their role as mediators of the immune response (humoral and cellular) on the other.

2. The Immunocompetent Lymphocyte - Its Migration Pathways and Immune Function

a) Migration pathways of the circulating lymphocytes - An understanding of lymphocyte recirculation in the body is essential in order to properly study the migration pathways and function of lymphoid cells involved in the immune response.

The studies of Gowans and McGregor in the rat (1) provided the first experimental demonstration of a massive recirculation of small lymphocytes and established that the main route from blood to lymph lay within the lymph nodes themselves. Ford and Gowans (49) found that specific depletion of the recirculating pool of lymphocytes in the rat by thoracic duct lymph drainage resulted in variable degrees of lymphoid depletion in the various organs. The periarteriolar lymphoid sheaths of the spleen and the cortical zones of lymph nodes were markedly depleted of small lymphocytes whereas the bone marrow and thymus content of lymphocytes was not affected (49). This would indicate that the bone marrow and thymus contain few or no circulating lymphocytes and that a large proportion of lymph node and spleen lymphocytes belongs to the recirculating pool. The circulation of small lymphocytes between the blood and the peripheral lymphoid tissues takes only a few hours and involves a population of non-dividing small lymphocytes with an average life span of several weeks (27, 49).

b) The migration pathways of bone marrow lymphocytes - Bone marrow lymphocytes continuously migrate out of the bone marrow to the peripheral lymphoid tissues (50). These cells, however, do not enter the recirculating pool of lymphocytes (50). Parrot (51) injected labelled marrow cells into irradiated mice and showed that the most prominent site of localization of the labelled small cells is the red pulp of the spleen, and not the recirculating traffic areas of lymph nodes and spleen.

It is not known whether bone marrow lymphoid cells migrate to the peripheral lymphoid tissues directly or by way of the central lymphoid organs, namely the thymus and the gut-associated lymphoid tissues. Ford (50) showed that chromosomally-marked bone marrow lymphoid cells injected into syngeneic irradiated mice go to the thymus, spleen and lymph nodes. The time sequence for the appearance of these cells in these organs was not reported. In a similar study it was demonstrated that dividing lymphoid cells migrated from the bone marrow to the lymph nodes via the thymus. The journey required a period of several weeks

during which time the proliferating cells probably underwent maturation (52, 53, 54). Micklem et al (55, 56) concluded from their studies that the bone marrow lymphocyte can migrate directly from the bone marrow to the lymph node without intermediary stops in the central lymphoid organs. Moreover, the same authors could not demonstrate migration of cells from the thymus to the bone marrow since no labelled donor thymus cells could be detected in the bone marrow of the recipient (56).

c) The migration of pathways of thymus lymphocytes - Contrary to the bone marrow, the thymus in the mouse and rat is a major site of production of recirculating small lymphocytes (57). There is evidence in the literature (58, 59, 60) that thymus lymphocytes are released into the blood either directly or via the lymphatics. Following the infusion of tritiated thymidine directly into the thymus of the adult rat, labelled small lymphocytes were seen leaving the thymus via the blood and lymphatics and then to localize in those areas of the spleen and lymph

nodes in which the recirculating cells predominate, namely the cortical areas of the lymph node and the periarteriolar areas in the white pulp of the spleen (59).

d) The migration pathways of gut-associated or lymph node cells - There are no data in the literature concerning the migration of appendix, sacculus rotundus, Peyer's patches or lymph node cells.

e) The possible significance of lymphocyte recirculation with respect to immunocompetence - In contrast to the situation in the bone marrow and the thymus, the response of peripheral lymphoid tissues to antigenic stimulation is largely dependent on the circulating pool of lymphocytes. The initial events following antigenic stimulation appear to be the redistribution of the recirculating lymphocytes and their transformation into blast cells in the peripheral lymphoid organs. Hall and Morris (61) found that the injection of both soluble and particulate antigens into the draining area of the sheep popli-

teal lymph node results in the cessation of the output of lymphocytes into the efferent lymph over the next six hours. However, during the following 72 hours, the number of cells in the efferent lymph was greatly increased compared with the normal situation. This was due to both an increase in the number of small lymphocytes and the appearance in the lymph of large basophilic blast cells. In a similar study, Ford and Gowans (49) have reported that the addition of sheep erythrocytes to the lymphocyte perfusate of an isolated rat spleen decreased the number of lymphocytes leaving the spleen. Austin (62) has shown that antigenic stimulation results in blast cell transformation in the lymphoid follicles in the spleen and lymph nodes. By using lymphoid cells labelled in vitro and in vivo, it was found that these follicles are within the migration pathway of the lymphoid cells, both in the spleen and in the regional lymph nodes (62, 63).

The most likely function of lymphocyte recirculation is that it facilitates the induction of the immune response by enabling a large proportion of the total cell population to make contact with a local depot of antigen

within a short period of time. Continuous migration of lymphocytes would also provide lymphocytes capable of being locally stimulated even though the antigen concentration in the circulation had fallen to sub-immunizing levels. Furthermore, if macrophages perform an essential preliminary role by processing the antigen, then the movement of lymphocytes past the relatively sessile macrophages would enable contact between these two cell types to take place and facilitate the transfer of information from the macrophage to the immunocompetent lymphocyte, thus initiating the sequence of events leading up to antibody formation (49, 50, 63, 64, 65).

B. The immunocompetent cell: Specific considerations

1. Immunocompetence of the various lymphoid organs

a) The cell transfer system - The reconstitution of immunocompetence in immunoincompetent hosts of allogeneic and syngeneic immunocompetent cells.

The transfer of the immune response to immunoincompetent recipients by the administration of lymphoid cells (66) has been extensively used for the study of (i) the kinetics of the primary and secondary immune responses; (ii) the identity and organ source of the immunocompetent cells; (iii) the site(s) in which immunocompetent cells acquire the capacity to initiate an immune response; and (iv) the extent of participation of the donor and recipient animals in antibody formation.

In general, cells from donor animals are inoculated into immunologically "crippled" recipients of the same species. These recipients may be newborn animals, x-irradiated adults or animals treated with a variety of immunosuppressant drugs (66). Cells for the transfer can be obtained from different organ sources and injected into the recipients intravenously or intraperitoneally. Prior

to transfer, stimulation of the donor cells can be accomplished by immunizing the donors, or by incubating the donor cells with the antigen in vitro. The transfer of normal donor cells is usually followed by immunization of the recipients at predetermined intervals of time. A variety of antigens - including bacteria, heterologous erythrocytes, serum proteins and hapten protein conjugates - have been employed successfully. Although successful transfer of the capacity to initiate a primary immune response has been reported (4, 67), the transfer technique has been used more extensively for the study of the capacity of immune cells to engage in a secondary response upon antigenic challenge, a response often referred to as the anamnestic or secondary response and attributed to cells possessing immunologic memory.

X-irradiation of recipient adults has been employed to abolish or minimize the host response to antigen, such as in the case of allogeneic transfers to prevent rejection of the donor cells. Studies with inbred mice have shown that irradiation of recipients permits greater

antibody synthesis by the transferred immune cells (68). Increasing the dose of x-irradiation permits increased immune responsiveness by the transferred cells, probably by preventing the antigen breakdown by the host cells.

b) Immunocompetence of thymus and bone marrow cells

i) The humoral immune response - Miller and Mitchell (4) observed that viable syngeneic thymus or thoracic duct lymphocytes could reconstitute to normal levels the plaque-forming capacity of spleens of neonatally thymectomized immuno-incompetent mice challenged with SRBC. No significant immunologic response was achieved by giving either syngeneic bone marrow cells, irradiated thymus cells, thoracic duct cells, thymus extracts or yeast. Spleen cells from reconstituted mice were exposed to anti-H2 sera directed against either the donor of the thymus or the thoracic duct cells or against the neonatally thymectomized host. Only isoantisera directed against the host could reduce the number of hemolysin-forming cells present in the spleen cell suspensions, indicating that the antibody-forming cells (AFC) are of host origin and are not derived

from the donor thymus or thoracic duct lymphocytes. Thymectomized, irradiated recipients were also used by the same investigators (5). The irradiated mice were protected with syngeneic bone marrow for a period of 2 weeks and injected with semi-allogeneic thoracic duct cells together with SRBC. These mice produced a greater number of plaques than irradiated mice which received the same number of thoracic duct cells without bone marrow. By using anti-H2 sera, it was found that the AFC in the spleens of these thymectomized irradiated hosts were derived not from the injected thoracic duct cells but from the injected bone marrow cells (4, 5). By using chromosomal markers (T6) in a strictly syngeneic system, Nossal et al (69) confirmed the above findings. Neonatally-thymectomized mice were restored immunologically by either transplants of thymus or thoracic duct lymphocytes and then injected with SRBC. The dividing AFC were found to be of host and not donor origin. When lethally irradiated mice were injected with mixtures of syngeneic thymus and bone marrow cells, one of which was chromosomally marked, all the AFC were found to be of bone marrow origin.

These investigators postulated that in the mouse, the thymus or thoracic duct lymphocytes "recognize" the antigen and interact with it, and this latter reaction triggers off the differentiation of a bone marrow-derived precursor cell to a specific antibody-forming cell.

Similar findings were also reported by Davies et al (70, 71) and by Leuchars et al (72). In their system, chromosomally-marked mouse radiation chimeras were used. Although it could be shown that thymus-derived cells responded vigorously by mitosis to antigenic stimulation, they were not capable of antibody production. In contrast, bone marrow-derived cells did not respond with mitosis to antigenic stimulation during the first 3 days following exposure to antigen, but they were capable of limited antibody production. Antibody was maximally produced in recipients of both thymus and bone marrow cells.

Using both the Jerne plaque assay (73, 74) and the Playfair hemolytic foci assay (75), Claman et al (76, 77, 78) demonstrated that irradiated mice injected with suspensions containing both syngeneic thymus and marrow cells

and immunized with SRBC produced much more antibody to SRBC than did irradiated syngeneic hosts injected with either of these cell suspensions alone (76). Living syngeneic thymus cells were required since sonicated or irradiated mouse thymus cells or living allogeneic (rat) thymus cells were incapable of transferring immunocompetence (78).

Taylor (79), using a protein antigen, bovine serum albumin (BSA), also showed that a mixture of thymus and bone marrow cells has to be given to irradiated syngeneic mice in order to obtain an immune response. Reducing the number of each type of cell resulted in a diminished response. Interestingly, the author found that the administration of BSA to the donor mouse 24 hours before sacrifice resulted in failure of the donor thymus cells to interact with normal syngeneic bone marrow cells for the induction of the immune response in an irradiated recipient. Similar findings were reported recently by Many and Schwartz (80). The authors (79, 80) suggested that the thymic cell had been converted to a tolerant cell following the antigen injection.

The studies of Doria and Agarossi (81, 82) have shown that the anatomically intact thymus in the mouse has a direct influence on the precursors of the antigen-sensitive cells. They observed that lethally irradiated recipient mice of thymus cells from isogeneic bone marrow radiation chimeras, but not from normal or irradiated syngeneic mice, were found to be fully competent following antigenic stimulation. The studies of Ford and Micklem (83) shed further light as to the source of the immunocompetent cells. They showed that chromosomally-marked dividing donor bone marrow cells could be seen in the thymus of host mice radiation chimeras following antigenic stimulation and that irradiated recipients of thymus from such a donor could synthesize antibody unlike a recipient of normal thymus cells.

Several considerations must be kept in mind before accepting as fact the synergistic effect of thymus and bone marrow cells in the immune response to antigens. Synergism has been uniformly observed in the experiments which have involved the use of heavily irradiated recipients for in vivo culture of the injected cells (4, 76).

The addition of bone marrow cells could enhance the immune response by providing hemopoietic precursors and thus, in some way, prevent depletion of the precursors remaining in the host or, as stated by Radovich et al (84), by a non-specific effect of the bone marrow cells on the localization of antibody-forming cells in the spleen. Absence of bone marrow-thymus synergism has been observed by Craddock et al (29) using steroid-treated animals as recipients. Their findings suggest that the irradiated recipient animal, as opposed to the other recipient types used, is depleted of more immunocompetent cell types required for the successful mediation of the immune response and that one of these cells is probably provided by the transferred bone marrow.

ii) The cellular immune response - An in vitro model system simulating the in vivo thymus-bone marrow interaction has been described by Globerson and Auerbach (85). Sublethally irradiated mouse spleen organ cultures required the presence of normal bone marrow cells for lymphopoiesis to occur, which was enhanced by the

presence of thymus cells. Thymus and lymph node cells, in the absence of bone marrow cells, failed to induce lymphopoiesis in the irradiated mouse spleen cultures. These investigators (85) also used an in vitro assay system for the quantitation of the graft-versus-host reaction, as measured by the degree of induced splenomegaly in cultured spleen slices. Splenomegaly occurred when the spleens were grown for 2-3 days in the presence of thymus tissue but not when grown in the presence of a variety of other tissues (liver, kidney and spleen). This thymic activity was demonstrated to occur even across an intervening Millipore filter barrier. When spleen slices were exposed to lethal doses of irradiation, reactivation of immune competence did not occur unless both thymus and bone marrow cells were present. Using the same in vitro graft-versus-host system, Umiel et al (86) found that liver cells or thymus cells were incapable of inducing splenomegaly when taken directly from embryos. A combination of these two types of cells, however, was successful in inducing the splenomegaly.

In the rat, Goldschneider and McGregor (52, 87) have shown that the graft-versus-host reaction in vivo can be induced by the transfer to irradiated recipients of small lymphocytes derived from precursors present in both adult bone marrow and neonatal thymus, both of which are essential for the generation of the immunologically competent small lymphocytes. On the other hand, Stuttman and Good (88) were unable to demonstrate a synergism between the bone marrow and the thymus in the graft-versus-host reactions. The thymus cells alone, but not bone marrow cells alone, were capable of inducing the reaction. The latter authors suggested that contrary to the humoral response to SRBC, thymic-dependent immunological functions such as graft-versus-host reaction and delayed hypersensitivity reactions do not operate through a direct synergism between thymus and bone marrow cells.

c) Immunocompetence of thoracic duct lymphocytes - Rat and mouse thoracic duct lymphocytes are capable of restoring the primary humoral response with respect to

SRBC in lethally irradiated syngeneic hosts (89, 90, 4). The plaque-forming capacity of the irradiated recipient has been found to be equal to that observed in non-irradiated animals given SRBC only, though delayed by 24 hours. By isotopic and immunofluorescent labelings, it was demonstrated that the thoracic duct small lymphocytes, and not the medium or large lymphocytes, were the source of the plaque-forming cells (PFC) (89). Varying the dose of irradiation given to the recipients between 600 rad up to a lethal dose of 1000 rad did not diminish the magnitude of the immune response. The authors did not use higher doses of irradiation nor did they use other types of lymphoid cells (89).

d) Immunocompetence of spleen cells - Mouse spleen has been shown to contain the cells required for the induction of the primary immune response both in vivo (67) and in vitro (91). Syngeneic spleen cells from normal donors, but not from neonatally thymectomized mice, can restore immunological capacity to otherwise immunoincompetent thymectomized and irradiated adult

mice. However, the injection of either fetal liver cells or normal adult marrow cells by themselves does not result in restoration of immunocompetence (4, 9). Normal mouse spleen cell suspensions have also been shown to be capable of forming plaques when incubated in vitro with heterologous red cells (91, 92).

e) Immunocompetence of gut-associated lymphoid cells - Chicken bursal cells by themselves are not able to produce immunity in adoptive transfer systems (93) and do not contain plaque-forming cells when taken from chickens immunized with SRBC (17). Bursectomy at hatching, however, results in reduced immunoglobulin levels and diminished antibody response to several antigens if the chickens are tested at 6-12 weeks of age (3, 7). Bursectomized chickens reject skin homografts in a normal fashion, can exhibit normal delayed reactions to tuberculin and can initiate graft-versus-host reactions (3). Autologous bursal cells or grafts placed subcutaneously or in Millipore chambers fail to reconstitute the immune capacity of bursaless chickens (3, 7).

Limited experimental data suggest that the gut-associated lymphoid organs of the rabbit (appendix, sacculus rotundus and Peyer's patches) play a similar role to that of the chicken's bursa in immune functions (94). Neonatal extirpation of the gut-associated lymphoid organs in the rabbit followed by lethal total body irradiation and reconstitution by liver cells results in suppression of the antibody response to brucella abortus (94).

The above data would indicate that the gut-associated lymphoid tissues of birds and some mammals play a central lymphoid function and, to a certain extent, influence the differentiation of the immunocytes capable of producing antibodies.

f) Immunocompetence of blood leukocytes - Antibody-forming cells have been found in the blood following systemic immunization (95, 96). However, the question may be asked whether these cells had actually been stimulated while in the peripheral circulation or whether they represent

cells migrating to and from other lymphoid organs. A second unanswered question is whether the antibody found on or in the blood leukocytes represents actual active secretion by these cells or whether the antibody is passively adsorbed onto their surface. By electron microscopy, Hummeler et al (97) could identify the structural characteristics of antibody-producing cells in the peripheral blood of rabbits immunized with SRBC: lamellae of endoplasmic reticulum and many polyribosomes. Hirschhorn et al (98) have also shown that human peripheral lymphocytes possess the basic potentials to respond in vitro to various mitogens and to produce immunoglobulins. On the other hand, Roseman et al (99), have shown that all hemolytic plaques produced by peripheral blood cells of rats are false plaques formed by antibody adherent to the leukocyte-platelet aggregate.

g) Comparative studies of immunocompetent cells of the various lymphoid organs - The studies of several investigators - reviewed by Cochrane and Dixon (66) - have shown that the organ source of the immunocompetent

cells may vary from one species of animals to another, and in the same species, depending on the antigen used. Armstrong et al (100) assayed the number of immunocompetent cells responding to a purified protein of Salmonella adelaide flagellin in several lymphoid tissues of mice. The assay involved the transfer of cells of normal lymphoid organs into lethally irradiated mice and consisted of the analysis of the host spleen for its capacity to form loci of bacterial immobilization in vitro. The bone marrow, mesenteric lymph nodes and Peyer's patches all possessed immunocompetent cells. The thymus, on the other hand, possessed only a negligible number. Strober and Mandel (67) studied the ability of different lymphoid cell types to restore immunocompetence to sublethally irradiated recipients given alum precipitated tetanus toxoid. Rat spleen cells were found to be more effective than rat thoracic duct lymph cells in their capacity to transfer immunocompetence, whereas in the mouse, the thoracic duct cells were more effective. Cells which initiated the primary antibody response with respect to SRBC and BSA were found in

high concentration in thoracic duct lymph of the rat but not in the spleen (67, 101).

Willard and Smith (102) studied the capacity of syngeneic transplanted mouse lymphoid cells to reject homografted bone marrow cells. The following decreasing order of effectiveness of the transferred cells was observed: Leukocytes, lymph node cells, spleen cells and peritoneal exudate cells. Thymocytes and marrow cells were not effective. The failure of the isologous bone marrow to reject the homologous marrow in the lethally irradiated mouse could be due to proliferation of the hemopoietic cells of the isologous marrow along erythropoietic cell lines rather than along immunocompetent cell lines.

2. Macrophages, Antigen Reactive Cells (ARC) and Antibody Forming Cells (AFC) - Their interaction(s) and involvement in the humoral immune response - In recent years, several cytokinetic models have been described (103, 104, 105) to illustrate the cellular events in the immune response. In general, these models postulate that when an immunologically uncommitted progenitor cell is stimulated with antigen, it gives rise to specific antibody forming cells (AFC) which differentiate along an irreversible pathway and, at the same time, produce a new set of progenitor cells (memory cells) committed to the immunizing antigen. Upon second contact with the same antigen, these memory cells, in turn, give rise to AFC in the so-called secondary antibody response as well as to another generation of memory cells.

The progenitor cell has been given different names by different investigators: "X" cell by Sercarz and Coons (105), "Effector" cell by Claman et al (78), "PC_1" by Makinodan and Albright (103), "Antigen Reactive Cell" (ARC) by Miller and Mitchell (4), "Antigen Sensitive Cell" by Armstrong and Diener (100), "Antigen Sensitive Unit" or

"Precursor of Plaque Forming Cell (P-PFC)" by Schearer et al (106) and "Reactor Cell" by Davies et al (70).

The above model probably constitutes an over-simplification of a very complex series of events and interactions. However, the recent work of many investigators (4, 70, 78, 92, 107) has indicated that the cellular system responsible for the production of hemolysin, in response to the injection of sheep red blood cells (SRBC), consists of at least two and probably three separate cell types. These are the antigen processing cell or the macrophage, the ARC, and the AFC.

a) The role of the macrophage in the immune response - The work of Adler et al (108) and of Fishman (109) emphasize the importance of the antigen processing step by macrophages and the transfer of informational signal to the immunocompetent cell. Lymph node fragments from nonimmune rabbits produced specific antibody in response to their exposure to a ribonucleic acid fraction extracted from peritoneal exudate cells which had been incubated with the antigen (108, 109). Feldman and

Gallily (110) induced antibody formation to Shigella in lethally irradiated mice by inoculating them with macrophages obtained from immunized animals combined with normal lymph node or thoracic duct lymphocytes. The injection of either the immune macrophages or lymphocytes alone did not evoke an immune response.

Mitchison (111, 112) studied the properties of the antigen retained by the macrophages following incubation with antigen in vitro and concluded that the retention of small quantities of antigen by macrophages plays an essential role in some but not all types of immune responses. He demonstrated that these macrophages enhance the capacity to induce a primary response, but are not required for a secondary response and can immunize after the induction of a state of specific immune paralysis. The macrophages had to be viable in the sense that they could be inactivated upon heating to 48°C, but they were unaffected or even enhanced in their reactivity if subjected to X-irradiation (550 R). The cells were effective only if injected into syngeneic hosts. Macrophages

taken from paralysed hosts were active (112); however, if normal macrophages were given to paralysed or immature mice, the latter regained immune competence (112), thus strongly implying that the non-responding cell in the tolerant mouse is not the macrophage.

In in vitro systems, the primary immune response has been shown by Adler et al (108) and by Mosier (6) to require the presence of macrophages. On the other hand, Simons and Fitzgerald (113) have shown that macrophages are not required for the induction of the secondary immune response in vitro.

Hersh and Harris (114) observed that macrophages are necessary for the blastogenic response of peripheral blood lymphocytes to antigen in vitro. The reaction was prevented if the lymphocyte preparation was first depleted of its macrophage content by perfusion of the cells through glass bead columns. The response was restored when the purified lymphocytes were cultured with antigen in the presence of isologous macrophage monolayers.

The mechanism by which macrophages function in the initiation of the humoral immune response is essentially

unresolved. Whether they deliver a highly immunogenic processed antigen, a messenger RNA or both to the ARC, or whether they simply lower the antigen concentration until the latter reaches a threshold level for the ARC is still debatable (109, 112).

b) The role of the ARC in the immune response - As shown in Chapters III A.2. and III B.1., in the mouse, bone marrow and thymus cells by themselves failed to induce SRBC plaque forming cells in irradiated recipients if each type of cell is individually transferred. It has subsequently been shown that the thymus provides cells that react with the antigen prior to the antibody forming step by the bone marrow cells. The interaction between the antigen reactive cell derived from the thymus and the antibody forming cell derived from the bone marrow is essential for successful induction of the primary immune response (4, 5, 6, 70, 76, 115, 116).

The following protocols have been used to study the role of the ARC in the immune response:

(1) Transfer of a known number of ARC to an immunoincompetent (irradiated or tolerant) recipient. The number of cells that form antibody is estimated from the amount of antibody formed in the recipient. This method, which has been used extensively by Makinodan et al (117), provides an approximation only and the dynamics of the population of transferred cells can be deduced only by sophisticated mathematical techniques.

(2) Detection of AFC, following the in vivo administration of ARC, by the localized hemolysis in gel technique developed by Jerne et al (73).

(3) An assay developed by Kennedy et al (118) for the detection of antigen sensitive cells in spleens of irradiated mice following the administration of spleen or lymph node cells. The foci of hemolysis developed in the spleens are considered to result form the proliferation of antigen sensitive precursors of hemolysin-producing cells (118). Using this technique, Kennedy et al (118) have shown that the spleens of irradiated recipients of spleen and lymph node cells, but not of bone marrow, thymus and fetal liver cells, form hemolytic foci.

(4) A technique for direct quantitation of stem cells developed by Till et al (119). The method is based on the

observation that mouse hemopoietic tissue contains a class of cells which, on exposure to an antigen, gives rise to macroscopic colonies in the spleens of irradiated mice. These colonies have been shown by Becker et al (120), using direct cytological means, to derive from single donor cells, and are called "colony-forming cells." The latter were found to have extensive proliferative capacity and are also capable of differentiation.

(5) A system described by Playfair et al (75), similar to that developed by Till et al (119). It was shown that each hemolytic focus is composed of the progeny of a single precursor cell which is restricted to form a single type of antibody and to localize in certain areas of the white pulp of the spleen.

(6) A technique designed by Armstrong and Diener (121) for the enumeration of ARC in spleens of mice responding to Salmonella protein antigen. The ARC injected into lethally irradiated hosts embed in the spleen and respond to antigenic stimulation, by proliferating and differentiating into colonies. Such colonies can be

detected by their ability to produce antibody which immobilize the indicator bacteria.

The events following interaction of ARC with the antigen were shown to be the rapid proliferation of ARC followed by their transformation into lymphoblastoid cells (70, 71). Administration of Vinblastin was shown by Syeklocha et al (122) to inhibit this step. Once the ARC are stimulated, they presumably settle in the lymphoid tissue, the cellular microenvironment of which provides the cell types necessary for the initiation of further events. Proliferation and division of these activated ARC or another cell type, the AFC (4, 71) may continue and spread concentrically and serve as the progenitor cells for the plasma cell series, resulting ultimately in the production of specific antibodies (104, 123, 124). Whether or not the cell types involved, namely the ARC and AFC, are derived from a single cell lineage or from two or more separate cell lines is not yet established.

c) The role of the AFC in the immune response - There is good evidence in the literature, as reviewed by Gowans and McGregor (1), indicating that the ancestor of the AFC is a lymphoid cell. Nossal et al (125) have shown that lymphocytes from thoracic duct lymph can transform into AFC. As stated previously (see Section B.1), the bone marrow and several embryonic organs of the mouse, but not the thymus, provide precursors of cells capable of forming hemolytic plaques in vitro and of synthesizing immunoglobulins and specific antibody. The spleen cells in the mouse are capable by themselves of transferring plaque-forming capacity, indicating that it either contains both ARC and AFC or that it contains a single cell type which acquires antibody forming capacity following interaction of its precursor with thymus-derived elements (see Section B.1). These mouse spleen cells have been shown by Shearer et al (106, 126, 127) to constitute antigen sensitive units (ASU). These units are composed of highly differentiated cells which are committed to produce an unipotent population of immunocytes capable of synthesizing antibody molecules of a

single class or allotypic variant and with a specificity directed to a single antigenic determinant. The ASU present in normal and immune populations of cells can generate either direct plaque-forming cells, indirect plaque-forming cells or cluster-forming cells. Moreover, Shearer and Cudkowicz (106) have shown that the antigen sensitive units formed in irradiated mice by interaction of marrow and thymus cells were similar both quantitatively and qualitatively to those of intact mice. In particular, they were specialized for the molecular class (IgM or IgG) and function (lysis or agglutination) of the antibody secreted by their descendant immunocytes. It was shown by the same investigators (106) that it is the bone marrow AFC and not the thymic ARC which is responsible for the class differentiation of the immune response, that two or more different marrow-derived AFC cooperate with the ARC and SRBC in forming the functional splenic ASU and that interaction between the activated ARC and the bone marrow AFC in vivo requires the presence of antigen and is a non-repetitive event, i.e. the same

ARC does not continue to interact with several precursors of PFC in succession. Whether the ARC is committed to a specific antigen prior to its contact with that antigen or whether a single ARC can interact with two different non-cross-reacting antigens is unclear. The in vitro work of Dutton and Mishell (91) and the in vivo work of Shearer et al (126) have shown that the ARC are pre-committed cells and that the AFC are unipotent. Trentin et al (128), on the other hand, have shown that the bone marrow immunocompetent cells are pluripotent, but once they are induced to transform to actual AFC, they lose their pluripotentiality.

d) Postulated mechanisms of cell to cell interactions - The interaction of two or more cell types is presumably required for the induction of the primary response. As discussed above (see Section B1), the interaction of the thymic ARC with the bone marrow AFC in the mouse is a requisite for the successful induction of the immune response. How does this interaction take place? Is there a need for a third cell type? These are two questions that have not as yet been answered.

Sharp and Burwell (129) and Schoenberg et al (130) have observed cytoplasmic connections between macrophages and lymphocytic cells in vivo. The frequency of such interactions appeared to increase after antigenic stimulation. McFarland et al (131) have described lymphocytes interacting with macrophages by means of a "uropod" in mixed leukocyte cultures. If the function of the macrophage is the processing of the antigen, direct cell to cell contact may be required to permit the passage of information from the macrophage to another cell type. The specific interaction of cells has been assumed to be mediated by antibody and antigenic determinants on the cell surface. This has been postulated to occur by several ways: An antigenic determinant on the surface of one cell might interact with antibody bound to the surface of a second cell; or two cells with antigenic determinants on their surfaces might interact with antibody serving as a link; or two cells with antibody fixed to their surfaces might interact with the antigen molecule linking them together. Mosier (6) has in fact demonstrated

that the AFC arise in areas of splenic cell clusters, that cluster formation is antigen specific and that it appears to be mediated by antigen and/or antibody on the surface of the interacting cells. All of these cellular interactions would be blocked by excess extracellular antibody which may explain why the primary immune response can be inhibited by the administration of antiserum prior to immunization with the specific antigen.

3. Fractionation of Immunocompetent Cells - The ability to segregate populations of immunocompetent cells from other cell types is essential for the proper investigation of the intracellular processes which take place during the immune response. Many approaches have been taken to isolate these cells, and to correlate structure to function. The following methods have been used:

a) Separation by size by means of glass wool or glass bead columns;

b) Separation on a density gradient using albumin, sucrose or dextran gradients. The cells separate on the basis of their sedimentation rate and density;

c) Separation on the basis of surface charge. The techniques used are electrophoresis and counter current distribution;

d) Separation by differential migration in vivo.

a) Fractionation by glass beads and glass wool columns - Shortman (132) and Rabinowitz (133) separated small lymphocytes by size filtration or on the basis of their active adherence to glass bead columns. Plotz and Talal (134) fractionated rat spleen antibody-forming cells on glass bead columns. The fraction passing through the column consisted predominantly of small mononuclear cells and was depleted of antibody synthesizing cells as measured by the Jerne plaque technique. The fraction eluted from the beads by ethylenediaminetetraacetic acid was enriched in antibody synthesizing cells, granulocytes and large mononuclear cells. The in vitro antigen-induced blastogenic response of human leukocytes could also be abolished if the leukocytes were first passed through glass bead columns (114), and can be attributed to the removal of

macrophages by the columns. Cultures of those purified lymphocytes on macrophage monolayers generally restored the cell response to antigens. However, the glass bead column-purified lymphocytes transform as well as unfractionated cells if stimulated with the optimal dose of a non-specific stimulant such as PHA or antileukocyte antiserum (135). Nossal et al (125) obtained a fraction of small lymphocytes from rat thoracic duct lymph, following filtration of the cells through a glass bead column, which was found to be rich in cells capable of transferring immunocompetence to sheep red blood cells and graft-versus-host reactivity across an H-2 barrier. However, recipients of these small lymphocytes were capable of responding only poorly to Salmonella flagellin. When the fraction was prepared from mice pre-immunized against the Salmonella antigen, the thoracic duct small lymphocytes were capable of initiating a typical secondary response.

The use of antigen-coated glass beads as specific immunoadsorbants for antibody was first demonstrated by

Sutherland and Campbell (136). Recently, Wigzell and Anderson (137) passed immune mouse lymph node cells through a column of antigen-sensitized glass beads and observed that the cells which passed through the column were specifically deprived of immune reactivity. Cells synthesizing the specific antibody were found to stick to the antigen-coated glass beads and could be eluted. The specific retention of immune cells by antigen-coated columns was shown to be selectively blocked by the presence of free antigen molecules in the medium during filtration. Up to the present time, fractionation of normal (non-immune) lymphoid cells using the antigen-coated glass bead columns has not been reported.

b) Fractionation on a density gradient - Albumin density gradients have been used to study the distribution of antigen reactive cells as compared to antibody-forming cells. Haskill (138) reported changes in the density profile of antigen reactive cells in rat spleen following antigen stimulation. This change of profile

was noted as early as 10 hours after antigen administration and remained up to 90 days. Using the same technique, Haskill et al (139) have shown a very complex profile for the 19 S plaque-forming cells. A clear distinction between the antibody-forming cells of spleen and lymph nodes, as compared to those circulating in the blood and thoracic duct lymph, was also noted. The circulating antibody-forming cells could be enriched 100-fold by separation on a density gradient whereas no more than a two-fold enrichment was obtained with cells from spleen or lymph nodes.

Raidt et al (92) fractionated spleen cells of normal and immunized mice on albumin gradients. The fractions were assayed for antibody forming cells by the hemolytic plaque assay and for antigen reactive cells by the size of the response to antigen in an in vitro culture system. The majority of ARC were found in the more dense regions of the gradient. After in vivo stimulation, the AFC were found in the less dense region. The change in cell density occurred in the first 12-18 hours after antigenic

stimulation. Plaque-forming cells of immune mouse spleen have also been shown (140) to sediment more rapidly in sucrose gradient than did the bulk of the non plaque-forming cells (140).

The cell(s) capable of initiating graft-versus-host reactions have also been separated by using density gradient centrifugation in albumin gradients. Shortman and Szenberg (141), using fowl peripheral leukocytes, have shown that a minor population of lymphocytes are the active cells in the graft-versus-host reactions. The active cells, however, were not found to be homogeneous, in terms of density, since they could be obtained as a series of peaks. Using mouse spleen cells, Dicke et al (142) noted that fractions rich in lymphocytes displayed poor hemopoietic activity but were active in inducing graft-versus-host reactions. A second fraction obtained from the gradient showed a 10-fold increase in the concentration of colony forming units and more than a 10-fold decrease of graft-versus-host activity. Lymphocyte-rich fractions of rat bone marrow obtained by

glass-wool filtration and dextran-gradient centrifugation were most efficient in protecting lethally x-irradiated syngeneic rats (143). The lymphocyte-rich fractions of normal rabbit bone marrow obtained by centrifugation in a linear sucrose gradient has also been found to respond markedly with blastogenesis and mitosis to stimulation with antigens in vitro, whereas the lymphocyte-poor fraction responded minimally (144).

c) Fractionation by surface charge - Mouse spleen cells have been fractionated by means of counter current distribution (145). The results showed that fractions enriched with respect to granulocytes and stem cells could be separated from antibody-producing cells. On the other hand, Mel and Jullien (146) failed to detect enrichment of "colony forming units" in any of the cell fractions after differential electrophoresis of mouse marrow cells. However, enrichment was obtained following separation of the marrow cells by density gradient techniques (146).

By using cell electrophoresis, Sundaram et al (147) were able to show a drastic reduction in the mean mobility of immune rat lymph node cells when the cells were first treated in vitro with the same antigen used for immunization.

d) Fractionation by differential migration in vivo - Functionally-distinct, i.e., immunocompetent, lymphoid cells have different in vivo migrating capabilities as compared to other lymphoid cells, and this property has been used to distinguish them. Lance and Taub (148) transferred ^{51}Cr-labelled lymph node lymphoid cells serially to syngeneic mice and observed that these cells localize mainly in the lymph nodes. Thymocytes, on the other hand, migrated mainly to the spleen and, to a lesser extent, to lymph nodes.

4. The mechanism of antigen recognition by immunocompetent lymphoid cells - Since the response to antigen is characterized by the extreme specificity of the reaction,

it is likely that a sub-population of lymphocytes react with the appropriate antigen because of the presence of membrane-associated recognition sites that have structural complementariness to the antigen (111, 124, 149). These sites may consist of cell-bound antibody molecules. The binding of antigen by this cell through its reaction with this antibody receptor probably initiates the sequence of events leading to cell division and antibody formation.

There is ample evidence in the literature indicating the immunoglobulin nature of these surface receptors (150):

i) Human thoracic duct small lymphocytes show weak immunofluorescence for IgM and can be stimulated to transform to blast cells when cultured in vitro with anti-immunoglobulin serum (151).

ii) Rabbit peripheral lymphocytes can be stimulated in vitro to transform into blasts by means of antisera directed against allotypic and other Ig determinants (152). Transformation was shown to be strictly specific to identifiable allotypic determinants.

iii) Simons and Fitzgerald (113) have demonstrated that exposure of immune human peripheral lymphocytes to anti-lymphocyte antisera, anti-whole immunoglobulin antisera, anti-IgG antisera and anti-flagellar antisera can block the blastogenic response to the flagellar antigen.

iv) Stimulation by hapten-carrier molecules of immune cells can be inhibited by prior reaction of these cells with free hapten. Furthermore, such a hapten-immune cell complex will not stick to hapten-sensitized glass beads (111, 124).

v) Extracts of immune human tonsilar small lymphocytes have been shown to possess properties of immunoglobulins specific for the antigens to which the donors had previously been immunized (153).

vi) Bert et al (154) have demonstrated changes in the physical properties of normal peripheral lymphoid cells after exposure to species-specific anti-immunoglobulin serum. A marked reduction in the random migration of the cells in vitro was noted following incubation of the cells with goat anti-human immunoglobulin serum.

However, treatment of the cells with antiserum directed against rabbit immunoglobulins or human prealbumin did not have any effect.

Despite the rapidly accumulating evidence for the presence of immunoglobulin-like receptors on the surface of immune cells, the actual demonstration of immunoglobulins or immunoglobulin fragments on the normal cell surface and their composition has not yet been reported.

C. The lymphoid system in the tolerant animal

1. The site of the lesion in the tolerant state - Immune tolerance (or paralysis or unresponsiveness) is caused either by depleting the tolerant host of cell clones, central or peripheral, reactive to the tolerogenic antigen or by altering the reactivity of these cells so that they can no longer recognize the antigen (155). The central organs failed to respond due to either interference with the access of antigen to the reactive cells (afferent limb of the immune response) or interference with the access of antibody that is subsequently produced (efferent limb of the immune response). Evidence for failure at the cell level of the immune response has been shown by several investigators (105, 155, 156, 157, 158, 159). They have demonstrated that the immune response can be induced in the paralyzed host by the transfer of cells of normal lymphoid tissues (lymph node, thymus, or spleen cells) (160) and that lymphoid tissues from the tolerant donors fail to give a

response in irradiated recipients (161, 162). The studies of McGregor et al (90) have clearly demonstrated that the immuno-incompetent cell in the paralyzed animal is the small lymphocyte.

McCulloch and Gowans (163) have shown that populations of small lymphocytes from the thoracic duct of rats may show full immune reactivity or partial or complete tolerance towards either histocompatibility antigens or sheep erythrocytes depending on the immune status of the donor with respect to these antigens. Since thoracic duct lymphocytes contain both ARC and AFC (see Section B.1), it cannot be concluded from this study which type of cell is tolerant in the immune tolerant state. However, recent studies, using flagellar antigen (100), SRBC (80) and BSA (79) have shown that the ARC is the site of the lesion in the tolerant state in the mouse. That the macrophage is not the tolerant cell has been shown by the studies of Mitchison (112) and Harris (164) who observed that macrophages obtained from peritoneal exudates of tolerant animals can take up the tolerogenic antigen and can initiate a specific immune response in both tolerant and normal recipients.

2. Mechanisms of immune tolerance - The mechanism of induction of immune tolerance has recently attracted the attention of several investigators. The studies of Cohen and Thorbecke (165) have demonstrated that the ratio between the lymphoid cells and the number of antigen molecules injected into the neonate is a critical factor in the induction of tolerance. They failed to induce tolerance to a tolerance-inducing dose of BSA, if a large number of neonatal lymphoid cells (spleen or thymus) were given to the newborn syngeneic recipients following the administration of the antigen. This would indicate that the newborn animal needs a large pool of antigen reactive cells or antibody forming cells in order to elicit an immune response. The work of Mitchison (111) and of Frei et al (166) point to the importance of the physical state of the antigen for the successful induction of paralysis. Aggregate-free antigen prepared by ultracentrifugation (167) or by in vivo filtering (166) of the original antigen is tolerogenic even in doses which would normally be immunogenic.

The molecular processes involved in the induction of tolerance at the cellular level are unknown. It is postulated (149, 155) that in the tolerant animal there must be a loss of cells with "receptor sites" or that these sites are there but are not available to interact with determinants to which tolerance has been induced. The response of the immune apparatus in the direction of antibody formation or tolerance would therefore depend on some qualitative or quantitative features of the initial interaction between the antigen and the receptor.

3. Kinetics of immune tolerance - Mitchison (111), in investigating the target cell(s) in the immune response, immunized mice with 20-100 mg BSA at 0, 10 min., 2 hrs. and 24 hrs. prior to sacrifice and transferred their peripheral lymphocytes and spleen cells to irradiated or tolerant (specific) syngeneic mice. The latter were then immunized and their immune response measured. Exposure of the donor peripheral lymphocytes to the antigen for two hours or more in vivo prior to their transfer resulted in

the maximum degree of paralysis in the recipient. However, the donor spleen cells required a longer exposure time to antigen in vivo prior to transfer to mediate tolerance in the recipient. These results suggest that the lymphocytes undergo their critical interaction with the antigen in a short time. Contrary to the above results, Golub and Weigle (168) used a similar transfer system and reported that the lymphoid cells have to remain in contact with the tolerogenic antigen in vivo for at least 5 days in order to induce immune paralysis in an irradiated recipient.

D. The Role of the Bone Marrow Cell in Immunity

1. The origin of the lymphoid stem cells, the immunocompetent cells, and the macrophages

a) The organ of origin of the bone marrow lymphoid cells - Chromosomally-marked bone marrow cells, and not labelled lymphoid cells of other organs, when infused into syngeneic lethally irradiated mice are capable of populating the host's thymus and peripheral lymphoid organs for long periods (55, 56). The bone marrow cells of the recipients contain cells of recipient origin from days 1-4, which then decrease in number rapidly and are replaced by donor bone marrow cells so that by days 10-20 one hundred percent of the marrow is of donor origin. Contrary to the above, Tyan and Cole (169) have shown in mice that though bone marrow cells primarily populate the recipient's thymus and bone marrow, the peripheral lymphoid pool is not populated by lymphoid cells of bone marrow origin. The major criticism against these experimental protocols is

the abnormal situation created by the rapid intravenous injection of a large number of hemopoietic stem cells into lymphoid-depleted lethally-irradiated recipients.

Whether the bone marrow lymphocyte originates in situ, or from the circulating blood lymphocytes or from both is still a controversial subject. A number of studies have suggested that the marrow small lymphocytes originate in the peripheral lymphoid tissues and are transported via the blood to the marrow where they serve as blood cell precursors (170). This concept has been disputed by several other investigators (26, 171, 172, 173) who have shown that the majority of marrow small lymphocytes are formed in situ. By injecting ^{3}H-thymidine into adult guinea pigs and rats and following the degree of labelling of the cells in the various tissues by means of radioautography, labelled dividing lymphoid cells were seen first in the bone marrow prior to their appearance in the peripheral lymphoid tissues. Surgical ablation of peripheral lymphoid tissues does not affect the kinetics or degree of labelling of the lymphoid cells in the bone

marrow (173). By electron microscopic studies, lymphocytes have been seen crossing the sinusoidal endothelium of guinea pig bone marrow (172). The likely precursors of marrow lymphocytes were considered to be the "transitional cells" intermediate in size between blast cells and the small lymphocytes and have been shown capable of synthesizing DNA (26).

Whether the bone marrow stem cell is pluripotential or unipotential is still a controversial question. By transplanting labelled adult mouse bone marrow into lethally irradiated recipients, evidence has been obtained suggesting that the stem cell can differentiate into cells with erythropoietic, myelopoietic, lymphopoietic or plasmacytopoietic potentialities (174). The differentiation of the stem cell into any of these progeny appears to be dependent on the competing proliferative demands of the body (174). Contrary to these findings, Bennett and Cudkowicz (175) showed only a unipotential role of mouse bone marrow stem cells. By either enhancing or depressing erythropoiesis in recipient mice, there was no evidence

for the shunting of the transferred stem cells from or into the production of non-erythroid cells.

b) The bone marrow origin of immunocompetent cells - The observations that the bone marrow cells can populate all lymphoid tissues, particularly the thymus, prompted several investigators (104, 128) to study the relationship of the bone marrow cell to the source of immunocompetent cells. Cells from 4-13 marrow-derived hemopoietic colonies, upon transfer to irradiated mice, established a large population of immunocompetent cells reactive to a variety of antigens. The lymphoid tissues of the repopulated mice were of donor origin as shown by the presence of the T6 marker chromosome of the donor bone marrow cells in 100 percent of mitotic cells of the spleen, mesenteric nodes and bone marrow (128). However, these transferred bone marrow cells appear to require thymic elements in order to differentiate into "antigen target cells" (4, 104) and were shown to be antigen independent (127). Irradiated mice grafted with bone marrow

cells were found to have precursors of PFC in their spleens which were unable to elicit anti-SRBC immune responses (5). The missing elements for the restoration of immunocompetence were presumably functional antigen-reactive cells. These cells are probably derived from the thymus since grafts of thymocytes added to marrow cells render mice immunologically competent without delay (5, 76). Whether the ARC is thymus-derived or bone marrow-derived under thymic influence is not settled. Globerson and Feldman (176, 177) have demonstrated that the immune response obtained in thymectomized lethally-irradiated mice protected with syngeneic chromosomally-marked bone marrow and thymic grafts is a manifestation of the donor marrow cells and not of cells from the thymic graft. Moreover, it has been shown by several investigators that bone marrow cells seed into the thymus cortex throughout life but acquire the capacity to behave as antigen reactive cells only after their subsequent dissemination to the peripheral lymphoid organs (50, 123, 178). Osoba (179) has suggested that ARC are marrow-derived since they are

present in heavily irradiated thymectomized mice grafted with marrow cells and implanted with thymus enclosed in a cell-impermeable chamber. In such mice, new ARC were detected in sites other than the thymus. However, the kinetics of splenic plaque formation did not differ from those described in nonthymectomized marrow chimeras and chimeras with thymuses implanted under the kidney capsule. The conclusion was made that a humoral factor, elaborated by the thymus, controlled the differentiation of marrow cells into ARC.

The direct involvement or influence of the thymus in the differentiation of bone marrow cells is illustrated by the studies of Doria and Agarossi (81, 82) and Agarossi and Doria (180). They have shown that the recovery of the immune response to SRBC in lethally irradiated mice transplanted with either syngeneic or allogeneic bone marrow cells is conditioned by the host thymus. Donor mitotic cells of bone marrow origin were found in the host thymus of mouse radiation chimeras. Thymus cells at different intervals following establishment of the chimeric state

were transferred together with SRBC to lethally irradiated syngeneic mice. Thymus cells from young chimeras were better able to transfer plaque forming capacity than cells from old chimeras. Syngeneic chimeras were more effective than allogeneic chimeras. This would indicate the presence of immune competent cells (both ARC and AFC) in the thymus of antigenically-unstimulated bone marrow-induced chimeras and that these cells are more efficient in antibody production when stimulated in syngeneic, rather than allogeneic, hosts.

How does the bone marrow render the thymus immunocompetent? Two possible interactions may occur: Differentiation of marrow cells into ARC which, on antigen stimulation, transform to AFC; or differentiation of marrow cells into thymocytes which, following interaction with antigen, stimulate the bone marrow cells to produce antibody.

The relationship between the bone marrow cells which form colonies in lethally-irradiated recipients and cells which constitute the immune cell system is not well understood. As shown above, the injection of chromosomally-

marked bone marrow cells into irradiated recipients results in a large number of cells exhibiting this marker in the thymus. These thymic cells, when transplanted into other irradiated syngeneic recipients, are capable of forming spleen colonies and of reconstituting immune competence. This latter property cannot be instituted with the transfer of normal thymus cells. No attempt has been made to determine whether the bone marrow-derived cell present in the thymus is the cell that produces the plaques. These data therefore indicate that the hemopoietic colony-forming capacity and immunocompetence derive from different cells, but these precursors originate from the same stem cell.

The direct demonstration that the transplanted chromosomally-marked bone marrow cell is the cell responsible for antibody production was shown conclusively by Wu et al (54, 181). They infused syngeneic chromosomally-marked bone marrow cells into lethally-irradiated recipients. The same chromosome marker was found in the hemopoietic colonies of the spleen, in the thymus and in the popliteal lymph nodes. Following the foot-pad injection

of SRBC, more than 50 percent of the dividing cells in the regional lymph node carried the donor chromosome marker and were capable of forming hemolytic plaques in vitro. On the other hand, chromosomally-marked popliteal lymph node cells of mice not given SRBC did not divide and formed the average number of background hemolytic plaques.

c) The bone marrow origin of the macrophages - The bone marrow has been shown to be the main source of macrophage precursors in inflammatory reactions and in peritoneal exudates. Volkman and Gowans (182), applying ^{3}H-thymidine labelling to the "skin-window" technique, established that the exudate macrophages in foci of sterile inflammation are derived from a rapidly dividing precursor present in the bone marrow. The failure to change the character of the exudate by previous thoracic duct drainage or whole body x-irradiation accompanied by bone marrow shielding excluded the possibility that invading macrophages could have been derived to any appreciable

extent from thoracic duct lymphocytes. Spector et al (183), by using a combination of tritium and colloidal carbon labelling techniques to label dividing precursors of macrophages, identified the highly phagocytic bone marrow-derived circulating monocytes as the antecedent of the majority of exudate macrophages. These findings were confirmed by using mouse radiation chimeras. In these models, peritoneal macrophages were shown to carry the chromosome marker.

Further evidence in favor of the bone marrow origin of the macrophage stems from the work of Virolainin (184). Radiation chimeras were induced with bone marrow with the marker T6-T6 and lymphoid cells from a genetically different donor carrying a different marker. All macrophages of the chimera carried the T6-T6 chromosome marker only. This indicates that lymphoid tissues outside the marrow, namely thymus, lymph node and peritoneal exudate cells, do not contain precursor cells of macrophages (184, 185, 186, 187).

The origin of the macrophages in visceral organs is still controversial. Virolainin (184), using a chromosome

marker in an in vitro culture system of a pure population of macrophages, has demonstrated that not only peritoneal macrophages but also those present in bone marrow, spleen, lymph node and thymus are derived from bone marrow. Pulmonary alveolar macrophages, however, were shown to have both bone marrow and pulmonary origins (188).

2. The role of the bone marrow in humoral immunity and immunoglobulin production - As discussed in Sections B.1, B.2, and D.1, the lymphoid cells destined to produce antibodies appear to be derived from the bone marrow and apparently differentiate into immunocompetent cells under the influence of the thymus or the bursal equivalent. In the rabbit, the sacculus rotundus, appendix and Peyer's patches, apparently play a role comparable to the bursa of Fabricius in the chicken (94). Though there is no evidence in the literature to suggest that bone marrow cells actually populate the gut-associated lymphoid tissues, it has nevertheless been assumed that the func-

tion of these latter organs, like that of the thymus in the mouse (123), is to modify stem cell differentiation along the lymphocyte pathway.

The in vitro studies on rabbit bone marrow cells have clearly suggested that the bone marrow supplies the immunocompetent cells in the mature adult rabbit. Singhal and Richter (46) have shown that only bone marrow from normal unimmunized rabbits can react with blastogenesis and mitosis in the presence of a number of protein antigens. Lymph node, spleen or thymus cells of normal rabbits also undergo transformation in vitro. Bone marrow cells, but not the other lymphoid cells, of rabbits immunized with an antigen on the other hand, lost their capacity to react in vitro to the immunizing antigen but not to a non-cross-reacting antigen. Chapman et al (189) have demonstrated that the rate of DNA synthesis in cultures of bone marrow cells obtained from hyperimmunized rabbits was depressed if incubated with either the immunizing antigen or PHA.

Human bone marrow cells cultured in vitro have been shown to secrete immunoglobulins and antibodies (190, 191).

Bone marrow plasma cells, but not bone marrow lymphocytes, showed positive fluorescent staining to immunoglobulins G, M and A (191). Furthermore, bone marrow of patients with cold hemagglutinin syndrome can synthesize cold hemagglutinin in vitro (192). Through combined in vitro culture studies and immunofluorescent staining of bone marrow cultures obtained from patients with multiple myeloma and Waldenstrom's macroglobulinemia, it was observed that the plasma cells of the bone marrow can synthesize monoclonal immunoglobulins with the same electrophoretic mobility and the same characteristics of heavy and light chains as those in the serum (193).

Studies concerned with investigating the role of the bone marrow in humoral immunity have emphasized the importance of the immune status and the genetic constitution of the bone marrow donor in order to elicit successful immune responses in the recipient. Several investigators have shown that the antibody response can be successfully obtained in the irradiated mouse if the latter is injected with bone marrow obtained from immunized but not from normal

donors (194, 195). Lesser amounts of antibody are formed if the genetic strain difference is increased between the donor and the recipient (196, 197). Doria et al (198) have in fact shown that the capacity of long-term mouse radiation chimeras to produce agglutinins directed to SRBC was greater in syngeneic than in allogeneic combinations. Higher primary antibody responses were obtained when irradiated recipients were given syngeneic, rather than allogeneic, bone marrow. However, syngeneic bone marrow did not stimulate or hasten the recovery of the ability to elicit secondary antibody responses in previously immunized irradiated mice.

Papermaster (104) has postulated, on purely theoretical grounds, that the differentiation of the bone marrow stem cell into antibody-producing cell consists of a number of irreversible steps. At some point during differentiation the stem cells come under the influence of the thymus and acquire the ability to recognize an antigen, probably by acquiring unique receptors on the cell surface with which the antigen interacts. Once the cell has interacted with the antigen to which it is directed, the further

differentiation of the antibody-forming cells (AFC) will result in cells producing antibody, of the same specificity, directed to this antigen. It has been suggested by several investigators that the division of the AFC continues until either the level of the antigen decreases below the level necessary for further stimulation (199) or until the level of antibody is high enough to exert a repressive function (200).

3. The role of the bone marrow in cellular immunity - The successful induction of the delayed hypersensitivity reaction (DH) requires the cooperation of two cell types: A thymic-derived cell for the initial sensitization steps and a bone marrow-derived cell for the subsequent cellular infiltrative reactions (37, 38).

In rats (37), neonatal thymectomy prior to sensitization with the antigen inhibits the subsequent delayed hypersensitivity reaction. However, the animals can become sensitized if they are injected with sensitized syngeneic lymphoid cells. On the other hand, thymectomy

of adult Lewis rats following sensitization with tubercle bacilli does not affect their ability to develop delayed skin reactions upon skin testing with PPD (37). These data indicate that in order to elicit a DH reaction the thymus is required for the active but not for the passive sensitization reaction.

The results of several investigators (36) indicate that the majority of cells infiltrating the sites of the DH reactions are not actively-sensitized cells. The experiments of McClusky et al (201), in which passive transfer of cells was combined with thymidine labelling of donor or recipient cells, demonstrated that 80-90 percent of the cells infiltrating the sites of specific skin reactions were host derived. These latter cells have been shown to be bone marrow derived and constitute a non-specific component of the reaction. They are phagocytic and resemble macrophages which appear in sites of non-specific inflammation. The experiments of Lubaroff and Waksman (37, 38) demonstrate that the successful passive transfer of tuberculin hypersensitivity with sensitized lymph node cells to thymectomized irradiated recipients depends on the simul-

taneous or prior injection of normal bone marrow cells (37). Normal thymus, spleen, lymph node or peritoneal exudate cells, even at high doses, could not be substituted for the bone marrow in producing the tuberculin reaction (37). These experiments indicate that once sensitization has occurred, a bone marrow cell and not a thymic cell is required for the manifestation of the reaction. The precise origin of the cells infiltrating the skin was investigated by the administration of allogeneic bone marrow to the thymectomized irradiated rats prior to the administration of the sensitized lymph node cells. Fluorescein-conjugated antiserum against the cells of the bone marrow donor was then applied to the biopsies of the sites of skin reaction. The majority of cells were shown to be derived from the infused marrow. The relative percentages of marrow-derived and lymph node-derived cells in the tuberculin reaction remained the same during the 9-24 hour period following skin test (38).

Histologic studies (202) and studies with tritiated thymidine suggest that a similar mechanism must be involved

in other reactions similar to the tuberculin-induced reaction (203), such as contact allergy (201), auto-allergic lesions (204), skin homograft reactions (205) and disseminated lesions of adjuvant arthritis (206).

4. The role of the bone marrow in the graft-versus-host reaction - As has been discussed previously, the lymphocytes are the effector cells in the graft-versus-host reaction (GVHR) (1, 2). The primary source of these competent lymphocytes has been shown to be the bone marrow (87, 141, 142). These lymphocytes are probably also present in smaller numbers in the peripheral lymphoid tissues (16), the thymus (88) and the circulating lymphocyte pool (87, 207). The model system used to demonstrate the presence of reactive cells in the circulating pool is as follows: Thoracic duct cells were obtained from F1 hybrid rats (intermediate host) which had been inoculated at birth with parental strain bone marrow cells. The thoracic duct cells were then transferred into a second F1 hybrid recipient of a different genotype. A GVHR was regularly observed in these latter recipients. However,

the capacity of the thoracic duct cells of the F1 intermediate host to transfer GVHR was diminished if spleen cell and not bone marrow cells were initially injected into the donor. Thoracic duct cells from normal uninoculated F1 hybrids failed to give the reaction in the recipients. Bone marrow cells from parental rats either depleted of small lymphocytes by chronic drainage from a thoracic duct fistula or made tolerant to the intermediate F1 host are less effective, if compared to bone marrow cells from normal donors, in causing homologous disease in the secondary F1 hybrid recipients (207). This would indicate that it is the bone marrow cell and not the circulating small lymphocyte or the lymphocyte residing in the peripheral lymphoid tissues which can best transfer GVHR in the rat.

Allogeneic adult bone marrow cells have also been shown to be capable of inducing GVHR in the mouse. Tyan and Cole (169) were able to show that a significant number of deaths occurred among F1 hybrid hosts when they had received spleen cells from parental mice which had been injected

with adult bone marrow. Spleen cells from mice which had not been injected with the marrow were less effective. There were no deaths among the mice which received spleen cells of donors injected with parentàà thymus cells.

That the bone marrow lymphocyte is the cell responsible for the GVHR in the mouse is shown by the cell fractionation studies of Dicke et al (142). Mouse spleen cells were fractionated on a discontinuous albumin gradient and the hemopoietic capacity and GVHR activity of the cell fractions obtained were studied in lethally irradiated F1 hybrid mice. A cell fraction rich in blast cells showed a 10-fold increase in the concentration of colony forming units (index of hemopoiesis) and a more than 10-fold decrease in GVH activity. No secondary disease was observed in allogeneic irradiated mice which received this fraction. A second fraction, composed mainly of lymphocytes, was very poor in reconstituting hemopoietic activity but was very active in inducing GVHR.

Although the thymus gland in the mouse is essential for the induction of cellular immunity, its role in the development of the GVHR is debatable. Simmons et al (208) have demonstrated that allogeneic bone marrow is capable of mounting a prompt and vigorous GVHR resulting in the death of the host even in the absence of the host thymus. Field and Gibbs (209) have shown that thymectomy increases the susceptibility of F1 hybrid rats to GVHR induced by the intraperitoneal injection of parental strain spleen cells. The capacity of thymocytes to induce GVHR in appropriate recipients is controversial since it has been observed that thymocytes can (210) and cannot (16) transfer GVHR to appropriate recipient hosts. Lymphoid cells (spleen and circulating lymphocytes) from neonatally thymectomized mice are less efficient as compared to cells from non-thymectomized donors in causing GVHR upon transfer to lethally irradiated recipients (211).

In contrast to these effects of thymectomy, ablation of the bursa of Fabricius in the chicken with subsequent loss of the bursal-dependent population of cells does not

lead to a decreased capacity of the circulating lymphocytes to exert GVHR in recipient animals (15).

5. The role of the bone marrow in immune tolerance - Investigations concerned with the types of cells affected in the tolerant mouse have disclosed that the antigen reactive cell is probably the unresponsive cell in the tolerant state (see Chapter III.C). Recently, however, Playfair (212) has shown that cells derived from the bone marrow in the mouse are the tolerant cells in cyclophosphamide-induced tolerance to SRBC. Argyris has reported that tolerance to a skin graft can be transferred by either a thymus graft or bone marrow cells to an irradiated recipient (213). The possibility that the immune defect in tolerance is at the central, rather than the peripheral, level cannot therefore be disregarded.

The role of the bone marrow in immune tolerance has been further enhanced by the studies of Uphoff (214, 215). She incubated mouse bone marrow cells in vitro in the presence of allogeneic erythrocytes. The bone marrow cells

and the erythrocytes were then injected into lethally irradiated syngeneic recipients. These latter animals could reject syngeneic skin grafts and also succumbed to graft-versus-host reaction. The cells failed to protect the syngeneic host against the lethal effect of irradiation. Furthermore, the in vitro-treated bone marrow cells no longer recognized the allogeneic erythrocyte donor as foreign since they could protect the allogeneic recipient against lethal irradiation. They were also unable to induce secondary disease in the allogeneic host, a disease induced in 100 percent of irradiated mice injected with allogeneic bone marrow cells not incubated in vitro with the erythrocytes. These results suggest that during the in vitro incubation of the allogeneic erythrocytes with the bone marrow cells, the latter are modified in such a way that they no longer recognize the allogeneic strain as foreign nor the syngeneic strain as self. However, it must be stressed that the universality of this generalization may be in doubt in view of the specific source of the erythrocytes required to be incubated with the bone marrow cells and the absence of confirmatory findings.

6. The use of bone marrow in transplantation - The use of bone marrow transplants for various blood dyscrasias was first attempted late in the nineteenth century (216). The benefits derived from it were rather equivocal. The concept of hematopoietic tissue transplantation as a post-irradiation therapeutic measure has, however, endured. Shielding of the exteriorized spleen in mice subjected to lethal irradiation results in enhanced survival (217). Similar enhancement in survival has been obtained by implantation of hematopoietic tissue (infant spleen) into lethally-irradiated animals shortly after exposure to radiation. Rekers and co-workers (218), in 1950, attempted marrow transplantation in irradiated dogs and obtained slightly favourable results as reflected by small differences in mortality and hematologic responses between experimental and control animals. Lorenz and co-workers (219), in 1951, demonstrated the protective effects of the post-irradiation injection of isologous bone marrow in 70-95 percent of lethally-irradiated mice and guinea pigs. They subsequently extended their studies by showing the therapeutic

value of homologous and heterologous bone marrow transplants in irradiated mice, although these were less effective than isologous tissues. Histologic and hematologic studies showed that the rapid destruction of host hematopoietic tissues by ionizing radiation was not prevented by shielding of the limbs prior to, or by the administration of donor hematopoietic cells intravenously subsequent to irradiation. However, these maneuvers permitted more rapid and extensive regeneration of hematopoietic tissues than would normally occur. The mechanism by which these procedures exerted this regenerative effect was considered to be either via the release of a humoral stimulating factor by the protected or donor bone marrow tissues or the seeding of cellular elements from these tissues. Evidence has since accumulated in favour of a definite role for the donor bone marrow. Lindsley and co-workers (220), using differences in red blood cell antigens as a marker system, demonstrated a progressive increase of donor type RBC in the circulation of irradiated rats treated with homologous bone marrow.

Nowell and co-workers (221), utilizing the difference in alkaline phosphatase activity between the mature neutrophils of rats and mice as a marker, demonstrated the presence of rat cells in lethally irradiated mice protected by the injection of rat bone marrow. Within 14 to 28 days after treatment, essentially all neutrophil cells in the bone marrow, blood and spleen were of rat origin. Makinodan (222) postulated the presence of rat erythrocytes in mice treated with rat-marrow due to their failure to respond immunologically to injections of rat RBC. Rat erythrocytes were detected in the circulation one week after treatment and within 60 days they constituted 100 percent of the circulating RBC. In a homologous mouse bone marrow transplant experiment, Ford and co-workers (223) used a chromosome marker to identify dividing donor cells in the bone marrow, spleen, lymph node and thymus of the protected animals. Within five days, almost all of the dividing cells in these tissues were identified cytologically to be of donor origin and remained so for the period of observation,

which was 386 days (223). These initial results provided an impetus for more extensive investigations involving bone marrow transplantation in animals and man. Seller and Polani (224) successfully treated mice with a genetically determined macrocytic anemia with allogeneic bone marrow hemopoietic cells. The grafted marrow proliferated, allowing the recipients to develop a normal blood picture.

Certain types of immune deficiency syndromes in man with a probable defect at the stem cell level have recently been treated by bone marrow grafting. Gatti et al (225) reported the successful treatment of a 5-month old male with sex-linked lymphopenic immunological deficiency. Both cellular and humoral immune functions in the recipient were reconstituted following the injection of immune competent cells from peripheral blood buffy coat and bone marrow of a sibling donor. Fatal graft-versus-host disease was circumvented by matching the donor cells with the patient's cells with respect to the HL-A locus, as determined by both mixed

lymphocyte culture and lymphocytotoxid assays. Bach et al (226) have successfully treated Wiskott-Aldrich syndrome in a 2-year-old boy by means of a closely-matched bone marrow transplant. Six weeks after transplantation, there was evidence of chimerism, production of isohemagglutinins and improvement in the patient's clinical state.

The technique of tissue grafting would appear to open a new approach in the treatment of otherwise fatal immune deficiency diseases. The advances in our knowledge concerning the role of the bone marrow in the maturation of immunocompetence will certainly also prove to be of immense value in the treatment of a wide variety of diseases. The problems of rejection and graft-versus-host disease, respectively, should be overcome by proper tissue-typing, immunosuppression therapy and/or the induction of tolerance to the donor tissue.

CHAPTER IV

MATERIALS AND METHODS

A. Materials

Animals

Adult, 4 to 6 pounds outbred New Zealand white rabbits were purchased from a local dealer. They were maintained in a well-ventillated, temperature-controlled animal room and were fed and watered at regular intervals of time.

Antigens

1. Red blood cells - Sheep red blood cells (SRBC), horse red blood cells (HRBC) and rat red blood cells (RRBC) were obtained as sterile suspensions in Alsever's solution and were stored at 4°C. Red cells were washed in saline (0.9 percent sodium chloride) before use.

2. Red blood cell stroma - Sheep and horse red cell stroma were prepared by centrifugation of the red cells

at 2,000 rpm for 10 minutes following lysis with distilled water. The sediment, consisting of the red cell stroma, was suspended in a volume of Medium 199 (Microbiological Associates, Bethesda, Md.) equivalent to the original cell concentration. The stroma was then solubilized by ultrasonic disintegration at 16,000 cycles per second using a Fisher ultrasonic probe. The solubilized sonicates of sheep and horse red cells are referred to as SCS and HCS, respectively.

3. Human serum albumin (HSA) - (Hyland Laboratories, Los Angeles, Calif.).

4. Bovine gamma globulin (BGG) - (Pentex Incorp., Kankakee, Ill.).

Antisera

1. Anti-SRBC and HRBC antisera - Antisera to SRBC and HRBC were prepared in rabbits by the intravenous administration of one ml of a ten percent suspension of

the red cells at weekly intervals for three weeks. The rabbits were bled 7-10 days following the last injection and the sera obtained following centrifugation of the clotted bloods were stored at -10°C until used.

2. Anti-allotype antisera - Anti-allotype antisera were kindly provided by Dr. S. Sell, Department of Pathology, University of Pittsburgh, Pittsburgh, Pa. They were produced in rabbits by immunization with antigen-antibody complexes prepared by incubation of the antigen with antiserum of a rabbit with a different allotype according to the method described by Sell and Gell (152).

3. Complement - Commercial dried guinea pig complement (Hyland Laboratories, Los Angeles, Calif.) was used through the study. It was dissolved and diluted in saline (0.9 percent sodium chloride) prior to use.

4. Media - Medium 199 with bicarbonate (Microbiological Associates, Bethesda, Md.) containing penicillin

(100 units per ml of medium) and streptomycin (100 ug per ml of medium) is referred to in the text as Med-PS. The penicillin and streptomycin stock solutions were obtained from Microbiological Associates, Bethesda, Md., USA. For in vitro cultures, normal rabbit serum (NRS) (Microbiological Associates) is added to Med-PS to yield a final serum concentration of 15 percent. This final mixture is referred to as Med-PS-NRS.

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

6. Glass beads - Superbrite glass beads, type 100-5005, obtained from the Minnesota Mining and Manufacturing Company, St. Paul, Minn. were boiled in nitric acid for 15 minutes following which they were washed with phosphate buffered saline (pH 7.0) (PBS) until the pH of the wash solution was 7.0.

B. Methods

Preparation of cell suspensions

Rabbits were sacrificed by the intravenous injection of nembutal (50 mg per kg body wt). The lymphoid organs - spleen, lymph nodes (popliteal and mesenteric), appendix, sacculus rotundus and thymus - were rapidly extirpated and individually placed in Med-PS contained in sterile disposable plastic tubes (Falcon Plastics, Los Angeles, Calif.). The organs were then lightly passed through a wire mesh (100 mesh) so as to provide cell suspension. The cells are then centrifuged at 800 rpm for 5 minutes and suspended in Med-PS at various cell concentrations.

Circulating lymphocytes were obtained by bleeding from the heart (45 ml) into a syringe containing 100 units heparin and dextran (x 15 ml), molecular weight 250,000 (Dextran 250, Pharmacia, Uppsala, Sweden). The blood-dextran mixture was then divided into sterile plastic tubes and kept at 37°C for 20 minutes at a 60 degree angle to the horizontal. The lymphocyte-rich upper layer

was then decanted, washed and suspended in Med-PS in the required cell concentration.

Bone marrow cells were obtained from rabbits that have just been sacrificed by the intravenous administration of nembutal (50 mg per kg body wt). The femur and tibia were cleaned and split with a bone cutter and the marrow was suspended in sterile disposable plastic tubes (Falcon Plastics, Los Angeles, Calif.) containing normal rabbit serum (NRS). The tubes were shaken vigorously for a minute and centrifuged at 800 rpm for 5-10 minutes. The fatty upper layer was decanted and the cells were suspended in Medium 199 containing penicillin (100 units per ml), streptomycin (100 µg per ml) and NRS (15 percent) (Med-PS-NRS). The cells were washed once more and resuspended in Med-PS-NRS to the required cell concentration.

Cell-Culture technique and determination of radioactive thymidine incorporated by the cultured cells incubated with the isotope

The cells to be cultured were suspended in a concentration of 10^6 per ml in Med-PS-NRS and 4 ml aliquots of

the cell suspension were transferred into sterile Falcon plastic tubes. Where stated, the antigen or PHA was added at the beginning of the culture. The tubes were capped and exposed to an atmosphere of 4 percent CO_2 in air in an incubator at 37°C for three days. Tritiated thymidine ($T\text{-}H^3$) (2 uc, spec. activity 1 C/mM) (Schwarz Bio-Research, Inc., Orangeburg, N.J.) was added to the tubes 18-24 hours prior to the termination of culture at which point 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 percent 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 the antigen or other stimulants to that incorporated in its absence.

Dye Exclusion Test

The viability of various cells was determined by the dye exclusion test using 0.1 percent trypan blue. A drop of the dye was added to one ml of the cell suspension and the latter was then analyzed in a hemocytometer. Cells that took up the dye are considered to be dead cells. The viability of the cells, on the basis of 200 cells counted, was recorded as percent of dead cells.

Irradiation of Rabbits

The rabbits were subjected to doses of irradiation ranging from 800r to 1400r whole body irradiation, using a Cobalt 60 source under the following conditions: skin-source distance 200 cm, field size 50 x 50 cm, colarimeter size 20 x 20 cm and output 6.97r per minute.

In Vitro Irradiation of Bone Marrow Cells

The bone marrow cells were suspended in Med-199 in a screw-capped flask in variable cell concentrations. They were exposed to 4000r or 10,000r irradiation, using a Cobalt 60 source, at a rate of 99.6r per minute, under the following conditions: 280 Kv peak at 18 ma, half-value layer 1.2 cm copper and focal surface distance 50 cm. Following irradiation, the cells were analyzed for viability by the dye exclusion test and for their capacity to undergo blastogenesis and mitosis following stimulation with PHA.

Induction of Immunological Tolerance

Neonatal rabbits were injected subcutaneously at days

2 and 5 of age with 100 mg HSA or BGG. They were left undisturbed with their mothers. All the rabbits were bled at 6 weeks of age and tested for circulating antibody and antigen by the hemagglutination and hemagglutination inhibition techniques, respectively (see below). Those rabbits that did not show presence of both antigen and antibody were used for the experiments.

Inactivation of Bone Marrow Cells

Normal rabbit bone marrow cells were either heat-killed by incubation in a 60°C water bath for one hour, or sonicated using an ultrasonic disintegrator (Fisher Ultrasonic Probe) at 16,000 cycles per second for one minute.

Hemolytic Plaque Technique

The technique used is that described by Jerne and Nordin (73) with slight modifications. Agarose was used in place of agar as described in the original procedure. The spleen cells (0.1 ml), washed sheep red cells (0.1 ml

of a 10 percent suspension) and the agarose (1 ml of a 0.5 percent solution) were mixed thoroughly in a 46°C water bath and layered into the Petri dishes (2 inches diameter) containing a thin basal layer 2 cc of 1.4 percent agar and they were allowed to stand at 37°C for 2 hours. One ml of commercial guinea pig serum (Hyland Laboratory, Los Angeles, Calif.), diluted 10-fold, was then added. The plates were left at 37°C for another hour and the plaques were counted with the aid of a magnifying lens. The results are expressed as the number of plaque-forming cells per 10^6 splenic lymphoid cells plated. The variation in the number of plaques observed in duplicate assays was consistently less than ± 10 percent from the mean.

To determine the antibody allotype produced by the spleen cells, the cells were incubated with anti-allotype antiserum (Anti - A_1 or Anti - A_2) or normal rabbit serum (NRS) and SRBC in the agar phase prior to the addition of complement. The antisera and NRS were all diluted in a one percent solution of human serum albumin (Hyland

Laboratories, Los Angeles, Calif., USA). The technique is essentially that described by Chou et al (227). The results are also expressed as the number of plaques per 10^6 spleen cells incubated.

Tanned Cell Hemagglutination Technique

The technique used is essentially that described by Boyden (228). The red cells were tanned by adding 6 ml of a 2.5 percent suspension of sheep red cells to 6 ml of a 1:20,000 preparation of tannic acid, freshly prepared from a 1:100 stock solution of tannic acid in phosphate buffered saline (PBS) (Phosphate buffer, pH 7.2:saline - 1:1). The cells were incubated for 10 minutes at 37°C. The tube was then 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, in the appropriate concentration, 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 percent. 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 were 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 inverse of the maximum dilution of the antiserum capable of effecting agglutination of the sensitized erythrocytes.

Determination of the Antigen Concentration by the Hemagglutination-Inhibition Technique

Doubling dilutions of the serum to be tested for the antigen were prepared using PBS as diluent.. Then, to each one-half ml of the serum is added half ml of a known dilution of the specific antiserum. The tubes were left at room temperature for a few minutes and then one tenth ml of the sensitized sheep red cells was added to each tube. The rest of the procedure is as above. In the presence of antigen, the red cells fail to agglutinate. The first tube showing positive hemagglutination is considered the end point. The exact amount of antigen is calculated from the control tubes in which a known amount of the antigen is added to the same dilution of the antiserum.

Fractionation of Bone Marrow Cells on Antigen-Sensitized Glass Columns

The bone marrow cell suspension was adjusted to a concentration of 10^8 cells per ml in Med-199 containing 15 percent NRS. These cells were then passed through a column of antigen-sensitized glass beads prepared according to the technique of Wigzell and Andersson (137). The antigen, HCS or SCS, processed from 25 ml packed red cells (or 250 mg HSA), was added to 25 ml glass beads in 25 ml PBS and incubated at 45°C for one hour and then left overnight at 4°C in the presence of 10 percent NRS. The antigen-sensitized beads were then poured into a glass column (20 cm long by 1.5 cm internal diameter) and washed with approximately 10 volumes PBS. As will be seen below, no antigen could be detected in the final effluent wash using the technique of inhibition of specific agglutination of antigen-sensitized red blood cells (see above). The normal bone marrow cells (300 to 500 x 10^6 cells) in 3 to 5 ml of medium 199 were then applied to the head of the glass bead column and passed through

the column with Med-199 as eluting fluid. When cells could no longer be recovered in the effluent, the glass beads were placed in a sterile 250 ml flask and vigorously shaken for 5 minutes. The cells in the supernatant which were eluted from the antigen-sensitized glass beads (eluate) were centrifuged and washed with Med-199. The cells in both the effluent and eluate were suspended in Med-199.

Determination of Cell Morphology

Smears of the unfractionated bone marrow, effluent cells and eluate cells obtained by fractionation on antigen-sensitized glass beads were prepared on cleaned glass slides and stained with hematoxylin and eosin or giemsa stains. They were analyzed for their morphological and staining characteristics under the microscope.

In Vitro Stimulation of Bone Marrow Cells

Bone marrow cell suspension was prepared as described above. The cell count was adjusted to a cell

concentration of 10^7 per ml. Ten ml of the cell suspension were transferred into sterile screw-cap flasks to which were added a variable number of SRBC (10^7 to 10^9 cells). The cells were incubated in an atmosphere of 4 percent CO_2 in air for 1 to 24 hours at 37°C, following which they were washed twice and injected, in variable numbers, into irradiated recipient rabbits.

CHAPTER V

EXPERIMENTAL PROCEDURES, RESULTS AND DISCUSSION

A. Preliminary Studies to Determine the Optimal Conditions for the Various Experimental Procedures

The initial sequence of experiments was carried out in order to establish baseline values with respect to the various parameters utilized in the experimental protocols outlined below. They constitute the framework upon which the interpretations of the results of the subsequent experiments are based.

1. The Plaque Forming Ability of the Different Rabbit Lymphoid Tissues and the Optimal Conditions for the Formation of Hemolytic Plaques in Vitro - Rabbits were injected intravenously with either 10^6, 10^7, 10^8, or 10^9 SRBC and sacrificed 7 days later. Cell suspensions of the various lymphoid organs were prepared and were analyzed for plaque-forming capacity with respect

to SRBC plated in constant or varying concentrations with respect to lymphoid cells.

As can be seen in Table 1, only the spleen cells of immunized rabbits were capable of forming hemolytic plaques with SRBC. The maximum immune reactivity of the spleen was attained between days 6 and 8 following immunization. Very few plaques could be detected before day 3 and after day 12.

As can be seen in Table 2, the administration of 10^9 SRBC resulted in the production of the maximum number of plaques by the spleen cells on the seventh postimmunization day. The maximum number of plaques was observed when 2×10^6 to 8×10^6 splenic cells were plated. Greater numbers of splenic cells (24×10^6 or 32×10^6) tended to exert an inhibitory effect on the system resulting in an almost complete inhibition of plaque formation (Table 3).

On the basis of these initial experiments, it can be seen that the optimal number of hemolytic plaques would be obtained by plating 2×10^6 to 8×10^6 spleen cells of rabbits given 10^9 RBC 6 to 8 days prior to sacrifice.

2. Cross-Reactivity between Antigens used in the Various Experiments

a) Cross Reactivity between Sheep Red Blood Cells (SRBC) and Horse Red Blood Cells (HRBC) - Normal rabbits were injected intravenously with either SRBC and/or HRBC (10^9 cells) and bled at intervals of time. The serum samples were tested for their agglutinin titers to these two red cell preparations. Several rabbits were sacrificed on day 7 and their spleen cells were examined for their plaque-forming ability and for their capacity to incorporate tritiated thymidine in the presence of the specific antigen(s) in vitro.

As can be seen in Table 4, rabbits immunized with SRBC formed antibodies essentially only to SRBC while rabbits immunized with HRBC formed antibodies almost entirely directed to HRBC. The plaque-forming capacity of the spleen cells of rabbits immunized with SRBC produced plaques to SRBC only while spleen cells of rabbits immunized with HRBC produced plaques directed to HRBC

only (Table 4). Similarly, spleen cells of rabbits immunized with SRBC incorporated radioactive thymidine only when incubated with SRBC stroma in vitro, whereas spleen cells of rabbits immunized with HRBC incorporated tritiated thymidine only when incubated with HRBC stroma (Table 5).

b) Cross-Reactivity between Human Serum Albumin (HSA) and Bovine Gamma Globulin (BGG) - As can be seen from Table 6, there was almost no cross-reactivity detected between HSA and BGG antigen-antibody systems using the passive hemagglutination technique.

From the above, it can be seen that the extent of cross-reactivity between the two types of red cells is less than 0.1 percent. A similar situation exists between HSA and BGG.

TABLE 1

PLAQUE FORMING CAPACITY OF DIFFERENT LYMPHOID ORGANS OF RABBITS IMMUNIZED WITH SHEEP RED BLOOD CELLS (10^9 CELLS)

Time after Antigen Administration (Days)	Cells of Lymphoid Organ Plated (No. of Plaques per 10^6 Lymphoid Cells)					
	Spleen	Thymus	Bone Marrow	Lymph Node	Sacculus Rotundus	Appendix
3	5	<1	<1	1	3	1
5	15	<1	<1	2	1	1
6	54	2	<1	2	2	2
7	73	<1	2	7	2	1
8	66	<1	1	<1	1	1
10	31	<1	2	<1	1	<1
12	8	<1	<1	1	4	<1

TABLE 2

THE PLAQUE-FORMING CAPACITY OF SPLENIC LYMPHOID CELLS OF RABBITS IMMUNIZED WITH VARYING DOSES OF SHEEP RED CELLS SEVEN DAYS PRIOR TO SACRIFICE

No. of Sheep Red Cells Administered	No. of Plaques per 10^6 Spleen Lymphoid Cells Plated (Day 7)
10^9	71
10^8	47
10^7	5
10^6	1

TABLE 3

RELATIONSHIP BETWEEN NUMBER OF SPLENIC LYMPHOID CELLS PLATED AND NUMBER OF PLAQUES OBTAINED FROM SPLEEN OF SAME DONOR

No. of Splenic Lymphoid Cells Plated ($\times 10^6$)	No. of Plaques per 10^6 Splenic Lymphoid Cells*
32	1
24	7
16	28
8	62
6	67
4	69
2	69

* Spleen Cells obtained from rabbit immunized 7 days earlier with 10^9 S-rbc intravenously

TABLE 4

CROSS REACTIVITY BETWEEN HORSE AND SHEEP RED BLOOD CELLS.

IN VIVO RESPONSE IN NORMAL RABBITS

Cells Used for Immunization (1×10^9)	Hemagglutination Titer on Day				No. of Plaques per 10^6 Splenic Lymphoid Cells on Day 7 Incubated with	
	7		14			
	Anti-H-rbc	Anti-S-rbc	Anti-H-rbc	Anti-S-rbc	H-rbc	S-rbc
S-rbc	10	640	40	25,600	7	71
H-rbc	1,280	0	40,000	80	97	5

TABLE 5

CROSS REACTIVITY BETWEEN HORSE AND SHEEP RED BLOOD CELLS.

IN VITRO RESPONSE OF IMMUNE RABBIT SPLENIC CELLS

Cells Used for Immunization	Specific Incorporation* of Radio-Active Thymidine by the Immune Spleen Cells** in the Presence of	
	H-rbc Stroma	S-rbc Stroma
S-rbc	1.2	11.0
H-rbc	13.0	0.8

* Ratio of radio-active thymidine uptake by spleen cells in presence of antigen to that taken up in the absence of the antigen.

** Normal rabbits were given the red cells i.v. on day 0 and sacrificed on day 7. Spleen cells (4×10^6) were incubated in vitro with either type of red cells for 3 days.

TABLE 6

THE CROSS REACTIVITY BETWEEN HUMAN SERUM ALBUMIN (HSA) AND BOVINE GAMMA GLOBULIN (BGG)

Antisera tested*	Hemagglutination titers** of the antisera following incubation with sheep red cells sensitized with	
	HSA	BGG
Rabbit anti-HSA	16000	40
	1280	0
Rabbit anti-BGG	20	25600
	0	8000

* Sera obtained from rabbits immunized with either 25 mg of HSA or BGG.

** Hemagglutination titers less than 10 are considered to be negative.

B. The Source of the Antigen Reactive Cell in the Normal Rabbit

1. Experimental Procedures - A general outline of the experimental procedures carried out in this investigation is presented in Figure 1. Lymphoid cells were obtained from a normal rabbit by the techniques described in Materials and Methods.

The lymphoid cell suspensions were injected, in varying numbers, into rabbits which had just been exposed to 800r total body irradiation, using a Cobalt-60 source. The rabbits were also injected with the antigen at the time of cell transfer. They were either sacrificed seven days later and the spleen cells were analyzed for their content of hemolytic plaque-forming cells (see Materials and Methods), or they were bled at intervals of time and the circulating antibody titers determined by direct agglutination of the immunizing red cells or by the agglutination of antigen-sensitized sheep red cells (see

Materials and Methods). The results are expressed as the number of plaques per 10^6 splenic lymphoid cells and as the reciprocal of the hemagglutination titer, respectively.

In other experiments, the normal lymphoid cells were injected into 8-week-old rabbits made tolerant to HSA. The recipients were also injected with 25 mg HSA and bled at intervals of time thereafter. The serum samples obtained were analyzed for their content of circulating anti-HSA antibodies, using the passive hemagglutination technique (see Materials and Methods).

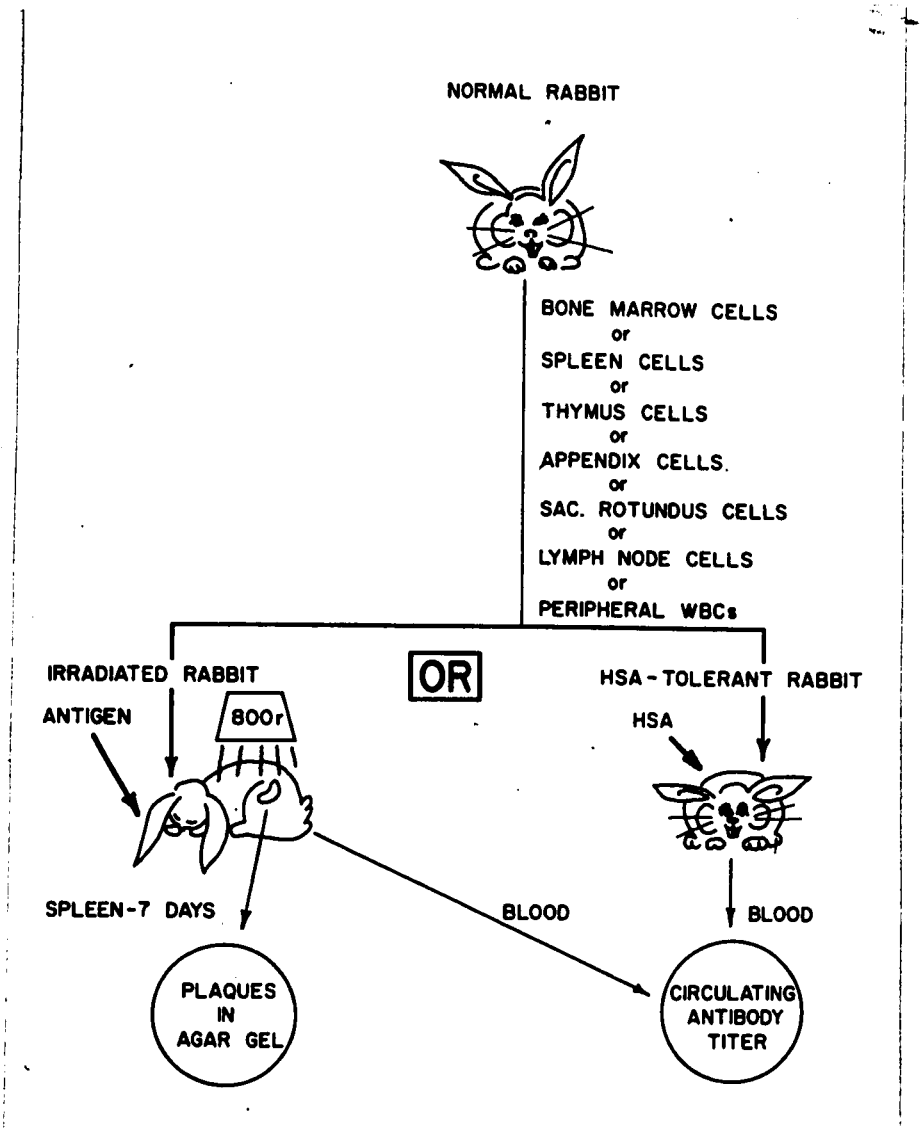

Figure 1. PROTOCOL FOR THE DEMONSTRATION OF THE ORGAN SOURCE OF THE ANTIGEN REACTIVE CELL(S) IN THE RABBIT

2. Results - As can be seen in Table 7, normal bone marrow cells, peripheral blood lymphocytes and a mixture of rabbit sacculus rotundus and appendix cells consistently transferred plaque-forming capacity to irradiated recipient rabbits with respect to SRBC. The lymph node lymphocytes (popliteal plus mesenteric) did so to a much lesser extent. The spleen and thymus could not transfer immunocompetence. However, when the cells from individual donor organs were injected, it was observed that the sacculus rotundus and mesenteric lymph node, and not the appendix and popliteal lymph node could transfer plaque-forming capacity with respect to SRBC (Table 8). When HRBC were used as immunizing antigen, only the bone marrow could transfer immunocompetence; the other organs capable of transferring immunocompetence with respect to SRBC, the sacculus rotundus, blood and mesenteric lymph node, were incapable of doing so with respect to HRBC (Table 9).

Similarly, only the bone marrow cells could transfer humoral antibody-forming capacity with respect to all the

antigens tested (Tables 10 and 11) whereas sacculus rotundus and blood lymphocytes could transfer immunologic reactivity with respect to SRBC only.

Results of a similar nature were recorded in the tolerant recipients. Only recipients of normal allogeneic bone marrow cells could successfully mount an immune response directed to the tolerogenic antigen (Table 12). The tolerant rabbits which have received lymphoid cells obtained from the other lymphoid organs remained tolerant following cell transfer.

TABLE 7

THE PLAQUE-FORMING CAPACITY, WITH RESPECT TO S-RBC, OF SPLEENS OF IRRADIATED RABBITS INJECTED WITH NORMAL ALLOGENEIC LYMPHOID CELLS AND S-RBC

Experiment Number	No. of Plaques per 10^6 Splenic Lymphoid Cells of Irradiated Rabbits* Injected with Lymphoid Cells Obtained from the Following Organs					
	Bone Marrow	Thymus	Spleen	Lymph Node (Popliteal & Mesenteric)	Sacculus Rotundus and Appendix	Blood
1	76	13	7	16	69	29
2	84	11	5	11	52	34
3	59	4	9	18	31	42
4	69	7	12	29	84	51
5	92	18	9	34	79	47
6	61	7	6	32	51	18
7	64	2	13	41	57	23
8	59	6	15	29	90	60
9	60	21	33	40	62	72
10	75	12	4	6	85	100
Mean	70	10	11	26	66	47

* The rabbits were subjected to 800r total body irradiation followed by the intravenous administration of 3-5 x 10^8 lymphoid cells and 10^9 S-RBC. The rabbits were sacrificed 7 days later and their spleens were analysed for plaque-forming capacity.

TABLE 8

THE PLAQUE-FORMING CAPACITY OF SPLEENS OF IRRADIATED RABBITS INJECTED WITH NORMAL ALLOGENEIC LYMPHOID CELLS AND S-RBC

Experiment Number	No. of Plaques per 10^6 Splenic Lymphoid Cells of Irradiated Rabbits* Injected with Lymphoid Cells Obtained from the Following Organs					
	Bone Marrow	Spleen	Popliteal Lymph Node	Mesenteric Lymph Node	Sacculus Rotundus	Appendix
1	82	14	6	30	65	8
2	94	13	7	27	58	8
3	74	9	8	23	59	18
4	96	17	13	39	68	17
Mean	86	13	8	29	62	13

* The rabbits were subjected to 800r total body irradiation followed by the intravenous administration of 3-5 x 10^8 lymphoid cells and 10^9 S-RBC. The rabbits were sacrificed 7 days later and their spleens were analyzed for plaque-forming capacity.

TABLE 9

THE PLAQUE-FORMING CAPACITY OF SPLEENS OF IRRADIATED RABBITS INJECTED WITH NORMAL ALLOGENEIC LYMPHOID CELLS AND S-RBC AND H-RBC

Lymphoid Cells Transferred	No. of Plaques per 10^6 Splenic Lymphoid Cells of the Irradiated Recipient Rabbit* Incubated with	
	S-RBC	H-RBC
Bone Marrow	63**	64
Sacculus Rotundus	54	6
Blood	84	2
Mesenteric Lymph Node	26	3
Popliteal Lymph Node	5	6
Nil	4	1
Nil	80***	58***

* Rabbits were subjected to 800r total body irradiation and then given 3-5 x 10^6 lymphoid cells, 10^9 S-RBC and 10^9 H-RBC intravenously. The rabbits were sacrificed 7 days later for the plaque assay against both S-RBC and H-RBC. Each value represents the mean of duplicate determinations.

** Each value represents the mean of three different experiments.

*** Non-irradiated rabbit, immunized with S-RBC and H-RBC (control).

TABLE 10

HEMAGGLUTINATION TITERS OF IRRADIATED RABBITS INJECTED WITH NORMAL ALLOGENEIC LYMPHOID CELLS, S-RBC, H-RBC AND R-RBC

Day of Bleeding Following Irradiation and Immunization	Hemagglutination Titers of Irradiated Rabbits* Injected with Cells Obtained from the Following Organs								
	Bone Marrow			Sacculus Rotundus			Blood		
	Anti-S-RBC	Anti-H-RBC	Anti-R-RBC	Anti-S-RBC	Anti-H-RBC	Anti-R-RBC	Anti-S-RBC	Anti-H-RBC	Anti-R-RBC
7	80	40	20	160	10	0	320	20	10
14	5,120	2,560	640	2,560	80	10	5,120	160	40

* The rabbits were subjected to 800r total body irradiation followed by the intravenous administration of 3-5 x 10^8 lymphoid cells and 10^9 S-RBC, H-RBC and R-RBC.

TABLE 11

HEMAGGLUTINATION TITERS OF IRRADIATED RABBITS INJECTED WITH NORMAL ALLOGENEIC LYMPHOID CELLS AND HSA AND BGG

Day of Bleeding Following Irradiation and Immunization	Hemagglutination Titers of Irradiated Rabbits* Injected with Cells Obtained from the Following Organs					
	Bone Marrow		Sacculus Rotundus		Blood	
	Anti-HSA	Anti-BGG	Anti-HSA	Anti-BGG	Anti-HSA	Anti-BGG
7	320	640	0	10	0	40
14	5,120	2,560	0	10	10	40
21	5,120	2,560	0	40	10	160

* The rabbits were subjected to 800r total body irradiation followed by the intravenous administration of 3-5 x 10^8 lymphoid cells and 25 mg of HSA and BGG.

TABLE 12

THE ANTIBODY-FORMING CAPACITY OF HSA-TOLERANT RABBITS

INJECTED WITH NORMAL ALLOGENEIC LYMPHOID CELLS AND HSA

Day of Bleeding Following Cell Transfer and Immunization	Hemagglutination Titers* of Tolerant Rabbits** Injected with Normal Lymphoid Cells Obtained From the Following Organs							
	Bone Marrow (4×10^8 Cells)	Thymus (3×10^8 Cells)	Spleen (3×10^8 Cells)	Lymph Node -Popliteal (2.5×10^8 Cells)	Lymph Node -Mesenteric (2.8×10^8 Cells)	Sacculus Rotundus (3.5×10^8 Cells)	Appendix (3.5×10^8 Cells)	Blood (4×10^7 Cells)
7	40	0	0	0	0	10	0	20
14	320	0	0	0	0	10	0	40
21	1,280	20	0	0	10	20	20	40
28	640	20	40	0	0	20	20	40

* The hemagglutination titer is defined as the inverse of the maximum dilution of the antiserum capable of effecting agglutination of HSA-sensitized sheep red blood cells. Titers less than 10 are considered to be negative.

** Rabbits were made tolerant to HSA by the subcutaneous administration of 100 mg HSA on days 2 and 5 of life. They were injected intravenously with the allogeneic lymphoid cells at six weeks of age along with 25 mg HSA.

3. Discussion - These experiments were carried out in order to ascertain the role of the different lymphoid tissues as a source of antigen reactive cells (ARC) in the normal rabbit. The work of Singhal and Richter (46) has demonstrated that only bone marrow lymphocytes of normal rabbits possess the capacity to react in vitro with antigens to which the cell donor had not previously been exposed with blastogenesis and mitosis (46). The present experiments confirm this conclusion using a strictly in vivo system. With four of the five antigens tested, only the bone marrow could transfer immunocompetence to both irradiated and specifically tolerant recipients. In the latter case, the recipients had been made tolerant to HSA and were tested for humoral antibody formation to HSA following cell transfer.

Of all the lymphoid tissues other than the bone marrow, only the sacculus rotundus and mesenteric lymph node cells could restore immunocompetence in an irradiated recipient with respect to only one of the antigens used, the SRBC. In every case where the sacculus and mesenteric node cells could successfully transfer immunocompetence,

the peripheral leukocytes in the blood were also capable of manifesting this activity (Table 8). These results suggest that the bone marrow serves as the prime source of ARC and as the only source with respect to most antigens but that the sacculus rotundus and mesenteric node may also possess ARC in the case of certain antigens. However, another explanation for these findings may be that the cells in the sacculus rotundus, mesenteric node and blood, reactive toward the SRBC, may not be ARC but memory cells or AFC. The SRBC, but not rabbit RBC or rabbit tissues, possess the Forssman antigen, an antigen present in many microorganisms, plants and animal cells (229). It is thus highly probable that the "normal" rabbits used as cell donors may previously have been actively immunized to the Forssman antigen and therefore to an antigen present in the SRBC. It is probable that the gut-associated lymphoid tissue, such as the sacculus rotundus and the mesenteric lymph node, would be continuously exposed to it, and that it would also circulate in the blood. Therefore, the ARC directed toward the Forssman antigen would have vacated the bone marrow and migrated

to these organs where they would react with the Forssman antigen. An immune response to the Forssman antigen would likely ensue in these gut-associated lymphoid tissues with antibody-forming cells and memory cells subsequently detected in these sites. These cells might also spill over into the circulation and would therefore be detected in the blood. Similar findings were also reported by Armstrong et al (230). By using an antigen (Purified protein of Salmonella adelaide polymerized flagellin) to which mice had probably been exposed to previously, they demonstrated that not only bone marrow but also mesenteric lymph nodes and Peyer's patches contain ARC. No attempt was made to study the circulating lymphocytes in their system.

The demonstration that only the bone marrow serves as the original organ source of the ARC serves to point out the functional heterogeneity which exists among lymphocytes in the different lymphoid organs. Furthermore, if, as has been demonstrated by numerous investigators, lymphocytes constantly migrate between the different lymphoid organs (27, 50, 62, 87), they are,

nevertheless, very selective as to their destinations or else there would be a uniformity of function among the lymphocytes of all the lymphoid organs. Certainly, the immunologically-competent ARC does not appear to be inclined to migrate from the bone marrow. However, the bone marrow is an open, and not a closed, system and a small number of cells must therefore vacate the marrow and settle in the peripheral lymphoid organs. It is therefore not surprising that threshold primary immune responses have been induced with normal rabbit lymph node (231, 232) and spleen (233) tissue cultures in vitro, indicating the presence of only a small number of ARC, and/or possibly only a small number of AFC, as well.

It might have been anticipated that ARC would be found in organs other than the bone marrow, albeit in lesser numbers, since one would not expect the cells to originate and die in the same organ. If such a state of affairs exists, it may lead to destruction of the entire clone(s) of ARC, if the balance between proliferation and transformation into ARC, on the one hand, and death of old

ARC, on the other, does not operate on the feed-back principle. If a tendency such as this develops in the neonatal state, with destruction of clones of ARC, the AFC, the cells which synthesize the immunoglobulins, would no longer be stimulated and a state of congenital hypogammaglobulinemia or agammaglobulinemia such as is characterized in the Bruton type agammaglobulinemia, may ensue. On the other hand, excessive proliferation of clones of ARC may lead to overstimulation of the AFC, with abnormal formation of immunoglobulins which may present clinically as multiple myeloma or macroglobulinemia. It may be, however, that the ARC are not killed in the bone marrow but that, when they reach senility, they migrate out of the bone marrow and die elsewhere. At this point in their life-cycle, they may no longer function as ARC and would therefore not be detected by the cell transfer technique utilized.

C. The Antigen Reactive Cell in the Immune Response

1. Experimental Procedures and Results - The experimental protocol is shown in Figure 2. Essentially bone marrow cells were transferred to 800r irradiated allogeneic recipients together with SRBC. Seven days later the recipients were sacrificed and their spleens were assayed for hemolytic plaques (see Materials and Methods).

a. Establishement of the Optimal Conditions for the Experiments

1) The Optimal Number of Bone Marrow Cells to be Transferred - As can be seen from Table 13, 5×10^8 normal bone marrow cells was the optimal number required to be transferred in order to confer the maximal plaque-forming capacity to the spleen cells of the irradiated recipients. Threshold activity was obtained following the transfer of 0.5 to 1×10^8 bone marrow cells whereas

a lesser number of bone marrow cells could not confer plaque-forming activity to an irradiated recipient rabbit (Table 13).

2) The State of Viability of the Bone Marrow Cells Transferred - As can be seen in Table 14, spleen cells of irradiated rabbits which had been injected with normal rabbit bone marrow cells gave many plaques, whereas spleen cells of irradiated rabbits which had been injected with either the heat-killed or sonicated preparations of the bone marrow did not give a greater than background number of plaques.

Insofar as the viability of these two latter cell preparations, prior to their administration into irradiated recipients, is concerned, no intact cell could be observed in the sonicate prepared from 0.5×10^9 cells. Using the dye-exclusion test as an indicator of viability, less than five percent of the cells in the heat-killed preparation were viable.

3) Interval Between Antigen Administration into Donor and Transfer of Bone Marrow Cells to Irradiated Recipients - Table 15 summarizes the results obtained with bone marrow cells transferred at different intervals of time following the administration of 1×10^9 SRBC to the donor rabbit. It is apparent that the maximum loss of capacity of the bone marrow to transfer plaque-forming ability is 24-48 hours following immunization with SRBC. However, most of the activity appears to be lost as early as 8 hours following the SRBC injection and does not reappear until 3-5 days following SRBC administration. The reactivity of the bone marrow transferred after day 5 following immunization generally exceeded that of normal bone marrow allografts.

4) Number of SRBC Injected into Donors - As can be seen in Table 16, bone marrow cells of donor rabbits given 10^8, 10^9, or 10^{10} SRBC were incapable of transferring plaque-forming capacity with respect to SRBC to the irradiated, recipient rabbits, whereas this activity was possessed by bone marrow cells obtained from

rabbits injected with 10^6 or 10^7 SRBC 18-24 hours prior to sacrifice.

5) Passive Immunization of Bone Marrow Donors - Several bone marrow donor rabbits were passively immunized with high-titered rabbit anti-SRBC antiserum twenty-four hours prior to sacrifice. The anti-SRBC titers in these donors was 1:320 at time of sacrifice. Recipients of these bone marrow specimens were injected with 10^9 SRBC and were sacrificed seven days later and their spleens were analyzed for plaque-forming capacity. As can be seen from Table 17, passive immunization of normal donor rabbits with homologous anti-SRBC antiserum did not affect the transfer of immunocompetence by their bone marrow cells with respect to SRBC in recipient rabbits.

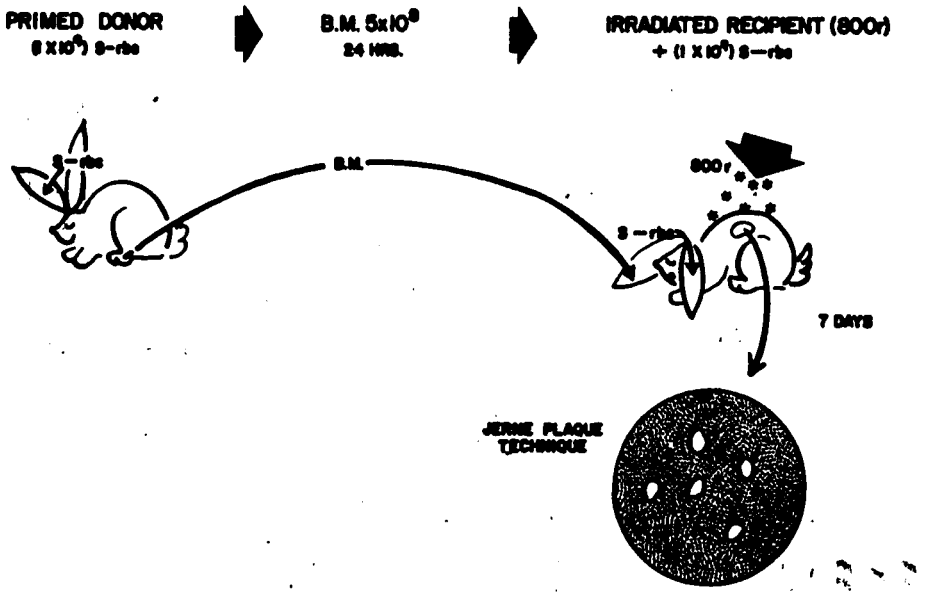

Figure 2. PROTOCOL FOR THE DEMONSTRATION OF THE ROLE OF THE ANTIGEN REACTIVE CELL IN THE IMMUNE RESPONSE

TABLE 13

THE RELATIONSHIP BETWEEN THE CAPACITY OF THE SPLEEN CELL IN AN IRRADIATED RABBIT TO PRODUCE HEMOLYTIC PLAQUES AND THE NUMBER OF NORMAL BONE MARROW CELLS INJECTED ALONG WITH THE ANTIGEN (S-rbc)

No. of Normal Rabbit Bone Marrow Cells Transferred to Irradiated Host* (Plus 10^9 S-rbc)	No. of Hemolytic Plaques per 10^6 Splenic Cells of Recipients on Day 7
5.00×10^8	68
2.00×10^8	30
1.00×10^8	12
0.50×10^8	12
0.25×10^8	1

* Normal rabbits were subjected to 800r total body irradiation and then given allogeneic bone marrow and 1×10^9 S-rbc.

TABLE 14

THE PLAQUE-FORMING CAPACITY OF SPLENIC CELLS OF IRRADIATED RABBITS INJECTED WITH SONICATED OR HEAT KILLED NORMAL ALLOGENEIC BONE MARROW CELLS

Cell Preparation Transferred to Irradiated Recipient (Equivalent to 0.5×10^9 Mononuclear Cells)	No. of Plaques per 10^6 Splenic Lymphoid Cells of Irradiated Recipient (Day 7)*
Whole, Normal Bone Marrow	54**
Sonicated, Normal Bone Marrow	1
Heat-Killed Normal Bone Marrow	4

* Recipients were subjected to 800 r total body irradiation prior to the intravenous administration of the bone marrow and the sheep erythrocytes (10^9 cells).

** Each value represents the mean of duplicate determinations; the values did not normally vary by more than ± 10 percent from the mean.

TABLE 15

RELATION BETWEEN TIME OF ANTIGEN ADMINISTRATION (PRIMING) AND THE CAPACITY OF PRIMED BONE MARROW CELLS TO CONFER PLAQUE-FORMING ABILITY TO THE SPLEENS OF IRRADIATED RECIPIENT RABBITS SEVEN DAYS LATER

Interval between Antigen Administration* into Donor and Transfer of Bone Marrow Cells to Irradiated Recipient	No. of Plaques per 10^6 Splenic Lymphoid Cells of Irradiated Recipient** (Day 7)
Two Hours	69***
Four Hours	71
Eight Hours	12
1 Day	4
2 Days	5
3 Days	11
5 Days	92
8 Days	81
10 Days	84

* 1×10^9 sheep red cells given intravenously

** Recipients were subjected to 800 r total body irradiation followed by the injection of 0.5×10^9 bone marrow cells and 1×10^9 sheep red cells.

*** Control values are 72 plaques for irradiated recipients given normal bone marrow and sheep rbc and 5 plaques for irradiated recipients given sheep rbc only.

TABLE 16

PLAQUE-FORMING CAPACITY OF SPLENIC LYMPHOID CELLS OF IRRADIATED RABBITS WHICH HAD BEEN INJECTED WITH ALLOGENEIC BONE MARROW FROM DONORS PRIMED WITH DIFFERENT DOSES OF S-RBC

No. of S-rbc Injected into Donors*	No. of Plaques per 10^6 Splenic Lymphoid Cells of Recipient Rabbits** on Day 7
10^{10}	4
10^9	8
10^8	7
10^7	23
10^6	35

* Donors were given S-rbc intravenously 24 hours prior to sacrifice.

** Rabbits were subjected to 800 r total body irradiation followed by the intravenous injection of 5×10^8 bone marrow cells and 1×10^9 S-rbc.

TABLE 17

THE PLAQUE-FORMING CAPACITY OF SPLENIC LYMPHOID CELLS OF IRRADIATED RECIPIENTS OF BONE MARROW CELLS OBTAINED FROM DONOR RABBITS PASSIVELY IMMUNIZED WITH ANTISERUM TO SHEEP RED BLOOD CELLS

Treatment of Bone Marrow Donor	Anti S-rbc Titer of Bone Marrow Donor at Intervals of Time Following Injection of the Antiserum			No. of Plaques per 10^6 Splenic Lymphoid Cells of Recipient Rabbits
	One Hour	4 Hours	24 Hours	
Rabbit Anti-Sheep-rbc*	320	320	160	64
None	-	-	-	67

* Rabbit was given 5 ml. of antiserum (titer 32,000) intravenously at time 0 and sacrificed 24 hours later.

From the above, it appears that 5 x 10^8 bone marrow cells have to be transferred to irradiated recipients in order to get maximum number of plaques in response to the injection of 1 x 10^9 SRBC. It can also be seen that the bone marrow cells obtained from a rabbit injected intravenously with 10^9, and not with anti-SRBC, 24 hours prior to sacrifice had lost the capacity to confer immunocompetence with respect to SRBC when transferred to an irradiated recipient.

This phenomenon of loss of immunocompetence following priming was studied in the following experiments.

In the experiments reported upon below, a primed rabbit is considered to be one injected with the antigen 18-24 hours prior to sacrifice.

b. <u>The Specific Loss of Antibody-Forming Capacity to Sheep Red Blood Cells by Primed Bone Marrow Cells</u> - Normal rabbits were injected intravenously with 10^9 SRBC and sacrificed twenty-four hours later. The bone marrow cell suspensions prepared from these rabbits were injected

into irradiated recipient rabbits along with 10^9 SRBC. The recipient rabbits were then divided into three groups. Group 1 were sacrificed seven days later and their spleen cells were analyzed for plaque-forming capacity. Group 2 were sacrificed at day 7 and the spleen cells were tested for their capacity to undergo blastogenesis and mitosis in the presence of the antigen in vitro. Group 3 were bled at intervals of time and the serum samples were analyzed for their anti-SRBC agglutinin titers by the conventional hemagglutination test.

As can be seen in Table 18, as few as 2×10^8 normal allogeneic bone marrow cells were capable of transferring plaque-forming capacity to irradiated recipients whereas as many as 9×10^8 primed allogeneic bone marrow cells were incapable of inducing responsiveness in spleens of irradiated recipients to any degree above background.

As can be seen in Table 19, the bone marrow cells of rabbits primed with SRBC twenty-four hours prior to sacrifice were unable to confer plaque-forming capacity to

spleens of recipient irradiated rabbits. On the other hand, the spleens of irradiated recipients of normal, allogeneic rabbit bone marrow displayed the same number of plaques as were obtained with spleens of normal rabbits immunized with SRBC and tested seven days later (compare Tables 1 and 19). Irradiated recipients given only SRBC or normal bone marrow did not exhibit plaque-forming capacity (Table 19).

As can be seen in Table 20, spleen cells of rabbits which had received normal allogeneic bone marrow cells were stimulated to undergo mitosis and blastogenesis and to incorporate tritiated thymidine when incubated with SRBC stroma in vitro, with a specific incorporation index of 61. On the other hand, spleen cells of rabbits which had received bone marrow primed to SRBC were incapable of responding in the presence of SRBC stroma (specific incorporation index of 0.9), although in this particular instance they were more stimulated by PHA than were the spleen cells of the rabbits which

had received the normal bone marrow cells, thus attesting to the viability of the cells in vitro (Table 20). Further evidence supporting the inability of primed bone marrow to transfer specific antibody formation to irradiated recipients is presented in Table 21. The recipients of normal bone marrow were able to respond with an apparently normal humoral immune response to SRBC whereas sera of recipients of SRBC-primed bone marrow possessed either no circulating anti-SRBC antibodies or possessed at most exceedingly low titers of antibody, which were delayed in onset.

TABLE 18

RELATION BETWEEN THE NUMBER OF NORMAL OR PRIMED BONE MARROW CELLS INJECTED AND THEIR CAPACITY TO CONFER PLAQUE-FORMING ABILITY TO THE SPLEENS OF IRRADIATED RECIPIENT RABBITS SEVEN DAYS LATER

No. of Bone Marrow Cells Transferred to Irradiated Recipient*	No. of Plaques per 10^6 Splenic Lymphoid Cells of Recipients* Receiving	
	Normal Bone Marrow	Primed Bone Marrow
9×10^8	72	11
5×10^8	71	N.D.**
2×10^8	30	N.D.
1×10^8	12	N.D.
0.5×10^8	12	N.D.

* Recipients were subjected to 800 r followed by the injection of bone marrow cells and 1×10^9 sheep red cells given i.v.

** N.D. = Not Done

TABLE 19

THE PLAQUE-FORMING CAPACITY OF SPLENIC LYMPHOID CELLS OF IRRADIATED RECIPIENTS SEVEN DAYS FOLLOWING THE ADMINISTRATION OF PRIMED (S-rbc) OR NORMAL ALLOGENEIC RABBIT BONE MARROW CELLS

Type of Bone Marrow Transferred (0.5×10^9 Lymphoid Cells)	No. of Sheep Red Cells Injected Into Irradiated Recipient	No. of Plaques per 10^6 Splenic Lymphoid Cells of Irradiated Recipients* (Day 7)
Primed: One Day**	10^9	4
Normal	10^9	72
None	10^9	5
Normal	None	5

* Recipients were subjected to 800 r total body irradiation prior to administration of sheep rbc or bone marrow cells.

** Bone marrow cells obtained from rabbit given 1×10^9 sheep red cells, IV, 24 hours previously.

TABLE 20

THE ANTIGEN-INDUCED IN VITRO INCORPORATION OF TRITIATED THYMIDINE BY SPLEEN CELLS OF IRRADIATED RABBITS GIVEN PRIMED (S-rbc) OR NORMAL ALLOGENEIC RABBIT BONE MARROW CELLS

Material added to Cell Cultures (4×10^5 Cells)	Incorporation of Tritiated Thymidine by Spleen Cells of Irradiated Recipients* of (Counts per Minute)	
	Normal Bone Marrow Cells	"Primed" Bone Marrow Cells**
Nil	51	96
PHA	793	1,119
S-rbc Stroma	3,149	89
Specific Incorporation***	61	0.9

* Recipients were subjected to 800 r total body irradiation and then injected with 0.5×10^9 bone marrow cells and 1×10^9 S-rbc. They were sacrificed 7 days later.

** Donor was primed by the i.v. injection of 1×10^9 S-rbc one day prior to transfer.

*** Ratio of H^3 uptake by spleen cells in the presence of S-rbc stroma to that incorporated in the absence of S-rbc stroma.

TABLE 21

THE HUMORAL IMMUNE RESPONSE TO SHEEP RED BLOOD CELLS OF IRRADIATED RECIPIENTS INJECTED WITH EITHER PRIMED (S-rbc) OR NORMAL ALLOGENEIC BONE MARROW CELLS

Recipient* Bled at Day	Hemagglutination Titers of Recipients Given	
	Normal Bone Marrow	Primed Bone Marrow
0	0	0
7	160	0
11	640	0
14	1,280	20
21	2,560	40
28	640	0
35	160	0
42	160	0
49	80	0
56	0	0

* Recipients were Subjected to 800 r Total Body Irradiation Followed by the Injection of 5×10^8 Bone Marrow Cells (Normal or Primed) and 1×10^9 S-rbc.

c. The Specificity of the Immune Response with Respect to SRBC and HRBC of Spleen Cells of Rabbits Injected with Bone Marrow Cells from Rabbits Primed with Sheep and/or Horse Red Blood Cells - Spleen cells of irradiated recipient rabbits injected with bone marrow cells of donors primed with SRBC or HRBC and immunized with both types of red cells produced plaques with respect to the heterologous antigen only, HRBC or SRBC, respectively (Table 22). Spleen cells of irradiated rabbits which had received bone marrow from donors primed with both SRBC and HRBC were unable to form plaques to either of the red cell preparations. These data were substantiated by in vitro experiments with spleen cells of irradiated recipients. Spleen cells of irradiated rabbits which had received bone marrow primed with respect to either SRBC or HRBC were capable of incorporating tritiated thymidine when incubated only with the heterologous antigen while spleen cells of irradiated rabbits which had been injected with bone marrow of

rabbits primed with both SRBC and HRBC were incapable of incorporating tritiated thymidine when incubated with either of the two red cell preparations in vitro (Table 23).

TABLE 22

THE SPECIFICITY OF THE HUMORAL IMMUNE RESPONSE AND PLAQUE-FORMING CAPACITY WITH RESPECT TO SHEEP AND HORSE RED BLOOD CELLS OF RECIPIENTS INJECTED WITH PRIMED (S-rbc AND/OR H-rbc) ALLOGENEIC BONE MARROW CELLS

Bone Marrow Donor Primed with	Hemagglutination Titer of Recipient* Serum at Time of Sacrifice		No. of Plaques per 10^6 Recipients'* Splenic Lymphoid Cells Incubated With	
	H-rbc	S-rbc	H-rbc	S-rbc
S-rbc	20	0	30	4
H-rbc	0	40	5	61
S-rbc & H-rbc	0	0	3	4
Not Primed	80	40	22	54

* Recipients were subjected to 800 r total body irradiation followed by the injection of 0.5×10^9 bone marrow cells from the specific donor and 1×10^9 S-rbc and H-rbc. All the rabbits were sacrificed on day 7.

TABLE 23

THE SPECIFICITY OF THE IN VITRO RESPONSE TO SHEEP AND HORSE RED BLOOD CELLS OF SPLENIC LYMPHOID CELLS OF IRRADIATED RECIPIENTS INJECTED WITH PRIMED (S-rbc OR H-rbc) ALLOGENEIC BONE MARROW SEVEN DAYS PREVIOUSLY

Bone Marrow Donor Primed with	Specific Incorporation* of Tritiated Thymidine by Recipient** Splenic Lymphoid Cells Incubated in the Presence of	
	H-rbc Stroma	S-rbc Stroma
S-rbc	5.6	0.8
H-rbc	1.2	3.7
S-rbc & H-rbc	1.2	0.6
Not Primed	4.8	3.7

* Ratio of radioactive thymidine uptake by the spleen cells in the presence of the antigen to that taken up in the absence of the antigen.

** Recipients were subjected to 800 r total body irradiation followed by the injection of 0.5×10^9 bone marrow cells from the specific donor and 1×10^9 S-rbc and H-rbc. All rabbits were sacrificed on day 7. Spleen cells (4×10^6) were incubated in vitro with either type of red cell stroma for 3 days. Radioactive thymidine was added to cultures on day 2.

2. Discussion - Using three criteria - the hemolysis in agar (plaque) technique, the humoral immune response and the in vitro blastogenic and mitotic response accompanied by tritiated thymidine incorporation in response to antigenic stimulation - it was unequivocally demonstrated that primed rabbit bone marrow is deficient in cells capable of either initiating or mediating the immune response to the specific antigen in an irradiated recipient. This conclusion is based on the results of approximately 50 experiments, all of which, without exception, support this interpretation. In each case, the bone marrow of rabbits injected with sheep red blood cells 18 to 24 hours prior to the transfer of their bone marrow to irradiated recipients failed to confer plaque-forming capacity with respect to sheep red cells in the recipients although the response to horse red cells, a non-cross-reacting antigen, was intact. The specificity of this loss in immunocompetence was corroborated by the failure of such recipient

rabbits to form humoral antibodies to sheep red cells following antigenic stimulation and by the inability of the recipient spleen cells to undergo blastogenesis and mitosis when incubated with the specific antigen in vitro. This deletion from the bone marrow of cells capable of reacting with sheep red cells was maximum 8-48 hours following the intravenous injection of the red cells (Table 15). Thus, one may conclude that a minimum of 8 hours were required for all the antigen-reactive cells directed to sheep red cells to interact with the antigen and to vacate the bone marrow (recruitment time).

Using an entirely different system in the rat, Ford (234) arrived at a similar conclusion. Irradiated rats were injected with syngeneic thoracic duct lymphocytes either simultaneously with or at varying times after the injection of sheep red cells. The optimal hemolysin response was obtained in the rats given the two cell preparations simultaneously. When the delay in lymphoid cell restoration was 12 hours or more, the latent period

of the hemolysin response with respect to the time of administration of the sheep red cells was prolonged and the entire hemolysin response was markedly diminished. These results implied that recruitment of antigen-reactive cells in the rat occurs only over 1 to 2 days after the injection of the sheep red cells (234), which is a time interval similar to the one found in the rabbit in the present investigation.

It was demonstrated that a definite relationship exists between the number of sheep red cells used for priming the donor and the degree of loss of capacity by the bone marrow to transfer immunocompetence to the specific antigen (Table 16). These results are essentially similar to those presented by Singhal and Richter with respect to various protein antigens (46) and can be explained on the basis of more efficient and/or more rapid depletion from the bone marrow of the specific, pre-committed cells as the number of sheep red cells injected is increased.

It was observed that the loss of capacity to transfer immunocompetence with respect to sheep red blood cells

was most conspicuous 1 to 2 days following the injection of this antigen into the prospective bone marrow donor. However, this ability of the bone marrow to transfer specific immunocompetence reappeared by day four following immunization of the donor (Table 15). This probably reflects the appearance of a circulating pool of maturing or mature immunocompetent cells, which should now probably be classified as antibody-forming cells.

Passive immunization of the bone marrow donor with high-titered antiserum to sheep red cells did not diminish the capacity of the donor bone marrow to transfer immunocompetence with respect to sheep red cells (Table 17). The data do not conflict with those of Wigzell (235), Dixon et al (236), Finkelstein and Uhr (237), Uhr and Moller (200), and Henry and Jerne (238), who showed that passively administered antibodies can inhibit antibody synthesis within the same animal following an antigenic stimulus. In our experiments, only the bone marrow recipients received the antigenic stimulus.

It was also demonstrated that viable bone marrow cells are required in the transfer of antibody-forming capacity to irradiated recipients. Neither sonicates nor heat-killed preparation of normal rabbit bone marrow were capable of transferring antibody-forming activity (Table 14). These findings, therefore, rule out any "adjuvant" effect by transferred cells, be they viable or not, and also rule out the possibility that cell extracts or cell-free preparations could transfer antibody-forming capacity of "information" to be subsequently taken up by host cells in a fashion similar to the totally in vitro system described by Fishman (109) and Adler et al (108).

These data, taken as a whole, strongly imply that the antigen-reactive cell in the rabbit originates in the bone marrow and that it migrates out of the marrow as soon as it interacts with the antigen. Once the antigen-reactive cells have vacated the bone marrow following interaction with the antigen, the bone marrow

becomes unreactive to this antigen and deficient with respect to the precursors of the antigen-reactive cells directed to the particular antigen.

Two questions stand out in light of the results reported in this section:

1. Do the antigen-reactive cells actually leave the bone marrow or do they become inactivated or "tolerant" to the antigen and therefore assume an incidental role in the immune response?

2. Do antibody-forming cells in the irradiated recipient represent transformed forms of the donor bone marrow antigen-reactive cells or do they represent recipient's lymphoid cells that have transformed into antibody-forming cells under the bone marrow influence?

The answers to these two important questions will be presented in Chapters V.D and V.H.

D. Donor Versus Recipient Source of Antibody Forming Cells: The Use of Allotypic Markers

1. Experimental Procedures and Results - The experimental design is seen in Figure 3. Bone marrow cells from normal rabbits with the allotype A_1, A_4 (or A_2, A_4, A_5) were injected intravenously into normal rabbits with the allotype A_2, A_4 (or A_1, A_4), which had just been subjected to 800r total body irradiation. The recipient rabbits were then also injected with 10^9 SRBC intravenously.

Seven days following cell transfer, the recipient rabbits were sacrificed and the spleen cells were analyzed for their capacity to produce direct plaques by the technique of Jerne et al in the presence of antiallotype antiserum directed against donor's or against recipient's cells. Controls were cells incubated with normal rabbit serum or with medium 199 only.

As can be seen in Tables 24 and 25, incubation of spleen cells with SRBC and anti-allotype antiserum

directed to the recipient genotype completely inhibited subsequent plaque formation following the addition of complement. However, incubation of the spleen cells with anti-donor allotype antiserum or normal rabbit serum had no inhibitory effect on the plaque-forming ability of the spleen cells.

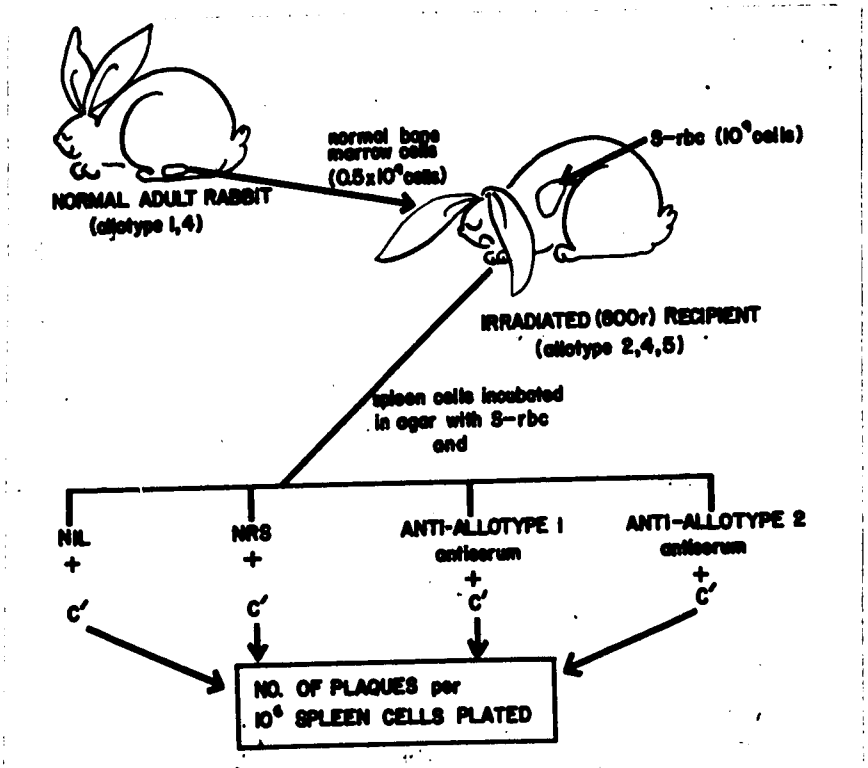

Figure 3. PROTOCOL FOR BONE MARROW CELL TRANSFER EXPERIMENTS USING IRRADIATED RECIPIENTS AND DONOR - RECIPIENT PAIRS OF DIFFERENT ALLOTYPIC GENOTYPES

TABLE 24

THE PLAQUE-FORMING CAPACITY OF SPLENIC CELLS OF IRRADIATED ($A_{2,4,5}$) RABBITS INJECTED WITH NORMAL ALLOGENEIC BONE MARROW CELLS ($A_{1,4}$) AND INCUBATED WITH ANTI-ALLOTYPE ANTISERUM IN VITRO

Cells Incubated In Vitro			No. of Plaques per 10^6 Splenic Mononuclear Cells of Irradiated Recipient (day 7)*
Allotype of Bone Marrow Donor	Allotype of Irradiated Recipient	Anti-Allotype Antiserum Incubated (0.2 ml)	
1, 4	2, 4, 5	Nil	52**
1, 4	2, 4, 5	NRS	45
1, 4	2, 4, 5	Anti-1	62
1, 4	2, 4, 5	Anti-2	6
1, 4	2, 4, 5	Nil	79
1, 4	2, 4, 5	NRS	72
1, 4	2, 4, 5	Anti-1	71
1, 4	2, 4, 5	Anti-2	1

* Recipients were subjected to 800r total body irradiation prior to the intravenous administration of the bone marrow and the sheep erythrocytes (10^9 cells).

** Each value represents the mean of duplicate determinations; the values did not normally vary by more than ±10 percent from the mean.

TABLE 25

THE PLAQUE-FORMING CAPACITY OF SPLENIC CELLS OF IRRADIATED RABBITS (A_1,A_4) INJECTED WITH NORMAL ALLOGENEIC BONE MARROW CELLS (A_1,A_2,A_4) AND INCUBATED WITH ANTI-ALLOTYPE ANTISERUM IN VITRO

Cells Incubated in Vitro		Other Reagents Incubated				No. of Plaques per 10^6 Splenic Mononuclear Cells of Irradiated Recipient (Day 7)*
Allotype of Bone Marrow Donor	Allotype of Irradiated Recipient	Anti-Allotype Anti Serum (0.2 ml)	Normal Rabbit Serum (NRS) 0.2 ml	Human Serum Albumin (HSA) 1 Percent 0.2 ml	Medium 199 (0.4 ml)	
A_1, A_2, A_4	A_1, A_4	Anti-1	—	HSA	—	2**
A_1, A_2, A_4	A_1, A_4	Anti-2	—	HSA	—	79
A_1, A_2, A_4	A_1, A_4	—	NRS	HSA	—	64
A_1, A_2, A_4	A_1, A_4	—	—	—	Med 199	94

* Recipients were subjected to 800r total body irradiation prior to the intravenous administration of the bone marrow and the sheep erythrocytes (10^9 cells).

** Each value represents the mean of duplicate determinations; the values did not normally vary by more than ±10 percent from the mean.

2. Discussion - It has been demonstrated in Chapters V.B. and V.C. that rabbit bone marrow is the major site for the antigen reactive cells and that primed bone marrow is incapable of conferring antibody-forming capacity to irradiated recipients. The interpretation offered is that the antigen-reactive cell, normally a resident in the bone marrow, rapidly vacates the bone marrow following contact with the antigen in vivo. This hypothesis, that normal bone marrow contains the antigen-reactive cells, necessitates the additional assumption that the irradiated recipient still possesses the antibody-forming cells. It was this latter hypothesis that was tested in the current investigation.

It was demonstrated that spleen cells of irradiated rabbits (recipients) which were injected with normal rabbit (donor) bone marrow cells and antigen (SRBC) gave many plaques in vitro. However, if the spleen cells were incubated with antiserum directed to recipient allotype, plaque formation was inhibited whereas incubation of the spleen cells with antiserum directed to donor allotype

had no inhibitory effect on the number of plaques formed. Since identical results were achieved with both the normal and converse situations (donor A_1, A_4 into recipient A_2, A_4, A_5 and donor A_1, A_2, A_4 into recipient A_1, A_4), it may be concluded that the inhibitory effects of the anti-allotype antiserum are specific and that the antibody-forming cell in the irradiated animal is of recipient, and not donor, origin.

It is interesting to note that neither "non-specific" inhibition nor "specific enhancement" of plaque formation by the anti-allotype antisera were observed in this investigation, although the antisera were used in varying dilutions. It has been reported that anti-allotype antiserum may, at certain concentrations, enhance the number of plaques when incubated with the cells to which it is directed (227, 239). However, incubation of the antibody-forming cells with the diluted anti-allotype antiserum generally results in an inhibition of plaque formation (227, 240, 241). It has also been reported that certain antisera possess "non-specific" inhibitory

activity in that they can inhibit plaque formation even if incubated with cells of a different allotype (242). These considerations must be taken into account and adequate controls must be performed in order to ensure correct interpretation of data obtained in experiments using anti-allotype antisera as markers.

Whether the antibody-forming capacity of an irradiated recipient animal which had been injected with homologous bone marrow or lymphoid cells is of donor and/or recipient origin is a question which has intrigued immunologists for several decades. However, the resolution of this problem has been difficult in view of the inability of the investigator to distinguish between the donor and recipient cells on a morphological or functional basis. The recent demonstrations by Oudin (243), Dray and Young (244), Sell (245), Dubiski et al (246), and Chou et al (247) that outbred rabbits can be distinguished from each other on the basis of antigenically-dissimilar immunoglobulin molecules (248) suggested a specific immunologic approach to the problem. In fact, Sell and Gell (152) have already

verified the potential immunological nature of such a system by demonstrating the induction of blastogenesis and mitosis in lymphocyte cultures incubated with specific anti-allotype antiserum. Chou et al (247) transferred various antigen-sensitized lymphoid cells to neonatal recipient rabbits and concluded that the antibody-forming cell in the neonatal host was of recipient origin, and not of donor origin. Antibodies in the circulation of the recipient were purified by the use of specific immunoabsorbents and were found to react only with anti-recipient allotype antiserum in vitro and not with anti-donor allotype antiserum. Nevertheless, immunoglobulins other than antibodies were found to be of donor cell origin. These investigators were unable to satisfactorily explain this dichotomy in their results. Mitchell and Miller (5), using the irradiated mouse as the recipient animal and the hemolytic plaque technique as the means of assay of immunologic activity, arrived at the same conclusion with respect to the recipient origin of the antibody-

forming cell. They observed that the plaques could be inhibited only by incubation of the spleen cells with anti-recipient lymphocyte antiserum and not with antiserum directed to donor lymphocytes. Results of a conflicting nature have been obtained by Harris et al (249, 250). They observed that the immune response to Shigella in the irradiated recipient mouse could be inhibited by prior immunization of the prospective recipient with donor white cells. They have also observed that in vitro plaque formation by spleen cells of one strain of mice could be inhibited by incubation of these cells with antiserum directed to these cells produced in another strain of mice (251). They also transferred rabbit lymph node cells of one allotype, following in vitro incubation with Shigella antigen, into irradiated rabbits of a different allotype (252). The antibodies detected in the circulation of the recipients were demonstrated to be of donor, and not recipient, origin. They therefore concluded that antibody formation is a property of the donor cells transferred

to the irradiated recipient. Our results using the rabbits are consistent with those of Mitchell and Miller (5) and Chou et al (247) in that they unequivocally demonstrate the host origin of the antibody-forming cell. Plaque formation was inhibited when spleen cells of irradiated recipients injected with allogeneic normal bone marrow and sheep erythrocytes were incubated with anti-serum directed to the recipient allotype prior to the addition of complement to the plates. No inhibition was obtained when the spleen cells were incubated with either anti-donor allotype antiserum or normal rabbit serum.

One possible explanation for the lack of correlation between our results, those of Mitchell and Miller (5), and Chou et al (247), on the one hand, and those of Harris et al (252), on the other, may be related to the type of antigen used. Harris et al have used the Shigella as antigen and have assumed that the immune response induced with it is a primary one. However, in all likelihood, the donor as well as the recipient

animals may have come into contact with Shigella antigens prior to the initiation of the experiment. Thus, the rabbit lymph node cells incubated with antigen and transferred to an irradiated recipient are, in fact, antibody-forming cells capable by themselves of initiating a secondary immune response in the irradiated recipient. On the other hand, the immune responses induced by sheep red cells and human serum proteins as antigens can be considered to be of a primary type.

Since the antibody-forming cell was demonstrated to be of recipient origin, one must necessarily assume that it is radio-resistant to 800r and that it is the antigen-reactive cell which is radio-sensitive. This interpretation is supported by the following findings: a) the irradiated rabbits given 800r irradiation, and injected with SRBC only, fail to exhibit an immune response (Table 19); b) the number of plaque-forming cells in the spleen of an irradiated recipient of normal allogeneic bone marrow cells is similar to the number observed in the spleen of a normal immunized rabbit (Tables 1 and 19);

and c) the bone marrow cells of irradiated rabbits lose their capacity to react with antigens in vitro and are incapable of transferring antibody-forming capacity to irradiated recipient allogeneic rabbits (See Table 46). Experiments performed by Harris et al (253) more than a decade ago also support our concept of the cellular events occurring during the primary immune response. They observed that the transfer of cells of the popliteal lymph node of a rabbit injected three days previously with Shigella antigen into normal or X-irradiated recipients resulted in the formation of antibodies. However, if the recipients were irradiated within one hour after receiving the primed lymph node cells, the immune response was markedly suppressed in comparison with that in a non-irradiated control. These results suggest that the immunologically important cell transferred is an irradiation-sensitive antigen-reactive cell, which had migrated out of the bone marrow to the peripheral lymphoid tissues. A scheme depicting the interrelationship of these two cell types in the induction of the primary immune response in the rabbit is presented in Figure 4.

Figure 4. THE POSSIBLE INTERRELATIONSHIP OF THE CELLS MEDIATING THE PRIMARY HUMORAL IMMUNE RESPONSE (A DIAGRAMATIC REPRESENTATION)

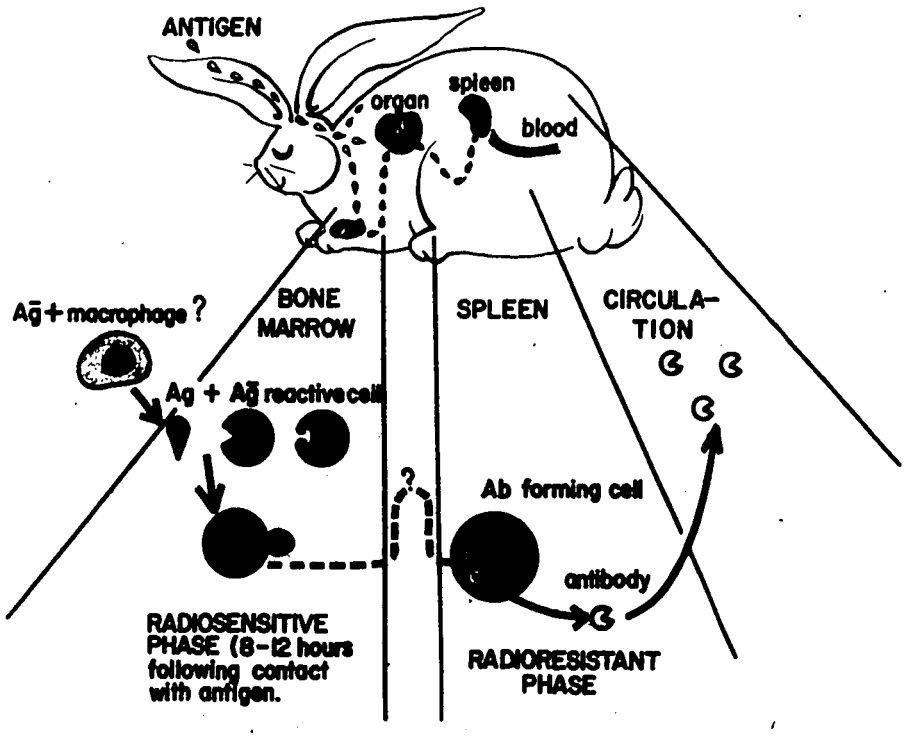

Figure 4. THE POSSIBLE INTERRELATIONSHIP OF THE CELLS MEDIATING THE PRIMARY HUMORAL IMMUNE RESPONSE (A DIAGRAMATIC REPRESENTATION)

E. Identification of the Antigen-Reactive Cell as the Tolerant Cell in the Immunologically-Tolerant Rabbit

1. Experimental Procedures - The protocol followed is presented in Figure 5. Rabbits were made tolerant to HSA or BGG by injecting them subcutaneously at age 2 and 5 days with a total of 200 mg of the antigen. At 10 weeks of age, several rabbits of each litter (2-4 rabbits) were each injected with 10 mg HSA or BGG intravenously. The humoral immune response was determined during the following four weeks using the passive hemagglutination technique (see Materials and Methods). The remaining rabbits of each litter (3-5 rabbits) were injected at ten weeks of age with either normal or primed allogeneic bone marrow. They were also injected with 25 mg HSA or BGG intravenously and the humoral immune response was followed by the passive hemagglutination technique. In other experiments, prospective normal recipient rabbits were subjected to 800r total body irradiation, using a Cobalt-60 source, prior to their receiving the bone marrow cells.

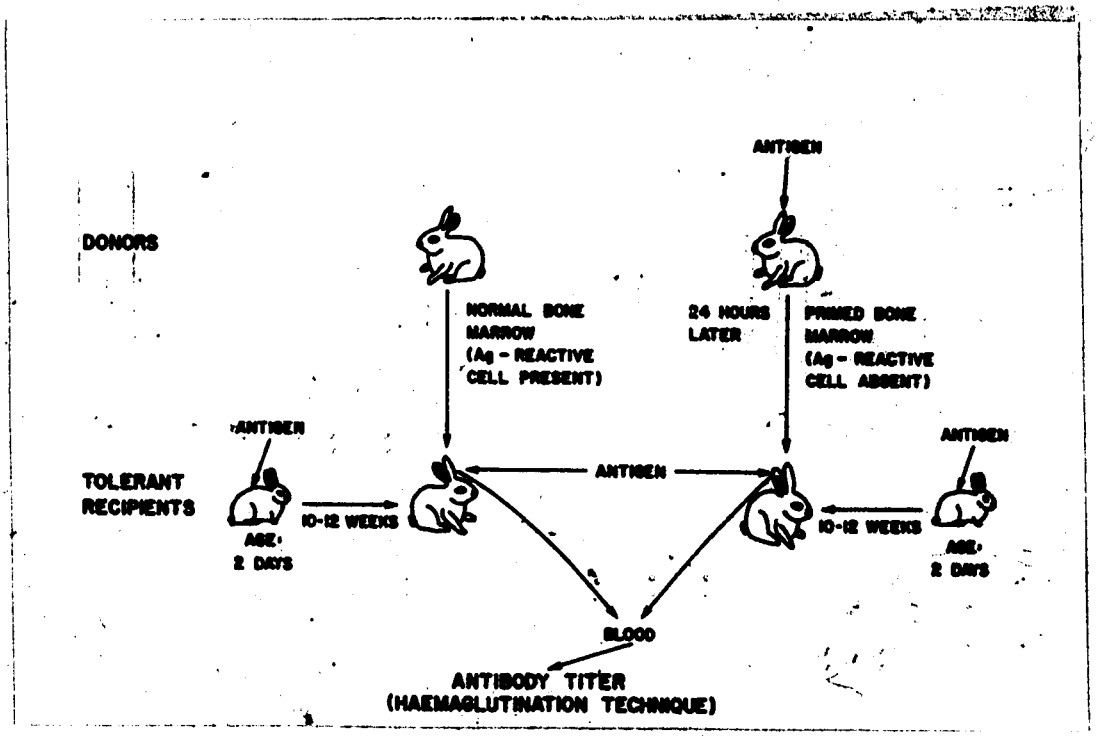

Figure 5. PROTOCOL OF PROCEDURES FOLLOWED FOR THE DEMONSTRATION OF THE ANTIGEN-REACTIVE CELL AS THE TOLERANT CELL IN THE IMMUNOLOGICALLY TOLERANT RABBIT

2. Results

a. The Immune Response in Normal, Irradiated and Immunologically-Tolerant Rabbits to HSA and BGG and The Specificity of the Antisera - This initial series of experiments was carried out in order to establish the non-responsiveness of the irradiated and tolerant rabbits. Normal rabbits responded briskly to immunization with either HSA or BGG, whereas neither irradiated nor immunologically-tolerant rabbits responded over a period of 40 days (Table 26).

b. The Failure of Primed Bone Marrow to Transfer Antibody-Forming Capacity to Tolerant Recipients with Respect to the Priming Antigen - Rabbits made immunologically-tolerant to HSA and given HSA-primed allogeneic bone marrow were incapable of giving an immune response following immunization with HSA but produced high-titered antisera if given normal allogeneic bone marrow cells and HSA (Table 27). The converse situation was true of rabbits

rendered immunologically-tolerant with respect to BGG and given BGG-primed allogeneic bone marrow. Immunization of these rabbits with BGG failed to elicit an immune response whereas recipients of normal allogeneic bone marrow cells gave good immune responses (Table 28).

The failure of the tolerant recipients of primed bone marrow to mount an immune response is reflected by the presence of free antigen in the circulation, which could be detected for three to four weeks following primary immunization (Tables 27 and 28).

It is interesting to note that the antibodies formed following secondary immunization of tolerant recipients of normal bone marrow, 38 days subsequent to primary immunization, were mercaptoethanol resistant. On the other hand, reimmunization at day 38 of tolerant recipients of primed bone marrow, which did not produce antibodies following initial immunization, now synthesized humoral antibodies which were mercaptoethanol sensitive (Tables 27 and 28).

When the tolerant recipients of primed bone marrow were tested for immunologic responsiveness toward the

specific and a non-cross-reacting antigen, it was observed that no immune response could be obtained with respect to the antigen used to prime the bone marrow donor, although a response to the non-cross-reacting antigen could be regularly obtained. HSA or BGG-tolerant rabbits given normal allogeneic bone marrow responded well to immunization with HSA or BGG (Tables 29 and 30). However, the HSA-tolerant recipient of HSA-primed bone marrow failed to respond to stimulation with HSA but responded well to BGG (Table 29), and the BGG-tolerant recipient of BGG-primed bone marrow cells failed to respond to stimulation with BGG but responded well to HSA (Table 30).

It should be pointed out that only the antibodies formed in tolerant recipients given specifically-primed bone marrow and antigen and re-immunized 38 days following primary immunization were mercaptoethanol sensitive (Tables 29 and 30). The antibodies formed following secondary immunization in tolerant recipients of normal allogeneic bone marrow or in recipients of primed bone marrow immunized with the non-cross-reacting antigen were all mercaptoethanol resistant (Tables 29 and 30).

Rabbits made tolerant to HSA and given either normal or BGG-primed bone marrow cells responded with antibody formation following immunization with either HSA or BGG (Table 31). Similarly, good immune responses to both antigens were elicited in BGG-tolerant rabbits given either normal or HSA-primed bone marrow cells (Table 32). In both cases, brisk secondary immune responses were obtained following re-immunization of the rabbits 38 days following primary immunization (Tables 31 and 32).

c. The Immune Response to HSA of Irradiated Rabbits Given Bone Marrow Cells from Either HSA-Primed or HSA-Tolerant Rabbits - Irradiated rabbits given HSA-primed or HSA-tolerant bone marrow failed to respond upon immunization with HSA whereas irradiated recipients of normal allogeneic bone marrow cells responded well (Table 33).

TABLE 26

THE IMMUNE RESPONSE IN NORMAL, IRRADIATED OR TOLERANT RABBITS FOLLOWING ADMINISTRATION OF HUMAN SERUM ALBUMIN (HSA) OR BOVINE GAMMA GLOBULIN (BGG)

Day of bleeding after intravenous administration of HSA or BGG (25 mg)	Hemagglutination titers* of serum samples obtained following immunization of					
	Normal Rabbits		Irradiated Rabbits**		Tolerant Rabbits***	
	Anti-HSA titers	Anti-BGG titers	Anti-HSA titers	Anti-BGG titers	Anti-HSA titers	Anti-BGG titers
0	0	0	0	0	0	0
7	20	0	0	0	0	0
14	2560	5120	0	0	0	0
21	1280	10,240	10	10	0	0
28	640	2560	20	0	0	0
40	160	1280	0	0	0	0

* The sera in varying dilutions were incubated with HSA-sensitized or BGG-sensitized sheep red blood cells. The titer represents the maximum dilution of the antiserum capable of effecting agglutination of the antigen-sensitized red cells. Titers less than 10 are considered to be negative.

** The rabbits were subjected to 800 r total body irradiation prior to the intravenous administration of HSA or BGG.

*** The rabbits were given 100 mg of HSA or BGG on days 1 and 3 of life. They were immunized with HSA or BGG during the sixth week of life.

TABLE 27

THE IMMUNE RESPONSE OF HSA-TOLERANT RABBITS GIVEN HSA-PRIMED OR NORMAL ALLOGENEIC BONE MARROW AND IMMUNIZED WITH HSA

Day of bleeding following bone marrow transfer and intravenous administration of HSA	Antibody and antigen levels in sera of HSA-tolerant rabbits* given bone marrow from			
	Normal donors		HSA-primed donors**	
	Anti-HSA titers***	Free HSA**** (ug/ml)	Anti-HSA titers***	Free HSA**** (ug/ml)
-3	0	0	0	0
5	10	ND+	0	0.2
8	320	ND	0	0.02
14	1280	ND	0	0.01
21	1280	ND	0	0.002
28	320	ND	0	0.0003
35	80	ND	0	0
38++				
42	1280	ND	0	0.01
49	2560 (2560)x	ND	40(0)x	ND
56	640	ND	20	ND

* Rabbits were given 100 mg HSA on days 1 and 3 of life. They were given either normal or primed allogeneic bone marrow at six weeks of age.

** Adult rabbits received 25 mg HSA intravenously 24 hours before sacrifice.

*** The antisera were incubated with HSA-sensitized sheep red cells. Titers below 10 are considered to be negative.

\+ Not done.

++ All rabbits received 10 mg HSA intravenously.

x Titers in brackets after treatment of serum with 0.1 molar 2-Mercaptoethanol.

**** Determined by the ability of the serum to specifically inhibit the agglutination of HSA-sensitized sheep red cells by specific antiserum.

TABLE 28

THE IMMUNE RESPONSE OF BGG-TOLERANT RABBITS GIVEN BGG-PRIMED OR NORMAL ALLOGENEIC BONE MARROW AND IMMUNIZED WITH BGG

Day of bleeding following bone marrow transfer and intravenous administration of BGG	Antibody and antigen levels in sera of BGG-tolerant rabbits* given bone marrow from			
	Normal donors		BGG-primed donors**	
	Anti-BGG titers***	Free BGG**** (ug/ml)	Anti-BGG titers***	Free BGG**** (ug/ml)
-3	0	0	0	0
5	0	0.002	0	0.1
8	80	ND+	0	0.06
14	4000	ND	0	0.007
21	1280	ND	0	0.0001
28	640	ND	0	0
35	160	ND	0	0
38++				
42	8000		10	ND
49	8000 (4000)x	ND	640(20)x	ND
56	2000	ND	320	ND

* Rabbits were given 100 mg BGG on days 1 and 3 of life. They were given either normal or primed allogeneic bone marrow at six weeks of age.

** Adult rabbits received 25 mg BGG I.v., 24 hours before sacrifice.

*** Titers below 10 are considered negative.

\+ Not done

++ All rabbits received 10 mg BGG intravenously.

x Titers in brackets after treatment of sera with 0.1 molar 2-Mercaptoethanol.

**** Determined by the ability of the serum to specifically inhibit the agglutination of BGG-sensitized sheep red cells by specific antiserum.

TABLE 29

THE IMMUNE RESPONSE OF HSA-TOLERANT RABBITS GIVEN HSA-PRIMED OR NORMAL ALLOGENEIC BONE MARROW AND IMMUNIZED WITH HSA AND BGG. THE SPECIFICITY OF THE IMMUNE RESPONSE

Day of bleeding following bone marrow transfer and intravenous administration of HSA and BGG	Hemagglutination titers of sera of HSA-tolerant rabbits* given bone marrow from			
	Normal donors		HSA-primed donors**	
	Anti-HSA titers***	Anti-BGG titers***	Anti-HSA titers***	Anti-BGG titers***
-3	0	0	0	0
8	160	40	0	10
12	640	2560	0	640
21	1280	1280	0	640
36	40	320	0	160
38+				
42	2560 (1280)++	1600 (1600)++	0	1280 (1280)++
50	1280	4000	40(10)++	320

* Rabbits were given 100 mg HSA on days 1 and 3 of life. They were given either normal or primed allogeneic bone marrow at six weeks of age.

** Adult rabbits received 25 mg HSA intravenously 24 hours before sacrifice.

*** Titers less than 10 are considered to be negative.

\+ All rabbits received 10 mg HSA and 10 mg BGG intravenously.

++ Titers in brackets after treatment of sera with 0.1 molar 2-Mercaptoethanol.

TABLE 30

THE IMMUNE RESPONSE OF BGG-TOLERANT RABBITS GIVEN BGG-PRIMED OR NORMAL ALLOGENEIC BONE MARROW AND IMMUNIZED WITH BGG AND HSA. THE SPECIFICITY OF THE IMMUNE RESPONSE

Day of bleeding following bone marrow transfer and intravenous administration of HSA and BGG	Hemagglutination titers of sera of BGG-tolerant rabbits* given bone marrow from			
	Normal donors		BGG-primed donors**	
	Anti-HSA titers***	Anti-BGG titers***	Anti-HSA titers***	Anti-BGG titers***
-3	0	0	0	0
8	80	40	20	0
12	1280	2000	640	0
21	640	2000	640	0
36	160	512	160	0
38+				
42	8000 (8000)++	4000 (2000)++	2560 (2560)++	0
50	2000	1280	1280	80 (10)++

* Rabbits were given 100 mg BGG on days 1 and 3 of life. Bone marrow transfer was done on the sixth week.

** Adult rabbits received 25 mg BGG intravenously 24 hours before sacrifice.

*** Titers less than 10 are considered to be negative.

\+ All rabbits received 10 mg HSA and 10 mg BGG intravenously.

++ Titer in bracket after treatment of sera with 0.1 molar 2-Mercaptoethanol.

TABLE 31

THE IMMUNE RESPONSE OF HSA-TOLERANT RABBITS GIVEN BGG-PRIMED OR NORMAL ALLOGENEIC BONE MARROW AND IMMUNIZED WITH HSA AND BGG

Day of bleeding following bone marrow transfer and intravenous administration of HSA and BGG	Hemagglutination titers of sera of HSA-tolerant rabbits* given bone marrow from			
	Normal donors		BGG-primed donors**	
	Anti-HSA titers***	Anti-BGG titers***	Anti-HSA titers***	Anti-BGG titers***
-3	0	0	0	0
8	40	320	10	10
12	1280	640	80	40
21	320	80	40	40
36	40	40	40	10
38+				
42	1280	160	80	160
50	320	80	20	40

* Rabbits were given 100 mg HSA on days 1 and 3 of life. They were given normal or BGG-primed allogeneic bone marrow at six weeks of age.

** Adult rabbits received 25 mg BGG intravenously 24 hours before sacrifice.

*** Titers less than 10 are considered to be negative.

\+ All rabbits were given 10 mg HSA and 10 mg BGG intravenously.

TABLE 32

THE IMMUNE RESPONSE OF BGG-TOLERANT RABBITS GIVEN HSA-PRIMED OR NORMAL ALLOGENEIC BONE MARROW AND IMMUNIZED WITH BGG AND HSA

Day of bleeding following bone marrow transfer and intravenous administration of HSA and BGG	Hemagglutination titers of sera of BGG-tolerant rabbits* given bone marrow from:			
	Normal donors		HSA-primed donors**	
	Anti-HSA titers***	Anti-BGG titers***	Anti-HSA titers***	Anti-BGG titers***
-3	0	0	0	0
8	40	80	80	40
12	80	320	160	320
21	40	80	20	40
35	10	80	0	0
38+				
42	2000	640	640	1280
50	1280	80	2560	1280

* Rabbits were given 100 mg BGG on days 1 and 3 of life. They were given normal or HSA-primed allogeneic bone marrow at six weeks of age.

** Adult rabbits received 25 mg HSA intravenously 24 hours before sacrifice.

*** Titers less than 10 are considered to be negative.

\+ All rabbits were given 10 mg HSA and 10 mg BGG intravenously.

TABLE 33

THE IMMUNE RESPONSE OF IRRADIATED RABBITS GIVEN EITHER NORMAL, HSA-PRIMED OR HSA-TOLERANT ALLOGENEIC BONE MARROW AND IMMUNIZED WITH HSA

Day of bleeding following bone marrow transfer and intravenous administration of HSA	Hemagglutination titers* of irradiated recipients** given bone marrow from		
	Normal donors	HSA-primed donors***	HSA-tolerant donors****
7	20	0	0
14	1280	0	0
21	800	0	0
28	320	0	20
42	10	0	0

* The antisera were incubated with HSA-sensitized sheep red cells. Titers less than 10 are considered to be negative.

** Recipients were subjected to 800 r total body irradiation followed by the intravenous administration of 5×10^8 bone marrow cells and 25 mg HSA.

*** Donors were given 25 mg HSA intravenously 24 hours before sacrifice.

**** Bone marrow obtained from six week old rabbits which had been injected with 100 mg HSA on days 1 and 3 of life.

3. Discussion - The data presented in the present investigation strongly indicate that the cell which is unresponsive in the immunologically-tolerant rabbit is the antigen-reactive cell and not the antibody-forming cell. This conclusion is based on the finding that, in the tolerant rabbit, antibody formation toward the tolerogenic antigen could be elicited if the recipients were given normal allogeneic bone marrow. This reconstitutive effect of the bone marrow, in an immunologic sense, was found to be specific since tolerant recipients of bone marrow obtained from donors primed with the tolerogenic antigen failed to form antibodies to this antigen but responded well following stimulation with a non-cross-reactive antigen. The specificity of the response in the tolerant recipient was further demonstrated by the fact that recipients made tolerant to one antigen (i.e. HSA) and given allogeneic bone marrow cells from a donor primed with a different antigen (i.e. BGG) responded with antibody formation when immunized with either of these two antigens. The interpretation of these latter findings

is that the antigen-reactive cells directed to HSA, to which the tolerant recipient was made unresponsive, were present in the BGG-primed bone marrow transferred and therefore the tolerant recipient could successfully mount an immune response to HSA. Furthermore, the tolerant recipients of normal allogeneic bone marrow, following re-immunization 38 days after primary immunization, possessed circulating antibodies which could not be inactivated by mercaptoethanol, thus indicating that these antibodies were of a "secondary" or 7S variety and not of a "primary" or 19S type. On the other hand, the tolerant rabbits given primed bone marrow and which did not respond following initial administration of the antigen, produced circulating antibodies following secondary immunization 38 days later which were all mercaptoethanol-sensitive and therefore can be classified as "primary" or 19S type antibodies. Thus, one may conclude that these latter tolerant recipients were indeed tolerant following administration of primed bone marrow and antigen and that it was not simply a matter of anti-

body having been synthesized but which could not be detected by the techniques utilized.

Several investigators have shown that the thymus is the source of ARC in the mouse (see Chapter III,B-2). That the ARC is the tolerant cell in the immunologically tolerant mouse is shown by several studies (79, 80, 212, 230, 254). In all these studies the authors speculated that the thymus or bone marrow cells were tolerant to the specific antigens prior to their transfer to the immunoincompetent recipient animals. However, a different interpretation must be considered. As will be seen in Chapters V,F and V,H, rabbit bone marrow ARC are not made tolerant following incubation, either in glass bead columns or in suspension, with relatively high concentrations of antigen, since these cells could then passively transfer immunocompetence to irradiated hosts with respect to the antigen(s) incubated. Thus, it is likely that the donor thymus or bone marrow cells of the above-mentioned studies had been depleted of antigen-reactive cells, rather than made tolerant, following contact with antigen

in vivo, in much the same manner as the rabbit bone marrow is depleted of ARC following the administration of the antigen.

The present results, demonstrating that normal but not primed bone marrow could facilitate an immunologic response of normal proportions in otherwise tolerant recipients, allow one to conclude that the cell which is immunologically unresponsive in the tolerant recipient is the antigen-reactive cell, which arises from cells normally residing in the bone marrow. The results also support the conclusion arrived at in Chapter V.D, based on investigations utilizing anti-allotype antisera to inhibit the formation of hemolytic plaques, that the bone marrow contains only antigen-reactive cells and is devoid of antibody-forming cells.

F. The Isolation of Specific Clones of Bone Marrow Antigen-Reactive Cells by Means of Antigen-Sensitized Glass Bead Columns

1. Experimental Procedures - The protocol of the experimental procedures is diagramatically presented in Figure 6. Normal rabbit bone marrow cells were obtained as described in Materials and Methods and 300 to 500 x 10^6 cells in 3 to 5 ml of medium 199 were then applied to the head of the glass bead column and passed through the column with Med-199 as eluting fluid. When cells could no longer be recovered in the effluent, the glass beads were placed in a sterile 250 ml flask and vigorously shaken for 5 minutes. The cells in the supernatant which were eluted from the antigen-sensitized glass beads (eluate) were centrifuged and washed with Med-199. The cells in both the effluent and eluate were suspended in Med-199 and injected into allogeneic adult rabbits which had just been subjected to 800r total body irradiation, using a Cobalt-60 source. Some rabbits were injected with either the effluent or eluate fraction, others were

injected with a mixture of effluent and eluate cells and the remaining animals were injected with varying numbers of the unfractionated bone marrow cell suspension (Figure 6). All the rabbits were injected intravenously with 0.5 to 1 x 10^9 SRBC or HRBC. Since only a small number of eluate cells were injected into the irradiated recipients, a subthreshold dose of the original unfractionated bone marrow cell suspension was also administered. All animals were sacrificed seven days later and their spleen cells were analyzed for their direct plaque-forming capacity by the hemolysis-in-gel technique.

Other irradiated rabbits were given bone marrow cells fractionated on the HSA-sensitized glass bead column (Figure 6) and 25 mg HSA intravenously. The rabbits were then bled at intervals of time and the sera were tested for their antibody content by the passive hemagglutination technique. Hemagglutinin titers under 10 are considered to be negative in all of the tables presented.

Smears of the unfractionated bone marrow, effluent cells and eluate cells were prepared and the viability of the various cell fractions was determined by the dye exclusion test.

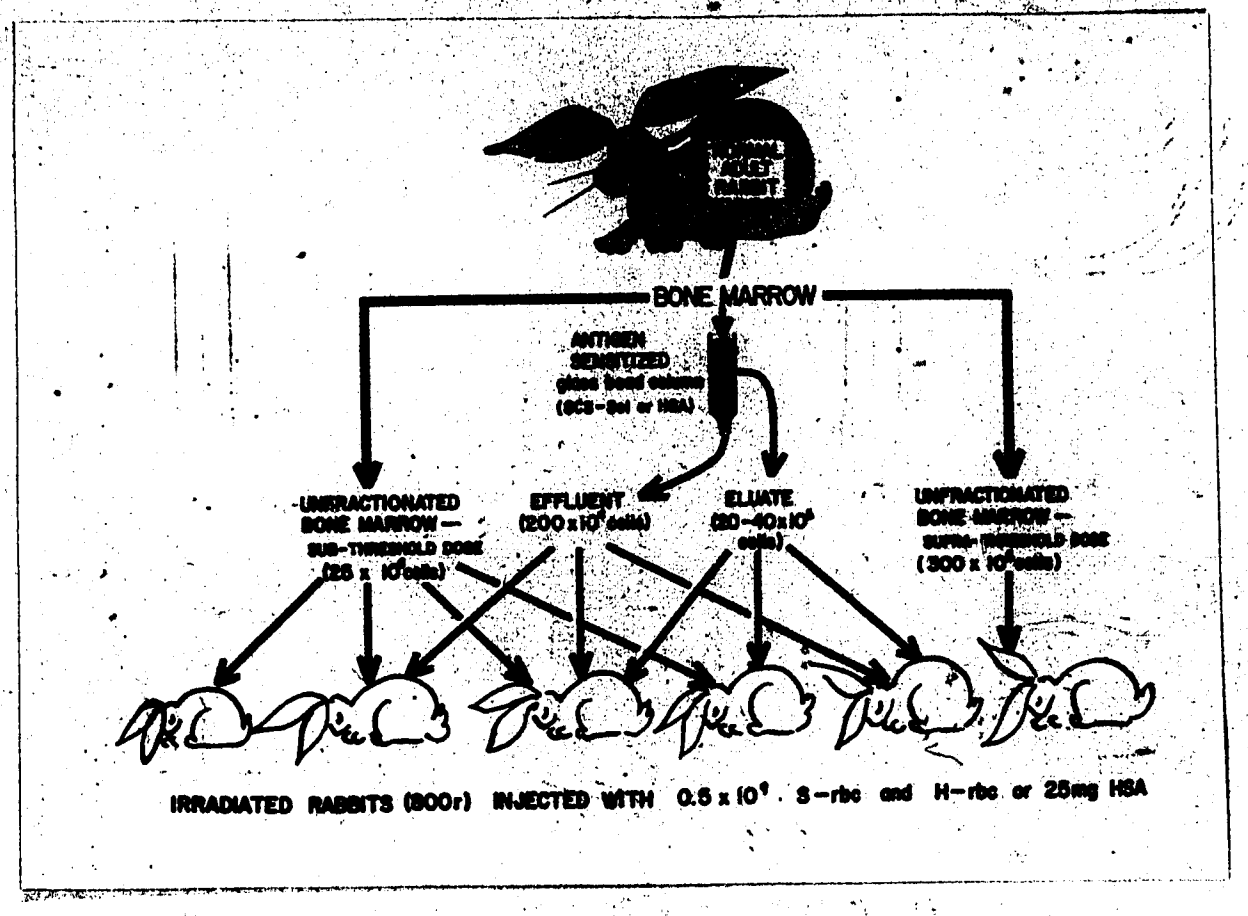

Figure 6. PROTOCOL FOR THE DEMONSTRATION OF THE SPECIFIC INTERACTION OF ANTIGEN WITH THE ANTIGEN-REACTIVE CELL IN VITRO

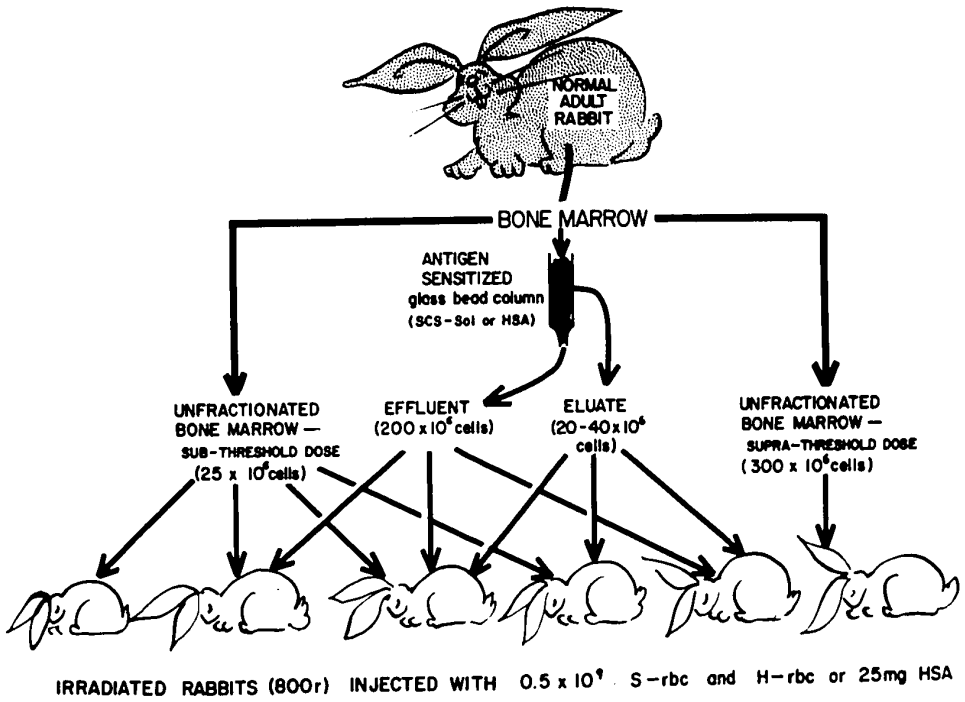

Figure 6. PROTOCOL FOR THE DEMONSTRATION OF THE SPECIFIC INTERACTION OF ANTIGEN WITH THE ANTIGEN-REACTIVE CELL IN VITRO

2. Results

a. Establishment of Optimal Conditions for the Fractionation of Normal Bone Marrow Cells on Glass Bead Columns and Transfer of Immunocompetence with Bone Marrow Cells. - As can be seen in Table 34, the total number of cells recovered following passage of normal bone marrow cells through either an antigen-sensitized or an unsensitized glass bead column was in the range of 40 to 55 percent of the original cell preparation. This range of recovery, which was obtained in a total of 20 experiments, represents the number of cells recovered in both the effluent and eluate. The vast majority of the cells recovered constituted the effluent fraction. The cells which were subsequently eluted from the glass bead column, by shaking the beads in Med-199 for 5 minutes, constituted only 1 to 10 percent of the original cell population, with a mean value of 3 to 5 percent. It should be noted that cells could be eluted from glass beads which were not originally sensitized with antigen (Table 34).

Insofar as the viability of the fractionated cells is concerned, the effluent cells were as viable as the original unfractionated bone marrow cell suspension (Table 35). The percentage of dead cells in the eluate fraction was, however, somewhat higher, probably reflecting the damage incurred while vigorously shaking the glass beads during the elution procedure (Table 35).

In order to establish baseline values for the following experiments, the minimum number of normal bone marrow cells required to be transferred to an irradiated host in order to confer plaque-forming capacity to the spleen cells of the recipient was determined. The administration of 5×10^8 cells has been found (Table 13) to confer maximum activity. Threshold activity was obtained following the transfer of 0.5 to 1.0×10^8 bone marrow cells whereas a lesser number of bone marrow cells could not confer plaque-forming activity to an irradiated recipient rabbit (Table 13).

b. Isolation of Antigen-Reactive Cells Directed to HSA by Passage of Cells Through an HSA-Sensitized Glass Bead Column. Passive Transfer of Specific Immunocompetence with these Cells. - Normal rabbit bone marrow cells were fractionated by passage through an unsensitized glass bead column, yielding effluent and eluate fractions. The latter cells were injected into irradiated recipient rabbits which were also immunized with 25 mg HSA. Only recipients of effluent cells formed circulating antibodies to HSA. At no time were antibodies found in the circulation of recipients of eluate cells (Table 36).

When the cells were fractionated using an HSA-sensitized glass bead column, results of an opposite nature were obtained. In this case, only irradiated recipients of eluate cells responded with an immune response to HSA whereas irradiated rabbits injected with effluent cells or effluent cells and a subthreshold dose of unfractionated bone marrow failed to respond upon immunization with HSA (Table 37). These experiments were repeated in an

identical fashion four times with essentially identical results.

The effect of the presence of free or excess antigen in the glass bead column on the subsequent fractionation of the bone marrow cells is presented in Table 38. In this case, all the immunocompetent activity, with respect to HSA, was localized to the effluent fractions. Recipients of eluate fractions did not respond to immunization with HSA.

c. Transfer of Immunocompetence with Respect to Red Cells to Irradiated Rabbits with Fractions of Bone Marrow Obtained by Passage of Bone Marrow Cells Through Red Cell-Sensitized Glass-Bead Columns - Initial experiments were aimed at demonstrating the lack of affinity of the antigen-reactive cells for unsensitized glass beads. Normal rabbit bone marrow cells were passed through an unsensitized glass bead column and the effluent and eluate cell fractions obtained were administered to irradiated recipients along with the red cells. Only

the spleen cells of recipients given either unfractionated bone marrow or the effluent cells of bone marrow were capable of producing plaques in vitro (Table 39). None of the recipients of the eluate fraction possessed this activity. Similarly, passage of cells through an HSA-sensitized glass bead column resulted in the localization of all the antigen-reactive cells directed to sheep red cells to the effluent fraction. Here as well, the eluate fraction could not transfer immunocompetence directed toward the sheep red cell (Table 40).

When the bone marrow cells were fractionated using a glass bead column sensitized with the solubilized sheep red cell stroma preparations (SCS), all the antigen-reactive cells directed to sheep red cells were retained in the column, since the effluent cells possessed no capacity to transfer plaque-forming ability to sheep red cells in irradiated recipients (Table 41). The cells eluted from the SCS-sensitized glass bead column possessed all the antigen-reactive cells directed toward sheep red cells, since they could confer considerable plaque-forming capacity to the spleens of irradiated recipients (Table 41).

The antigenic specificity of the cells retained by the antigen-sensitized glass bead columns was further established by fractionating bone marrow cells on two columns sensitized with soluble stromal preparations of two non-cross-reactive red cells species - sheep (SCS) and horse (HCS) erythrocytes. In each case (Tables 42 and 43), the sensitized glass bead column retained the antigen-reactive cells directed only to the antigen used to sensitize the column.

The passage of normal bone marrow cells through an HCS-sensitized column resulted in the retention on the column of horse, and not sheep, red cell-reactive cells (Table 44). However, passage of the effluent (sheep red cell sensitive) cells, recovered from the HCS column, through an SCS-sensitized column, resulted in the retention of sheep red cell-reactive cells by the glass bead column (Table 44).

In order to ascertain whether the capacity of the bone marrow antigen-reactive cells to react with antigen on the glass beads is dependent on the presence of other

"non-specific" cell types (i.e. macrophages), which are themselves removed by the glass beads, normal bone marrow cells were passed through an unsensitized glass bead column followed by passage of the recovered effluent cells through an SCS-sensitized column (Table 45). The unsensitized column did not retain any cells capable of transferring specific plaque-forming capacity; on the other hand, a second passage of the cells through the SCS-sensitized column resulted in the specific retention of cells capable of mediating an immune response to sheep red cells (Table 45).

d. Morphology of Cells Recovered Following Passage of Normal Bone Marrow Cells Through an Antigen-Sensitized Glass Bead Column - Normal bone marrow cell suspensions were passed through antigen-sensitized and non-sensitized glass bead columns and the fractions collected - the effluent and eluate fractions - and the original unfractionated bone marrow, were compared on the basis of their morphological characteristics. The

effluent cells appeared to be morphologically indistinguishable from the unfractionated bone marrow, both qualitatively and quantitatively (Figures 7 and 8). The effluent consisted of all the erythrocyte and leukocyte precursors, the blast cells, macrophage-like cells and mature erythrocytes and leukocytes. The eluate fraction of cells was far more uniform in composition (Figure 9) and consisted of only small mononuclear cells.

TABLE 34

EXTENT OF RECOVERY OF NORMAL RABBIT BONE MARROW CELLS FRACTIONATED BY PASSAGE THROUGH AN ANTIGEN-SENSITIZED GLASS BEAD COLUMN

Antigen Used for Sensitizing the Glass Beads	Cells Added to the Column		Cells Obtained in Effluent*		Cells Eluted from The Glass Beads**	
	No. of Cells Added to Column	Percent (Original)	No. of Cells	Percent of Original	No. of Cells	Percent of Original
BSA	5×10^8	100	2.0×10^8	40	7.5×10^6	1.5
S-rbc Stroma (SCS)	5×10^8	100	1.5×10^8	30	45.0×10^6	9.0
None	5×10^8	100	2.5×10^8	50	27.0×10^6	5.4

* Cells which passed through the column.

** Cells retained by the glass beads and recovered by shaking of the beads.

TABLE 35

THE VIABILITY OF THE RABBIT BONE MARROW CELLS RECOVERED FOLLOWING PASSAGE THROUGH AN ANTIGEN-SENSITIZED GLASS BEAD COLUMN

Antigen Used for Coating the Glass Beads	Viability of Cells (Percent Dead)*		
	Unfractionated Bone Marrow	Effluent Fraction**	Eluate Fraction***
BSA	11	11	16
S-rbc Stroma (SCS)	8	9	20
None	12	12	16

* Cells stained with trypan blue.

** Cells which passed through the column.

*** Cells retained by the antigen-sensitized glass beads and eluted by shaking.

TABLE 36

THE IMMUNE RESPONSE TO HSA IN IRRADIATED RABBITS GIVEN FRACTIONS OF NORMAL BONE MARROW PASSED THROUGH AN UNSENSITIZED GLASS BEAD COLUMN

Fraction of Bone Marrow Cells Transferred (No. of Cells)	Hemagglutinin Titers in the Irradiated Recipients* Subsequent to Transfer of the Bone Marrow (Days)				
	8	12	16	25	32
Eluate (2.5×10^6)	0**	0	0	0	0
Effluent (3×10^8)	10	80	160	40	0
Eluate (2.5×10^6) & Effluent (3×10^8)	0	160	160	80	0

* Rabbits were subjected to 800r total body irradiation followed by the intravenous injection of 25 mg HSA and the specified fraction of bone marrow.

** Titers less than 10 are considered to be negative.

TABLE 37

THE IMMUNE RESPONSE TO HSA IN IRRADIATED RABBITS GIVEN DIFFERENT FRACTIONS OF BONE MARROW OBTAINED BY PASSAGE OF CELLS THROUGH AN HSA-SENSITIZED GLASS BEAD COLUMN

Fraction of Bone Marrow Cells Transferred (No. of Cells)	Hemagglutinin Titers in Irradiated Recipients* Subsequent to Transfer of the Bone Marrow (Days)					
	8	12	16	24**	30	36
Unfractionated Bone Marrow (2.5×10^8)	0***	80	80	20	160	40
Effluent (1×10^8) & Unfractionated Bone Marrow (2×10^7)	0	0	0	0	0	40
Eluate (4×10^6) & Unfractionated Bone Marrow (2×10^7)	10	40	80	20	320	160
Effluent (1×10^8)	0	0	0	0	10	40
Eluate (4×10^6)	20	40	40	0	80	160
Unfractionated Bone Marrow (2×10^7)	0	0	0	0	0	0

* Rabbits were subjected to 800r total body irradiation followed by the intravenous injection of 25 mg HSA.

** Each rabbit received 10 mg HSA intravenously to induce a secondary immune response.

*** Titers less than 10 are considered to be negative.

TABLE 38

THE IMMUNE RESPONSE TO HSA IN IRRADIATED RABBITS GIVEN DIFFERENT FRACTIONS OF BONE MARROW OBTAINED BY PASSAGE OF THE CELLS THROUGH AN HSA-SENSITIZED GLASS BEAD COLUMN. EFFECT OF THE PRESENCE OF UNBOUND* HSA IN THE COLUMNS

Fraction(s) of Bone Marrow Cells Transferred (No. of Cells)	Hemagglutinin Titers in the Irradiated Recipients** Subsequent to the Transfer of the Bone Marrow (Days)					
	8	12	16	24***	30	36
Unfractionated Bone Marrow (1.5×10^8)	0+	80	20	0	80	40
Effluent (0.75×10^8) & Unfractionated Bone Marrow (2×10^7)	0	10	20	0	160	40
Eluate (7×10^6) & Unfractionated Bone Marrow (2×10^7)	0	0	0	0	0	10
Effluent (0.75×10^8)	0	20	10	0	80	40
Eluate (2×10^6)	0	0	0	0	0	0
Unfractionated Bone Marrow (2×10^7)	0	0	0	0	0	0

* Last wash of the glass bead column prior to passage of the bone marrow cells contained one microgram HSA per ml of the wash.
** Rabbits were subjected to 800r total body irradiation followed by the intravenous injection of 25 mg HSA.
*** Each rabbit received 10 mg HSA intravenously to induce a secondary immune response.
\+ Titers less than 10 are considered to be negative.

TABLE 39

THE PLAQUE-FORMING CAPACITY OF SPLEEN CELLS OF IRRADIATED RABBITS GIVEN DIFFERENT FRACTIONS OF NORMAL BONE MARROW CELLS OBTAINED BY PASSAGE OF THE CELLS THROUGH AN UNSENSITIZED GLASS BEAD COLUMN

Cell Fraction Transferred to Irradiated Recipient* (No. of Cells)	No. of Plaques per 10^6 Recipient Splenic Lymphoid Cells Incubated with Sheep Red Cells and Complement
Unfractionated Bone Marrow (4×10^8)	68
Combination of Eluate (13×10^6) and Unfractionated Bone Marrow (25×10^6)	<1
Combination of Effluent (10^8) and Unfractionated Bone Marrow (25×10^6)	33
Combination of Eluate (13×10^6), Effluent (10^8) and Unfractionated Bone Marrow (25×10^6)	31

* Rabbits were subjected to 800r total body irradiation followed by the intravenous injection of 10^9 S-rbc.

TABLE 40

THE PLAQUE-FORMING CAPACITY OF SPLEEN CELLS OF IRRADIATED RABBITS GIVEN DIFFERENT FRACTIONS OF NORMAL BONE MARROW CELLS OBTAINED BY PASSAGE OF THE CELLS THROUGH AN HSA-SENSITIZED GLASS BEAD COLUMN

Cell Fraction Transferred to Irradiated Recipient* (No. of Cells)	No. of Plaques per 10^6 Recipient Splenic Lymphoid Cells Incubated with Sheep Red Cells and Complement
Unfractionated Bone Marrow (25×10^6)	1
Combination of Eluate (17.5×10^6) and Unfractionated Bone Marrow (25×10^6)	5
Combination of Effluent Cells (260×10^6) and Unfractionated Bone Marrow (25×10^6)	68
Effluent Cells	45

* Rabbits were subjected to 800r total body irradiation followed by the intravenous injection of 10^9 S-rbc.

TABLE 4I

THE PLAQUE-FORMING CAPACITY OF SPLEEN CELLS OF IRRADIATED RABBITS GIVEN DIFFERENT FRACTIONS OF NORMAL BONE MARROW CELLS OBTAINED BY PASSAGE OF THE CELLS THROUGH A SHEEP RED CELL (SCS)-SENSITIZED GLASS BEAD COLUMN

Cell Fraction Transferred to Irradiated Recipient* (No. of Cells)	No. of Plaques per 10^6 Recipient Splenic Lymphoid Cells Incubated with Sheep Red Cells and Complement
Unfractionated Bone Marrow (5×10^8)	87
Combination of Eluate (22×10^6) and Unfractionated Bone Marrow (25×10^6)	49
Combination of Effluent (75×10^6) and Unfractionated Bone Marrow (25×10^6)	1
Combination of Eluate (22×10^6), Effluent (75×10^6) and Unfractionated Bone Marrow (25×10^6)	53

* Rabbits were subjected to 800r total body irradiation followed by the intravenous injection of 10^9 S-rbc.

TABLE 42

THE PLAQUE-FORMING CAPACITY, WITH RESPECT TO SHEEP AND HORSE RED CELLS, OF SPLEEN CELLS OF IRRADIATED RABBITS GIVEN BONE MARROW CELLS FRACTIONATED ON A SHEEP RED CELL (SCS)-SENSITIZED GLASS BEAD COLUMN

Cell Fraction Injected into Irradiated Recipient* (No. of Cells)	No. of Plaques per 10^6 Recipient Splenic Lymphoid Cells Incubated with	
	H-rbc	S-rbc
Unfractionated Bone Marrow (2.8×10^8 cells)	45	63
Unfractionated Bone Marrow (25×10^6 cells)	6	3
Eluate (10×10^6 cells)	4	37
Combination of Eluate (10×10^6 cells) and Unfractionated Bone Marrow (25×10^6 cells)	2	48
Effluent (1.5×10^8 cells)	40	5
Effluent (10×10^6 cells)	4	3
Combination of Effluent (1.5×10^8 cells) and Unfractionated Bone Marrow (25×10^6 cells)	49	9

* Rabbits were subjected to 800r total body irradiation followed by the intravenous injections of 10^9 S-rbc and 10^9 H-rbc.

TABLE 43

THE PLAQUE-FORMING CAPACITY, WITH RESPECT TO SHEEP AND HORSE RED CELLS, OF SPLEEN CELLS OF IRRADIATED RABBITS GIVEN BONE MARROW CELLS FRACTIONATED ON A HORSE RED CELL (HCS)-SENSITIZED GLASS BEAD COLUMN

Cell Fraction Injected into Irradiated Recipient* (No. of Cells)	No. of Plaques per 10^6 Recipient Splenic Lymphoid Cells Incubated With	
	H-rbc	S-rbc
Unfractionated Bone Marrow (4×10^8 cells)	53	72
Unfractionated Bone Marrow (25×10^6 cells)	4	2
Eluate (24×10^6 cells)	39	8
Combination of Eluate (24×10^6 cells) and Unfractionated Bone Marrow (25×10^6 cells)	47	3
Effluent (10^8 cells)	6	44
Effluent (24×10^6 cells)	3	4
Combination of Effluent (10^8 cells) and Unfractionated Bone Marrow (25×10^6 cells)	7	61

* Rabbits were subjected to 800r total body irradiation followed by the intravenous injections of 10^9 S-rbc and 10^9 H-rbc.

TABLE 44

THE PLAQUE-FORMING CAPACITY, TO SHEEP AND HORSE RED CELLS, OF SPLEEN CELLS OF IRRADIATED RABBITS GIVEN BONE MARROW CELLS FRACTIONATED INITIALLY BY PASSAGE THROUGH A HORSE RED CELL (HCS) SENSITIZED GLASS BEAD COLUMN FOLLOWED BY PASSAGE OF THE CELLS THROUGH A SHEEP RED CELL (SCS) SENSITIZED GLASS BEAD COLUMN

Normal Bone Marrow Cells Fractionated as Follows	Cell Fraction Injected into Irradiated Recipient* (No. of Cells)	No. of Plaques per 10^6 Recipient Splenic Lymphoid Cells Incubated With	
		H-rbc	S-rbc
Unfractionated Cells	Unfractionated Cells (200×10^6)	80	72
HCS Column			
Eluate Cells	Eluate of HCS Column (25×10^6)	69	8
Effluent Cells	Effluent of HCS Column (200×10^6)	9	52
SCS Column			
Eluate Cells	Eluate of SCS Column (20×10^6)	0	39
Effluent Cells	Effluent of SCS Column (120×10^6)	2	5

* Rabbits were subjected to 800r total body irradiation followed by the intravenous injections of 10^9 S-rbc and 10^9 H-rbc.

TABLE 45

THE PLAQUE-FORMING CAPACITY, TO SHEEP RED CELLS, OF SPLEEN CELLS OF IRRADIATED RABBITS GIVEN NORMAL ALLOGENEIC BONE MARROW CELLS FRACTIONATED BY PASSAGE OF THE CELLS THROUGH AN UNSENSITIZED GLASS BEAD COLUMN FOLLOWED BY PASSAGE THROUGH A SHEEP RED CELL (SCS) SENSITIZED GLASS BEAD COLUMN

Normal Bone Marrow Cells Fractionated as Follows	Cell Fraction Injected into Irradiated Recipient* (No. of Cells)	No. of Plaques per 10^6 Recipient Splenic Lymphoid Cells Incubated with S-rbc
Unfractionated Cells	Unfractionated Cells (46×10^6)	52
Unsensitized Column		
↓ Eluate Cells	Eluate Cells (18×10^6)	2
↓ Effluent Cells	Effluent Cells (200×10^6)	39
SCS Column		
↓ Eluate Cells	Eluate Cells (18×10^6)	28
↓ Effluent Cells	Effluent Cells (60×10^6)	3

* Rabbits were subjected to 800r total body irradiation followed by the intravenous injections of 10^8 S-rbc.

Figure 7. UNFRACTIONATED BONE MARROW. GIEMSA STAIN x 640

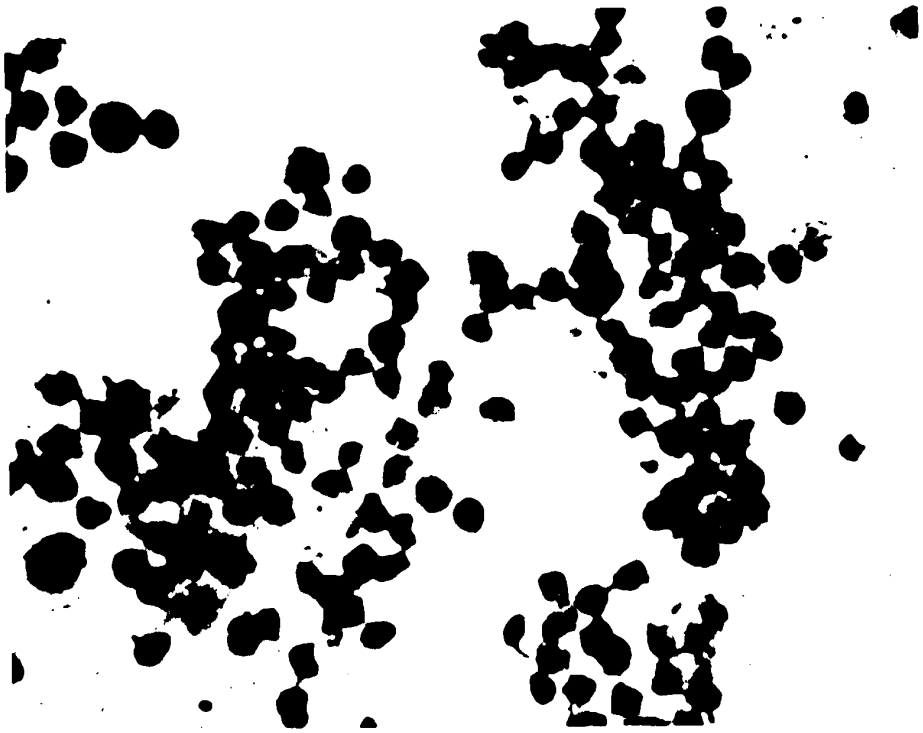

Figure 7. UNFRACTIONATED BONE MARROW. GIEMSA STAIN x 640

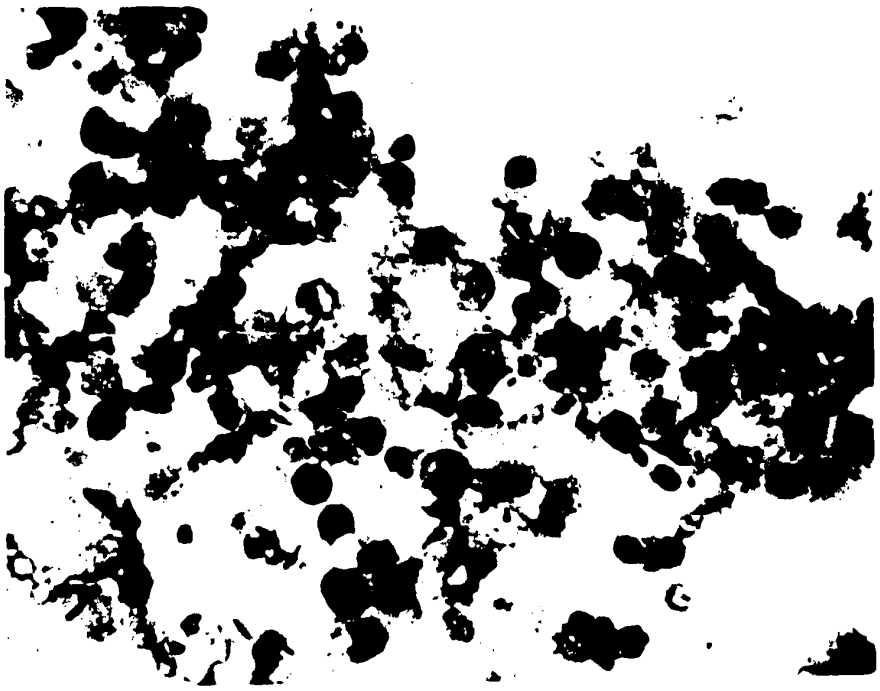

Figure 8. EFFLUENT FRACTION OF CELLS. GIEMSA STAIN x 640

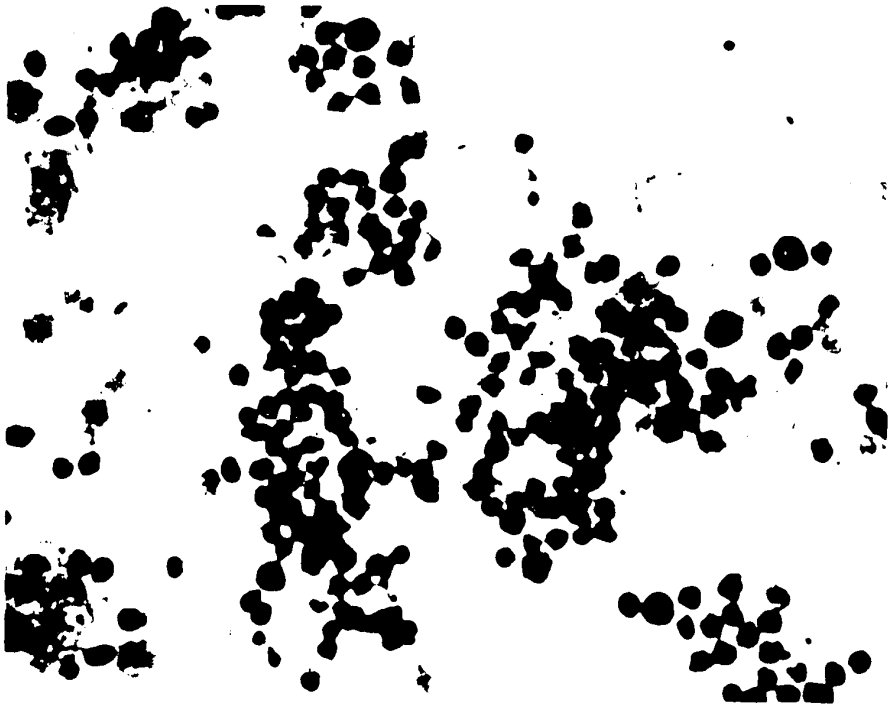

Figure 9. ELUATE FRACTION OF CELLS. GIEMSA STAIN x 400

3. Discussion - In the present investigation, it has been demonstrated that passage of normal rabbit bone marrow cells through an antigen-sensitized glass-bead column results in the retention of specific antigen-reactive cells capable of mediating an immune response in an irradiated recipient only towards the antigen used to sensitize the column. The cells which were not retained by the column (effluent) did not possess the capacity to transfer plaque-forming ability with respect to the specific red cell antigen used to sensitize the column, although they could facilitate a response to a non-cross-reacting red cell, following their transfer to irradiated recipients. Furthermore, effluent cells obtained from an HSA-sensitized glass bead column could not transfer humoral antibody-forming capacity with respect to HSA when transferred to the irradiated host. On the other hand, effluent cells from a non-sensitized column were capable of conferring antibody-forming capacity toward HSA or sheep red cells in irradiated hosts, as were bone marrow cells retained by and eluted from the

specific antigen-sensitized columns. The presence of residual antigen (i.e. HSA) in the medium bathing the antigen (HSA)-sensitized glass beads prevented the retention by the column of cells capable of mediating an immune response to the antigen (HSA) in the irradiated host (Table 38). This is probably due to the competition by free antigen and glass bead-adsorbed antigen for the antigen-reactive cells, the interaction probably occurring between the antigen and an antibody-like site on the surface of the antigen-reactive cell (see below). These experiments demonstrated that cells are specifically retained by the antigen-sensitized glass-bead columns since unsensitized glass bead columns did not retain any of the specific antigen-reactive cells. Furthermore, the transfer of immunologic activity by the eluted cells was shown to be specific, in that it was directed solely to the antigen originally used to sensitize the column. The converse is true for the effluent cells since they were unable to transfer immunocompetence with respect to the antigen used to sensitize

the glass beads. The magnitude of the immune response in the irradiated recipient given the eluate cells, which constitute 3 to 10 percent of the cells of the bone marrow, was on a level similar to that observed in recipients given the whole unfractionated bone marrow. This, in itself, is an unexpected finding when it is realized that as few as 20 x 10^6 eluate cells could confer an immune response in a recipient equivalent to that mediated by 200 to 500 x 10^6 unfractionated bone marrow cells. This demonstration of specific immunocompetence of the eluate cells is even more remarkable when it is realized that many lymphoid cells other than the specifically-bound antigen-reactive cells are non-specifically retained by the glass beads and are subsequently eluted along with the antigen-reactive cells. Wigzell and Andersson (137) arrived at a similar conclusion. Therefore, although the technique may not permit for the isolation of only specific antigen-reactive cells, it does permit a complete segregation of one species of antigen-reactive cells from all the others. Stimulation of the

cells by the specific antigen in vitro in the presence of tritiated thymidine followed by radioautographic analysis of the cell preparation may provide a more precise indicator as to the number of specific antigen-reactive cells in the eluate fraction.

No significant differences were observed in the immune responses of irradiated recipients given eluate cells alone or eluate cells plus a subthreshold dose of the unfractionated bone marrow. This latter dose of bone marrow cells was incapable, by itself, of mediating an immune response in an irradiated recipient. The reasons for the administration of this dose of bone marrow cells were: a) to decrease the severity of irradiation-induced morbidity and/or mortality in sublethally irradiated rabbits receiving only a small number of bone marrow (i.e. eluate) cells. We have observed that approximately 10-20 percent of the irradiated rabbits die if they are not protected with bone marrow. b) To provide the recipient with macrophages and/or other cell types which, although not the predominant cell(s) required for the

induction of the primary immune response, may nevertheless be necessary in small numbers in order to potentiate the immune response, or to function in a synergistic fashion. Macrophages have been shown to play an active role in promulgating the sequential steps leading to humoral antibody formation, especially in the primary response (108, 255, 256, 257, 258). It has also been demonstrated that macrophages are retained by the glass bead columns (114, 134, 135) and apparently are not eluted by the procedure utilized in this investigation. Furthermore, the glass-bead purified lymphocyte suspensions have been found to display a markedly reduced response to antigens, which could be restored to normal by the addition of macrophages to the system (114, 135). It was therefore felt that the administration of a subthreshold dose of unfractionated bone marrow along with the test cell preparation (eluate or effluent) might enhance the primary immune response in the irradiated recipient.

The results, however, do not lend support to these theoretical considerations. The immune response of

irradiated recipients given eluate cells and a sub-threshold dose of unfractionated bone marrow cells was only slightly enhanced as compared to that obtained in a recipient given eluate cells only. Furthermore, initial passage of the unfractionated cell suspension through a column sensitized with a non-cross-reacting antigen (Table 44) or an unsensitized glass bead column (Table 45) prior to passage of the cells through a specifically-sensitized column did not diminish the effectiveness of the eluate cells to transfer specific immunocompetence to mediated recipient rabbits. Since the initial passage of the cells should have depleted the cell suspension of macrophages or seriously reduced their number (114, 134, 135), it is obvious that the functionally-active macrophage does not participate in the interaction between the antigen-reactive cell and the antigen adsorbed onto the glass beads. Although one might be tempted to rule out any role for the macrophage in the induction of the primary response in the irradiated recipient rabbit, it would be necessary to establish unequivocally that the exposure to 800r irradiation killed all the macrophages

and their precursor cells in vivo and that no macrophages were transferred with the eluate cells. Although macrophages could not be distinguished, morphologically, in the eluate population of cells, it is possible, due to the manipulation of the cells, that they had assumed a different appearance. Furthermore, it has also been demonstrated that lymphocytes can transform to macrophages (259), thus rendering academic the entire question of the role of the macrophage.

Glass bead columns have been utilized by a number of investigators in attempts to separate morphologically-identical but functionally-different lymphocyte populations (114, 132, 133, 134, 135, 137). Plotz and Talal (134) passed immune mouse and rat spleen cells through glass bead columns and observed that only small, immunoincompetent mononuclear cells passed through, whereas the column retained the antibody-synthesizing cells, granulocytes and large mononuclear cells which could be eluted from the bead with ethylenediaminetetraacetate.

Oppenheim et al (135) and Hersh and Harris (114) have both observed that glass bead-purified human peripheral lymphocytes are capable of reacting to stimulation by PHA but are no longer capable of being stimulated by a number of antigens to which the original donors had been actively immunized and to which the unfractionated cells could react. The immunologic responsiveness of the lymphocytes could be re-established by the addition of macrophages. Nossal et al (125) also observed that column purified small lymphocytes, prepared from normal mouse thoracic duct lymph, could not transfer immunocompetence to a number of antigens in irradiated recipients, whereas the unfractionated cell suspensions could transfer antibody-forming capacity. However, column purified small lymphocytes obtained by fractionating immune mouse thoracic duct lymph could successfully transfer antibody-forming capacity to irradiated hosts. These findings suggest that the macrophage, which is normally retained by the glass bead column, is required along with the small lymphocyte (probably the antigen-reactive

cell) in the initiation of the primary immune response (260). This cell, however, does not appear to be necessary for the induction of the secondary immune response, since the antigen apparently reacts directly with the antigen-recognizing-antibody-forming (memory) cells (261, 262).

Wigzell and Andersson (137) fractionated immune lymph node cells using a specific antigen-sensitized glass bead column. They observed that antibody-forming cells, directed toward the immunizing antigen, were retained by the glass beads and could subsequently be eluted from the beads by shaking. The binding of the cells to the antigen-sensitized glass beads was found to be selective for the particular cell population since cells from an animal immunized to two different antigens were deprived of reactivity to only one of the antigens, that adsorbed onto the glass beads, following passage through the column. Thus, the specific interaction of the antigen-bead complex with the antibody-forming cell cannot be ascribed to passively-adsorbed

antibody by the cells. However, the great specificity of the interaction makes it mandatory to assume an immunologic nature for the interaction and it must be due to reaction of the glass-adsorbed antigen with specific antibody or antibody-like sites produced by, and retained on the surface of, the specifically-retained immune cells themselves. This explanation, however, might not be the only one, for it does not at all aid in elucidating the mechanism of interaction observed in the current investigation. Here, normal and not immune cells were utilized and it was a select population of normal bone marrow cells which was retained by the antigen-sensitized glass bead column. Since no immune response had previously been induced in the irradiated donor of these cells, the interaction of the normal bone marrow cells with the antigen adsorbed onto the glass bead column cannot be attributed to conventional antibody. However, the specificity of the interaction between the antigens and a particular immunologically-specific lymphoid cell necessitates the assumption of the presence of some

sort of immunoglobulin or antibody-like structure on the surface of the cell capable of "recognizing" the antigen and of reacting with it. This concept was originally presented by Ehrlich almost a century ago (263) as the "side chain theory" and has been discussed at length by Mitchison (111) and Gell (264). Recent investigations (153, 265) tend to confirm the validity of this concept. Daguillard and Richter (266) have observed that goat anti-rabbit immunoglobulin antiserum (GARIG) can stimulate normal rabbit lymphocytes to undergo blastogenesis and mitosis in the same manner as antigen can stimulate immune lymphocytes (266). Furthermore, rabbit peripheral lymphocytes incubated with GARIG are capable of conferring antibody-forming capacity directed to goat gamma-globulin when transferred to recipient rabbits previously made tolerant to goat gamma-globulin, whereas rabbit lymphocytes incubated with normal goat gamma-globulin do not possess this activity (266). These data suggest that GARIG exhibits a great affinity toward peripheral lymphocytes and imply that GARIG is

reacting with an immunoglobulin or immunoglobulin-like site on the surface of the cell. Paul et al (267) have also demonstrated a relationship between the affinity of an antigen for lymphoid cells and the capacity of the antigen to induce mitosis of the cells in vitro. Fidalgo and Najjar (265) arrived at a similar conclusion in investigations with dog lymphoid cells. Merler and Janeway (153) have eluted a material from immune human lymphoid cells which was capable of interacting with the antigen, thus suggesting that it is an immunoglobulin. It would therefore appear that normal, as well as immune, lymphoid cells possess immunoglobulins or immunoglobulin-like structures on their surfaces, which exhibit specific affinities toward particular antigens.

The above interpretation goes a long way toward an understanding of the mechanism of interaction between the antigen-reactive cell and the antigen. Certainly, the reaction of the normal bone marrow cells with antigen cannot be attributed to interaction of the antigen with

antibody-forming cells (which would be expected to possess specific immunoglobulin molecules on their surfaces) since previous investigations have disclosed that the immuno-competent cells residing in normal bone marrow are antigen-reactive cells and not antibody-forming cells (actual or potential) (Chapter V.B, C, and D). Thus, it would appear that the antigen-reactive cell exercises its function on the basis of specific antibody-like sites on its surface capable of reacting with the antigen.

G. Loss and Reappearance of Antigen-Reactive Cells Following Irradiation of Normal Adult Rabbits

1. Experimental Procedures - The protocols for the experimental procedures carried out in this investigation are diagramatically presented in Figure 10.

The rabbits were subjected to doses of irradiation ranging from 800r to 1400r whole body irradiation and then injected with 10^9 SRBC at varying intervals of time thereafter. The rabbits were sacrificed 7 days following immunization and their spleen cells were analyzed for antibody-forming capacity by the hemolysis-in-gel (plaque) technique, as described in Materials and Methods.

Other rabbits were irradiated and immediately given bone marrow cells (0.5×10^9 cells) obtained from normal or irradiated (800r) donors. These rabbits were also given SRBC intravenously at predetermined times thereafter and the immune response was determined by the direct agglutination technique and the hemolysis-in-gel (plaque) technique.

Primed bone marrow cells refer to cells obtained from a rabbit immunized with the antigen 24 hours prior to sacrifice.

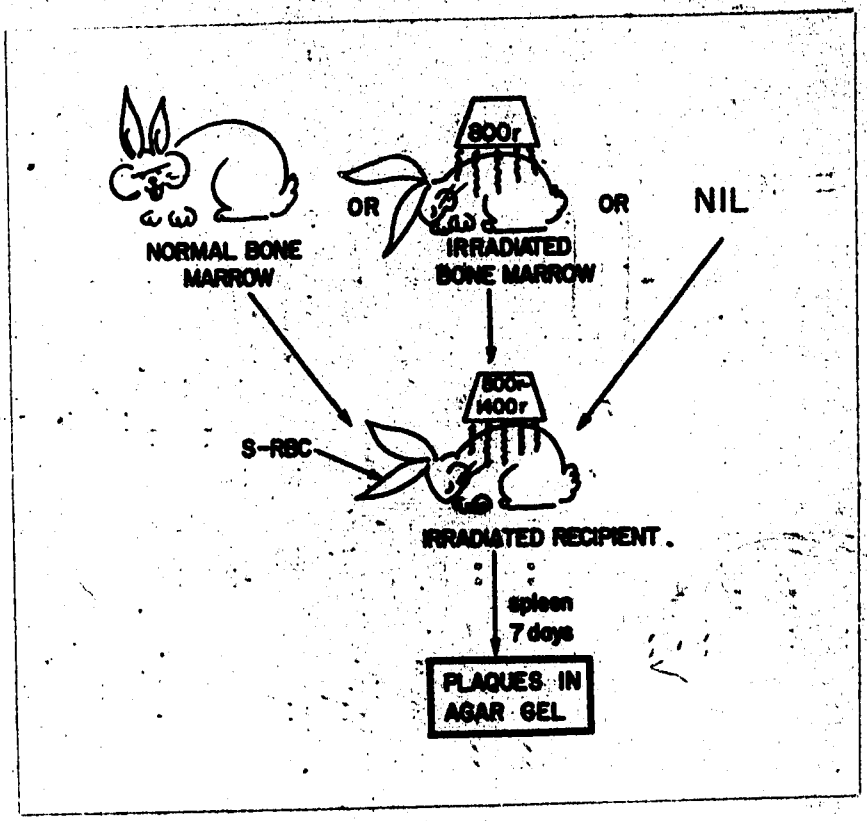

Figure 10. PROTOCOL FOR THE DEMONSTRATION OF RADIOSENSITIVITY OF THE ANTIGEN-REACTIVE CELL (ARC) IN RABBIT BONE MARROW

2. Results - As can be seen in Table 46, spleen cells of irradiated (800r) rabbits immunized with SRBC and either not given any cells or injected with bone marrow cells of primed or irradiated (800r) donor rabbits could not form hemolytic plaques when incubated with SRBC in agar gel. On the other hand, spleen cells obtained from an immunized unirradiated rabbit or an irradiated (800r) rabbit given normal allogeneic bone marrow cells and immunized with SRBC formed abundant plaques upon incubation with SRBC.

The effect of varying the dose of irradiation on the capacity of recipient rabbits of normal allogeneic bone marrow cells to elicit an immune response following stimulation with SRBC is presented in Table 47. A dose of 800r total body irradiation was the maximum the recipient could tolerate and be capable of forming antibodies whereas exposure of the recipient to 1000r or more resulted in complete inhibition of the immune response.

Rabbits exposed to 800r and immunized with SRBC at varying times thereafter were incapable of eliciting a

humoral immune response during the first three weeks following irradiation. Recovery of immune responsiveness occurred about 3 to 4 weeks following irradiation (Table 48). By 5 weeks, the capacity of the rabbit to respond greatly exceeded that of a normal unirradiated rabbit (Tables 48 and 49). However, if the irradiated rabbits were given normal allogeneic bone marrow cells immediately following irradiation, the response to SRBC was maximum at one week and diminished over the following two weeks (Table 49). The capacity of the spleen cells of the recipients to form plaques rose sharply by four weeks, and then returned to normal levels.

The bone marrow cells transferred from irradiated (800r) donors, immediately following irradiation, to irradiated (800r) recipients were incapable of conferring antibody-forming capacity to these animals. The capacity of the bone marrow to transfer immunocompetence was regained somewhat if transferred 2 or 4 weeks following irradiation. The bone marrow completely recovered the capacity to transfer antibody-forming capacity if transferred 6 weeks following irradiation (Table 50).

As can be seen in Table 51, rabbits subjected to 800r total body irradiation were incapable of responding with antibody formation until approximately 4 weeks had elapsed from the day of irradiation. By six weeks their capacity to synthesize humoral antibody had attained normal levels.

TABLE 46

THE EFFECT OF IRRADIATION AND THE ADMINISTRATION OF PRIMED OR IRRADIATED ALLOGENEIC BONE MARROW ON THE PLAQUE-FORMING CAPACITY OF SPLEEN CELLS OF IRRADIATED RECIPIENT RABBITS

Bone Marrow Donor Treated as Follows	Recipient Rabbit Treated as Follows	S-rbc (10^9 Cells) Injected in Recipient as Follows	No. of Plaques per 10^6 Splenic Lymphoid Cells Incubated with S-rbc*
-	Untreated****	Yes	72
-	Irradiated (800r)****	Yes	3
Normal Untreated	Irradiated (800r)	Yes	70
Irradiated (800r)**	Irradiated (800r)	Yes	2
Primed***	Irradiated (800r)	Yes	3
-	Untreated****	No	4

* Rabbits were sacrificed 7 days following the injection of the S-rbc. Each value represents the mean of duplicate determinations.

** Donor rabbits were subjected to 800r total body irradiation several hours prior to sacrifice.

*** Primed bone marrow was obtained from donor rabbits 24 hours following the injection of the S-rbc.

**** Rabbits were not injected with any donor cells.

TABLE 47

THE PLAQUE-FORMING CAPACITY OF SPLEEN CELLS OF RABBITS SUBJECTED TO VARYING DOSES OF IRRADIATION AND GIVEN NORMAL ALLOGENEIC BONE MARROW CELLS

Dose of Irradiation Administered	No. of Plaques per 10^6 Splenic Lymphoid Cells 7 Days Following the Administration of Bone Marrow* and S-rbc
Nil	70
800r	68
1,000r	2
1,200r	<1
1,400r	<1

* Normal allogeneic bone marrow (5×10^8 cells per recipient) and S-rbc (10^9 cells) were given intravenously immediately following irradiation. Each value represents the mean of duplicate determinations.

TABLE 48

THE PLAQUE-FORMING CAPACITY OF SPLEEN CELLS OF RABBITS INJECTED WITH SHEEP RED BLOOD CELLS (S-RBC) AT VARYING INTERVALS OF TIME FOLLOWING IRRADIATION (800r)

S-rbc (10^9 Cells) Injected at Following Days Subsequent to Irradiation	No. of Plaques per 10^6 Splenic Lymphoid Cells*
1	1
7	1
14	24
21	25
28	103
63	80
98	95
105	71

* The rabbits were subjected to 800r total body irradiation prior to the injection of the S-rbc. They were sacrificed 7 days later and tested for plaque-forming capacity to S-rbc. Each value represents the mean of duplicate determinations.

TABLE 49

THE PLAQUE-FORMING CAPACITY OF IRRADIATED (800r) RABBITS RECONSTITUTED WITH ALLOGENEIC NORMAL BONE MARROW AND GIVEN SHEEP RED CELLS (S-RBC) AT VARYING INTERVALS OF TIME

S-rbc (10^9 Cells) Given at Following Days Subsequent to Irradiation and Bone Marrow Transfer	No. of Plaques per 10^6 Splenic Lymphoid Cells of Irradiated Recipients of Normal Allogeneic Bone Marrow and S-rbc*
1	64
7	49
14	37
21	130
28	300
68	60
98	84

* Rabbits were subjected to 800r total body irradiation and injected with normal allogeneic bone marrow cells (5×10^8 cells) and S-rbc (10^9 cells). They were sacrificed 7 days later. Each value represents the mean of duplicate determinations.

TABLE 50

THE PLAQUE-FORMING CAPACITY OF SPLEEN CELLS OF IRRADIATED (800r) RABBITS GIVEN ALLOGENEIC BONE MARROW CELLS OBTAINED FROM IRRADIATED (800r) DONOR RABBITS

Irradiated Donor Sacrificed and Bone Marrow Transferred (5×10^8 Cells) at Following Days Subsequent to Irradiation	No. of Plaques per 10^6 Recipient* Splenic Lymphoid Cells Incubated with S-rbc
1	4
14	18
28	21
42	43

* Irradiated rabbits were sacrificed 7 days following the administration of the allogeneic irradiated bone marrow cells and the S-rbc (10^9 cells) and the spleen cells were analyzed for plaque-forming capacity. Each value represents the mean of duplicate determinations.

TABLE 51

THE ANTIBODY FORMING CAPACITY OF IRRADIATED RABBITS*

IMMUNIZED WITH SHEEP RED BLOOD CELLS (S-RBC)

Day of Injection of S-rbc Relative to Day of Irradiation (Day 0)*	Antibody Titers** at the Following Days After Injection of the Antigen		
	7	10	14
1	0	0	0
7	0	0	0
14	0	0	0
28	0	320	640
42	1,280	2,560	2,560

* The rabbits were subjected to 800r total body irradiation and injected with 10^9 S-rbc intravenously.

** Expressed as the inverse of the dilution of the serum capable of agglutinating sheep red blood cells. Titers less than 10 are considered to be negative.

3. Discussion - The present investigation was carried out in an attempt to answer the following questions: a) Is there a specific relationship between the degree of immunocompetence following total body irradiation and the presence of viable antigen-reactive cells (ARC); b) Do the ARC recover following immunosuppressive irradiation; c) If they do recover, does the rate of re-appearance of the ARC following irradiation correspond to the extent of recovery of immunocompetence; and d) Do the ARC reappear in the bone marrow, their original organ of habitation.

The initial experiments were carried out in order to establish the dose of irradiation which would be optimal for the series of experiments contemplated. Unirradiated or irradiated adult rabbits were injected with antigen (SRBC) along with bone marrow cells obtained from normal, irradiated (800r) or primed donors. When their spleen cells were analyzed for plaque-forming capacity 7 days later, only the spleen cells of the untreated immunized rabbits and the irradiated rabbits given normal bone

marrow cells responded. Therefore, irradiation of the donor with 800r was sufficient to deplete its bone marrow of ARC (Table 46). Furthermore, it was also demonstrated that the administration of 800r total body irradiation to the recipient rabbit was the maximum which the animal could tolerate and still possess antibody-forming cells (AFC) in its spleen 7 days later. Subjecting recipients of normal bone marrow cells (ARC) to 1000r or more prior to the cell transfer resulted in complete destruction of the antibody-forming apparatus in the recipient. The cells mediating this latter function have been demonstrated to be of host origin and are not contained in the bone marrow cells transferred (see Chapter V.D). The dose of irradiation administered (800r) in the subsequent experiments was, therefore, one which effectively destroys the ARC but does not harm the antibody-forming cells (AFC) to any significant degree. Therefore, the failure of such irradiated rabbits to respond with humoral antibody formation following antigenic stimulation can be attributed to the lack of immunologically-competent ARC. By cell transfer experiments, it

was observed that no ARC could be detected in the bone marrow of an irradiated (800r) rabbit immediately following irradiation whereas slight recovery of the ARC was evident by 2 weeks. The immune responsiveness of the irradiated (800r) rabbit was re-established to normal levels by 4 weeks following irradiation (800r) (Table 51) at a time when the ARC were shown to have reappeared in these animals (Tables 48 and 50). These findings were confirmed under somewhat different conditions (Table 49) in that irradiated rabbits were given normal allogeneic bone marrow cells and antigen. In this case, the plaque-forming capacity of the recipients' spleen cells diminished during the first three weeks following the transfer of the bone marrow cells, thus demonstrating the recovery of the antigen-recognizing apparatus in the irradiated rabbit followed by rejection of the transplanted allogeneic bone marrow cells. Complete recovery of the irradiated rabbits was not attained until 4 weeks post irradiation when the number of plaque-forming spleen cells detected was far greater than the number

observed in the unirradiated immunized rabbit. Thus, it would appear that the loss of immune responsiveness of the rabbits in the immediate post-irradiation period is a direct consequence of the lytic action of irradiation (800r) on the radiosensitive ARC and that recovery of the immune responsiveness is directly related to the re-emergence of the ARC population of cells.

Our present knowledge concerning the radiosensitivity of the various types of cells involved in the different phases of the immune response is still incomplete (268, 269). The immunosuppressive action of irradiation could variously be attributed to its effect on the macrophage and/or the antigen-reactive cell and/or the actual antigen-processing step and/or the antibody-forming cell. Harris and Noonan (270) have shown that rat peritoneal macrophages are less sensitive to 750r total body irradiation than the circulating leukocytes. Mouse macrophages, or at least those macrophages which exhibit immunologic functions were found to be radiosensitive to 550-600r

(255, 112). Rabbit immunologically-active macrophages were completely inactivated, following exposure in vivo to 750r (271). Hege and Cole (272) could not suppress the background antibody plaque-forming cells by 500r total body irradiation; a dose found to suppress cellular proliferation and the production of plaque-forming cells (273). Cells producing 19S antibodies were shown to be more sensitive to irradiation than those producing 7S antibodies (274). It has also been shown that the induction phase (latent period) of the immune response is radiosensitive, whereas the antibody-forming phase of the immune response is much more radioresistant (275). Our observations are in agreement with these findings as we have demonstrated that the ARC is radiosensitive to 800r whereas the AFC is much more radioresistant (see Chapter V.D). Davies et al (70) found that the response of the ARC to stimulation by antigen is characterized by brisk cell division and proliferation. These cells, and their progeny, display increased nucleic acid metabolism

and take deep basophilic stains (276). It is this phase of cell division which is very radiosensitive (277). On the other hand, the antibody-producing cells were found to be relatively radioresistant (275, 278).

The exaggerated immune response observed in the irradiated rabbits 4-5 weeks following irradiation would imply an abrupt recovery of the ARC population by transformation from irradiation-resistant precursor stem cells followed by synchronous division and proliferation of these cells to greater than normal levels. Feedback-inhibition, which undoubtedly operates at the cellular level to control the proliferation of the different cell lines, would then forestall any further proliferation of that particular cell line, with a return to normal levels by the eighth week following irradiation. This delayed quasi-stimulatory effect of irradiation on the immune response in thc rabbit has previously been observed by Dixon and McConahey (279), in an entirely in vivo system. They demonstrated that the immune response to a number of protein antigens could be enhanced by subjecting the immunized rabbits to 500r total body irradiation 2 hours to 2

days following the injection of the antigen. The enhanced immune response was attributed to depletion of the cells of the lymphoid tissues by the irradiation followed by a disproportionate proliferation of the antigen-stimulated immunocompetent cells which were resistant to the lytic effects of the irradiation. A similar enhancing effect of irradiation on the immune response has also been reported by Gengozian and Makinodan (280) and Morgan et al (281) who irradiated mice four to six days following injection of the antigen and by Vlahovic and Stankovic (282) who irradiated guinea pigs after injection of horse serum proteins. Taliaferro and Taliaferro (268, 275) noted that the immunodepressing effects of sublethal doses of irradiation were transient, with recovery commencing within one week following a low dose of irradiation and within several weeks following exposure to larger doses of irradiation. Recovery of immune competence was found to be uniformly followed by an overcompensatory proliferation of the lymphoid tissue and an enhanced immune response. However, Silverman and Chin (283) and Fitch et al (284)

were unable to substantiate the immuno-enhancing effect of irradiation on the primary immune response in the mouse.

The data presented in the present investigation confirms the greater radiosensitivity of the ARC as compared to the AFC in the normal rabbit. This would strongly imply that the suppressive effects of irradiation on the primary immune response in the rabbit can be directly attributed to inactivation of death of the unstimulated antigen-reactive cell population. The present data implicate only two cell types - the antigen reactive cell and the antibody forming cell - in the induction of antibody synthesis. The exact role of the macrophage, if any, in the facilitation of the immune response in the rabbit is still a controversial subject (112, 285).

H. The In Vitro Stimulation of Rabbit Bone Marrow Cells

1. Experimental Procedures - The protocols carried out in this investigation are diagramatically presented in Figure 11. Bone marrow cells were obtained from normal rabbits according to the technique described in Materials and Methods. Ten ml of the cell suspension were transferred into sterile screw-cap flasks to which were added a variable number of SRBC (10^7 to 10^9 cells) (Figure 11). The cells were incubated in an atmosphere of 4 percent CO_2 in air for 1 to 24 hours, following which they were washed twice and injected, in variable numbers, into irradiated recipient rabbits (Figure 11). Other irradiated rabbits were injected with unincubated bone marrow cells and SRBC. Seven days later, the rabbits were sacrificed by the intravenous administration of nembutal and the spleen cells were tested for their plaque-forming capacity in agar gel.

The bone marrow cells were also exposed to 4,000r or 10,000r irradiation, using a Cobalt 60 source. Following

irradiation, the cells were analyzed for viability by the dye exclusion test and for their capacity to undergo blastogenesis and mitosis following stimulation with PHA. (See Materials and Methods for the technique of cell culture, the dye exclusion test and the conditions of irradiation.)

In other experiments, the bone marrow cells were exposed to 4,000r or 10,000r irradiation either before or after incubation with 10^9 SRBC or HRBC for 24 hours. They were then injected into irradiated (800r) rabbits along with 10^9 HRBC or SRBC. The rabbits were sacrificed 7 days later and the spleen cells were analyzed for plaque-forming capacity.

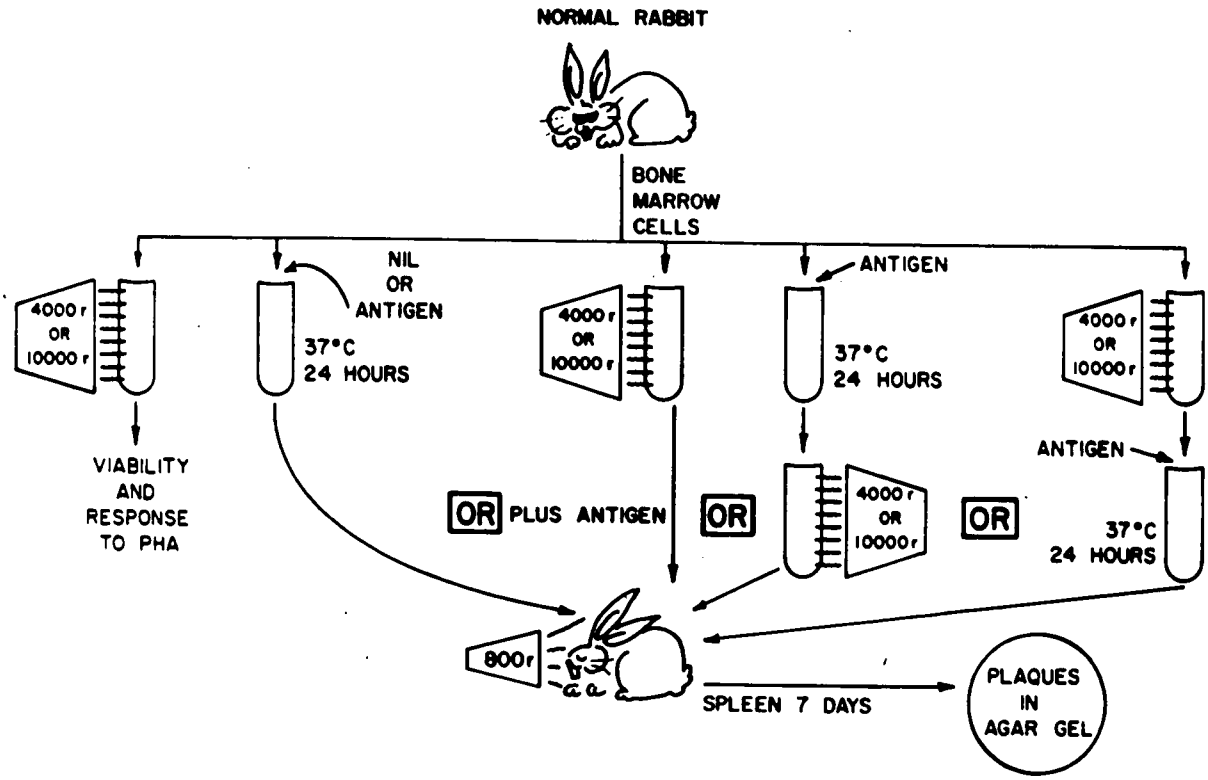

Figure 11. THE TRANSFER OF IMMUNOCOMPETENCE WITH NORMAL RABBIT BONE MARROW ANTIGEN-REACTIVE CELLS (ARC) INCUBATED WITH THE ANTIGEN BEFORE, AFTER OR IN THE ABSENCE OF IRRADIATION OF THE CELLS IN VITRO

2. Results - As can be seen in Table 52, it was necessary to inject 10^9 cells into the irradiated (800r) rabbit which had received allogeneic normal bone marrow cells in order to facilitate greater-than-background plaque formation by the spleen cells. The administration of 10^8 SRBC provided only a threshold stimulus whereas the injection of 10^7 SRBC resulted in plaque formation slightly below that of normal control levels, which normally average 6 ± 2 plaques per 10^6 splenic lymphoid cells. However, large numbers of plaques were detected in the spleens of irradiated (800r) rabbits injected with bone marrow cells which had been incubated with 10^8 SRBC for 24 hours at 37°C (Table 53). It was observed that incubation of the normal bone marrow cells for 24 hours resulted in their optimal facilitation to transfer immunocompetence (Table 54).

The number of dead bone marrow cells, as determined by the dye exclusion test, was identical in non-irradiated and irradiated (4,000r) cell preparations (Table 55). However, the irradiated (4,000r) cells failed to respond

to stimulation with PHA whereas the unirradiated cells responded in a normal fashion (Table 55). On the other hand, irradiated (4,000r) and unirradiated cell preparations were equally capable of transferring immunocompetence to irradiated (800r) recipients which had also been injected with SRBC (Table 56). However, cells exposed to 10,000r could not transfer immunocompetence. Neither could bone marrow cells exposed to 4,000r or 10,000r followed by incubation with the antigen for 24 hours in vitro (Table 57). It was, however, established that cells which had been incubated with SRBC for 24 hours and then subjected to either 4,000r or 10,000r were capable of transferring plaque-forming capacity to irradiated recipient rabbits (Table 57). It was also observed that cells incubated with SRBC (or HRBC) in vitro were still capable of transferring immunocompetence with respect to the non-cross-reacting antigen, HRBC (or SRBC), following transfer to irradiated (800r) recipient rabbits (Table 58).

TABLE 52

THE EFFECT OF ANTIGEN (S-RBC) DOSE ON THE PLAQUE-FORMING CAPACITY OF SPLEENS OF IRRADIATED RABBITS INJECTED WITH NORMAL ALLOGENEIC BONE MARROW CELLS

No. of S-rbc injected intravenously	No. of plaques per 10^6 splenic lymphoid cells of irradiated recipient rabbits*
10^7 cells	3
10^8 cells	12
10^9 cells	79

* The rabbits were subjected to 800 r total body irradiation and injected intravenously with 5×10^8 normal bone marrow cells and the specified number of S-rbc. The rabbits were sacrificed 7 days later and the spleens were tested for plaque-forming capacity.

TABLE 53

THE PLAQUE-FORMING CAPACITY OF IRRADIATED UNIMMUNIZED RABBITS INJECTED WITH ALLOGENEIC BONE MARROW CELLS PRIMED IN VITRO WITH VARIABLE AMOUNTS OF THE ANTIGEN (S-RBC)

No. of S-rbc used for in vitro priming of the bone marrow cells*	No. of plaques per 10^6 splenic lymphoid cells of irradiated recipient rabbits**
10^7	8
10^8	72
10^9	100

* In vitro priming of the bone marrow cells was done by adding the S-rbc to 10^9 bone marrow cells suspended in medium 199 containing 20 percent NRS. Duration of in vitro priming was 24 hours at 37°C.

** The rabbits were subjected to 800 r total body irradiation and then given the in-vitro primed bone marrow cells. They were sacrificed 7 days later and the spleens were analyzed for plaque-forming capacity.

TABLE 54

THE PLAQUE-FORMING CAPACITY OF SPLEEN CELLS OF IRRADIATED RABBITS (800r) INJECTED WITH ALLOGENEIC BONE MARROW CELLS PRIMED IN VITRO WITH ANTIGEN (S-RBC) FOR VARIOUS PERIODS OF TIME

Period of in vitro priming * (hrs)	No. of primed bone marrow cells transferred	No. of plaques per 10^6 splenic lymphoid cells of recipient rabbits (day 7)
1	5×10^8 cells	8
6	5×10^8 cells	18
24	5×10^8 cells	72
Nil	5×10^8 cells	9

* The normal bone marrow cells were incubated in vitro at 37°C with 1×10^8 S-rbc for the specified period of time prior to their transfer to irradiated (800 r) allogeneic rabbits.

TABLE 55

THE EFFECT OF IN VITRO IRRADIATION (4000 r) OF RABBIT BONE MARROW CELLS ON THEIR VIABILITY AND THEIR CAPACITY TO RESPOND IN VITRO TO PHYTOHEMAGGLUTININ (PHA)

Cells tested	Cell viability: Dye exclusion test (percent dead cells)	Response to PHA in vitro		
		Control (c.p.m.)	PHA (c.p.m.)	Specific incorporation *
Non-irradiated cells	14	4458	19010	4.2
Immediately after irradiation	15	810	832	1.0

* Defined as the incorporation of tritiated thymidine in the presence of PHA to that incorporated in the absence of PHA (control).

TABLE 56

THE EFFECT OF IN VITRO IRRADIATION OF NORMAL RABBIT BONE MARROW CELLS ON THEIR CAPACITY TO CONFER IMMUNOCOMPETENCE TO IRRADIATED ALLOGENEIC RABBITS

Cells Subjected to Following Dose of Irradiation	Duration of In Vitro Incubation Following Irradiation (Without Antigen)	No. of Plaques per 10^6 Splenic Lymphoid Cells of Irradiated Recipient Rabbit*
4,000r	Nil	76
	24 Hours	8
10,000r	Nil	8
	24 Hours	7
Nil	Nil	72
	24 Hours	58

* Rabbits were subjected to 800r total body irradiation and then injected intravenously with the bone marrow cells and 10^9 S-rbc. The rabbits were sacrificed 7 days later and the spleens were analyzed for plaque-forming capacity.

TABLE 57

THE EFFECT OF IN VITRO IRRADIATION ON THE CAPACITY OF NORMAL RABBIT BONE MARROW CELLS TO BE PRIMED IN VITRO AND TO TRANSFER IMMUNOCOMPETENCE TO IRRADIATED RECIPIENTS

Time of In Vitro Priming* in Relation to Irradiation of Bone Marrow Cells	Cells Subjected to Following Dose of Irradiation	No. of Plaques per 10^6 Splenic Lymphoid Cells of Unimmunized Irradiated Recipients**
Cells Primed for 24 Hours Immediately Following Irradiation	4,000r	13
	10,000r	2
Cells Primed for 24 Hours Prior to Irradiation	4,000r	61
	10,000r	64
Non-Irradiated Cells Primed for 24 Hours	Nil	78

* In vitro priming of the bone marrow cells was carried out by adding 10^8 S-rbc to 10^9 bone marrow cells suspended in Medium 199 containing 20 percent NRS. The cells were cultured at 37°C in an atmosphere of 4% CO_2 in air.

** Rabbits were subjected to 800r total body irradiation and then given the in-vitro primed irradiated bone marrow cells. They were sacrificed 7 days later and their spleens were analyzed for plaque-forming capacity.

TABLE 58

THE PLAQUE-FORMING CAPACITY OF SPLEEN CELLS OF IRRADIATED RABBITS INJECTED WITH ALLOGENEIC BONE MARROW CELLS PRIMED IN VITRO WITH EITHER S-RBC OR H-RBC. THE DEGREE OF COMMITTMENT OF THE ANTIGEN-REACTIVE CELL POPULATION PRIMED IN VITRO

Type of RBC Used for the In Vitro Priming of the Bone Marrow Cells*	Type of RBC Given to Irradiated Rabbits Following Bone Marrow Transfer	No. of Plaques per 10^6 Splenic Lymphoid Cells of Irradiated Recipient Rabbits**	
		S-RBC	H-RBC
S-RBC	H-RBC	70	34
H-RBC	S-RBC	160	37
NIL	H-RBC	4	82
NIL	S-RBC	68	5
NIL	H-RBC & S-RBC	54	25

* In vitro priming of the bone marrow cells was carried out by adding 10^8 RBC to 10^9 bone marrow cells suspended in medium 199 containing 20% NRS. Duration of in vitro priming was 24 hours at 37°C in an atmosphere of 4% CO_2 in air.

** The rabbits were subjected to 800r total body irradiation and then given the in-vitro primed bone marrow cells and 10^9 RBC. They were sacrificed 7 days later and the spleens were analyzed for plaque-forming capacity with respect to both S-RBC and H-RBC.

3. Discussion - The findings presented above generally confirm those reported in Chapter V.B, C, D, and F, with respect to the ability of normal bone marrow cells to transfer antibody-forming capacity to irradiated (800r) rabbits. These results have, moreover, been enhanced by the application of an in vitro system for the induction phase, thus abrogating the necessity of injecting the antigen into the irradiated bone marrow recipient. It has been unequivocally demonstrated that the ARC function in the immune response can be initiated by incubation with the antigen in vitro. Although the cells, following their incubation with the SRBC (or HRBC) were not freed of the SRBC (or HRBC) before their injection into the irradiated (800r) host, the immune response in the latter cannot be seriously entertained as having been stimulated by the transferred RBC since the number of RBC transferred could not have exceeded 10^8 cells, a number of cells which, by themselves, are not capable of directly stimulating the irradiated bone marrow recipient (Table 52). The failure of bone marrow cells incubated

with SRBC for 6 hours or less to transfer plaque-forming capacity to irradiated allogeneic recipients (Table 54) further attests to the validity of the interpretation given, that the capacity of the incubated bone marrow cells to transfer immunocompetence is incumbent upon their being stimulated by the SRBC during the in vitro incubation.

It has been demonstrated in Chapter V.C that bone marrow cells of rabbits injected 24-48 hours previously with an antigen lose the capacity to transfer immunocompetence with respect to this particular antigen but are quite capable of mediating the humoral immune response to other antigens in the recipient rabbit. It seemed plausible to speculate that the bone marrow contains the antigen-reactive cells (ARC) which vacate the bone marrow following contact with the antigen in vivo, leaving the marrow deficient in ARC capable of mediating a humoral immune response to the particular antigen upon cell transfer of this marrow to an irradiated recipient. However, an alternate hypothesis presented was that the bone marrow cells become "tolerant" following contact

with the antigen in vivo and are therefore incapable of transferring immune responsiveness. Various findings would mitigate against this latter suggestion. The experiments presented above demonstrate unequivocally that normal bone marrow cells can be incubated with a large number of SRBC in vitro without resulting in the induction of a state of tolerance in these cells. Certainly, the number of SRBC (10^8 cells) per bone marrow lymphoid cell (10^8 - 10^9 cells) in the incubate is many hundred-fold greater than that occurring in vivo following the intravenous administration of 10^9 SRBC. Horiuchi and Waksman (286) have observed that a state of temporary immunologic tolerance can be induced in adult rats injected intrathymically with the specific antigen. However, tolerance has not been shown to occur in the adult animal following the injection of an immunogenic dose of the antigen intravenously. Golub and Weigle (168) have demonstrated that a minimum of 4 to 5 days must elapse between the injection of the tolerogenic dose of the antigen and the actual induction of tolerance. Lymph node cells obtained from animals injected with the toler-

ance-inducing antigen could transfer antibody forming capacity to recipients with respect to this same antigen up to day 4 following the injection of the antigen into the cell donor. However, it has been observed in Chapter V.C. that the bone marrow cells in the rabbit lose the capacity to transfer antibody-forming capacity with respect to the immunizing antigen within 12 to 24 hours following the intravenous injection of an immunogenic dose of the antigen. They also lose their capacity to respond with blastogenesis and mitosis within 8 to 24 hours following the administration of the antigen, even if the latter is given in complete Freund's adjuvant, an immunization procedure almost guaranteed not to induce immunologic tolerance (46). On the basis of the evidence presented, it would not appear overly presumptuous to consider that the ARC in the bone marrow actually vacate that organ following exposure to antigen in vivo.

The finding that irradiation of the bone marrow cells failed to kill these cells, using the dye exclusion technique, but succeeded in rather dramatic fashion in inhibiting the cell response to PHA was certainly not anticipated. These observations imply that irradiation altered

the surface properties of the cells, rendering them incapable of responding to PHA, or that the irradiation actually killed the PHA-responsive cells. These latter cells must constitute no more than a minority of the lymphoid cell population in the bone marrow since the number of dead cells in the irradiated suspensions was not significantly greater than in the control, unirradiated preparations. In retrospect, this finding is not too surprising in view of the observation of Dukor and Deitrich (287) that only the thymus-dependent lymphoid cells, those capable of mediating cellular immunity, respond to stimulation with PHA. One might speculate as to the number of thymus-derived lymphoid cells in the rabbit bone marrow which cannot, in view of the viability studies performed, be large in number.

Even more surprising were the results of the experiments concerned with the in vivo incubation of irradiated rabbit bone marrow cells with the antigen, SRBC. Since the irradiated cells were capable of transferring plaque-forming capacity to irradiated (800r) recipients if injected immediately following in vitro irradiation, it must be

concluded that the immunocompetent cell in the bone marrow is not identical to the cell responding to stimulation with PHA, since it survived the 4,000r irradiation treatment. These findings are in line with those of Daguillard and Richter (266) who arrived at a similar conclusion using goat anti-rabbit immunoglobulin antiserum to stimulate rabbit lymphoid cells in vitro. Nevertheless, damage must have been incurred in the cells as a result of the irradiation treatment since cells first irradiated (4,000r) and then incubated with or without the antigen (SRBC) for 24 hours were incapable of transferring antibody-forming capacity.

The results serve to better understand why irradiation (500r - 700r) of a rabbit 24 hours following injection of an antigen does not prevent the immune response whereas irradiation of the animal either prior to or just after the administration of the antigen can depress or completely inhibit the immune response (268). Nettesheim and Hammons (288) and Miller (289) have presented evidence to show that the antibody-forming cell (AFC) is

irradiation-resistant. The cell affected by the dose of irradiation therefore appears to be the ARC in the bone marrow, a consideration substantiated experimentally in Chapter V.D. However, once the ARC has been stimulated by the antigen, its subsequent immunologic functions are not susceptible to the dose of irradiation (4,000r) which is otherwise capable of inactivating the antigen-unstimulated ARC. Furthermore, the antigen-stimulated ARC are not inactivated even if exposed to 10,000r irradiation in vitro. These results are in agreement with the findings of other investigators who showed that cells already engaged in several active metabolic functions are less susceptible to the lethal effects of irradiation than are resting cells. Howard (290) reported that the normal physiologic activities of mammalian cells are not affected by sublethal doses of irradiation whereas formation of new enzyme systems is highly radiosensitive. Jaroslow (291) has shown that DNA synthesis has to be inhibited prior to stimulation by the antigen in order to inhibit the immune response. Except for a few reports (292, 293), it is generally agreed (275, 280, 294-297)

that the AFC becomes radio-resistant once the antigenic stimulus has triggered the antibody-forming machinery. In fact, it has been observed that the incorporation of labelled amino-acids into the antibody molecules synthesized by irradiated (10,000r) immune mouse spleen cells in culture is qualitatively and quantitatively similar to that incorporated by non-irradiated control cell preparations (296).

It has also been demonstrated that different metabolic functions of the same cells are not equally affected by the same dose of irradiation. The essential metabolic functions of cells, such as oxidative phosphorylation (293) and DNA synthesis (298), but not phagocytosis (298), are completely inhibited by exposure of the cells to 700r in vitro. However, a dose of 4,000r is required in order to inhibit blastogenesis in the mixed human leukocyte culture (299) and 10,000r has been found to be required to inhibit spleen colony formation by irradiated (10,000r) spleen stem cells transferred to irradiated (850-950r) recipient mice (300).

The data presented also lend further support to the concept of the existence of pre-committed clones of ARC in the normal rabbit bone marrow. It has been demonstrated in Section F that passage of normal bone marrow cells through antigen-sensitized glass bead columns results in the specific retention of cells capable of transferring immunocompetence with respect to the antigen adsorbed onto the column. On the other hand, the cells which pass through the column are capable of mediating antibody-forming capacity to other, non-cross-reacting antigens. Nevertheless, the possibility exists that pre-commitment with respect to antigen B need not be absolute and that it could be enhanced with respect to antigen A, and conversely diminished with respect to antigen B, if the cells were stimulated with high concentrations of antigen A in vitro. Such was not the case, however. Incubation of the normal bone marrow cells with high concentrations of SRBC (or HRBC) for 24 hours in vitro did not diminish their capacity to transfer immunocompetence with respect to the second non-cross-

reacting antigen, HRBC (or SRBC). Thus, it appears that pre-committment is absolute and cannot be detectably altered by incubation of the cells with antigens other than those with which the cells are already committed to react.

These findings disagree somewhat with those of Perkins and Makinodan (301). The antibody-forming capacity of mouse spleen cells to rat red cells following transfer of the spleen cells into lethally-irradiated recipients declined as the time interval between cell transfer and in vivo stimulation with the antigen was lengthened. This decline was accentuated by exposing the spleen cells in vivo to a non-cross-reacting antigen, sheep red cells, immediately after transfer. However, the decline in antibody-forming capacity to rat red cells was not observed when spleen cells from immunized mice were used. These results suggested that exposure in vivo of immunologically competent cells precommitted to respond to one antigen, i.e. rat red cells, to a non-cross-reacting antigen can alter the subsequent responsiveness of the cells with respect to the specific antigen, the rat red cells (301). Since the hemopoietic

tissues in the irradiated recipient mouse must be considered to be obliterated by the lethal irradiation, it is probable that a substantial number of the transferred mouse spleen cells, which have been shown to have hemopoietic function (302, 303), may be diverted towards hemopoiesis in the absence of an antigenic stimulus. The original immunocompetent cells would therefore no longer be available for the production of specific antibodies. In fact, these investigators (301) found that administration of both bone marrow cells and spleen cells from the donor to the lethally-irradiated recipient resulted in a lesser reduction in the antibody-forming capacity of the transferred spleen cells, as compared to that in the recipient given normal spleen cells only. Another explanation for the dichotomy of our results and those of Perkins and Makinodan (301) is that in our system only the ARC were subjected to high concentrations of a non-cross-reacting antigen in vitro whereas the AFC were exposed to both specific and non-cross-reacting antigens simultaneously in vivo. On the other hand,

Perkins and Makinodan injected the non-cross-reacting antigen into the recipient animal, exposing both the ARC and the AFC to the antigen. The specific antigen was not injected until several days later. Since the mouse spleen consists of both antigen-reactive (ARC) and antibody-forming (AFC) cells (92, 126), one can only speculate as to whether the ARC and/or the AFC are affected in the Perkins and Makinodan model (301). Therefore, they may have induced a state of antigen competition at the level of the AFC which appears to be a pluripotential cell (103, 128, 304), and not at the level of the ARC (305), which appear to possess an inalterable commitment to the specific antigen (Chapter V.C, D and F). A third explanation to account for the differing results may be attributed to the high concentration (10^{10} RBC) of the non-cross-reacting antigen required by these latter investigators to produce a subsequent inhibition of response to the specific antigen. In our hands, this dose of antigen is lethal in a large proportion of the animals and may result in a "non-specific" general depression of immunocompetence (305, 306).

CHAPTER VI

GENERAL DISCUSSION

The experiments carried out in this investigation were based on a previous finding from this laboratory, that the normal rabbit bone marrow lymphocytes, but not the lymphocytes of any of the other lymphoid organs, could respond with blastogenesis and mitosis upon incubation with a host of antigens in vitro (46). It was also observed that the "immune" rabbit bone marrow cells could not respond in this way when taken from the immunized rabbit 12-48 hours following immunization and incubated with the specific immunizing antigen in vitro. On the other hand, the response to other non-cross-reacting antigens was unimpaired (46). On the basis of these and other findings, it was concluded that this in vitro response of the bone marrow lymphocytes represents an immunological reaction (46, 144). However, this conclusion was based essentially on a single protocol, the in vitro blastogenic response, and it required confirmatory evidence obtained by other procedures before it

could be accepted as established fact. Furthermore, one could not state whether it was the ARC and/or the AFC in the bone marrow which responded with blastogenesis in vitro.

On the basis of the experiments carried out in this study, one can categorically state that (a) the immunocompetent cell in the normal rabbit bone marrow is the ARC, and not the AFC; and (b) the bone marrow is the prime, if not the only, source of ARC in the rabbit.

A number of experimental protocols were utilized in the experimental procedures upon which these conclusions are based. Briefly, normal or "primed" cells from the various lymphoid organs (bone marrow, thymus, spleen, sacculus rotundus, appendix, lymph node and blood) were transplanted into irradiated or tolerant allogeneic recipient rabbits. The cells were either stimulated with the antigen(s) by in vitro incubation for 24 hours prior to their transfer or else they were stimulated following their transfer into the irradiated

recipient. The recipients were tested for their immune response by the capacity of their spleen cells to form hemolytic plaques in agar gel or by determining the circulating antibody titer by the hemagglutination technique. In initial experiments, it was established that only the spleen cells of the intravenously immunized rabbit are capable of producing hemolytic plaques in vitro. By the use of antiallotype antiserum as a marker, it was demonstrated that the antibody-forming cells in the irradiated recipient are of recipient and not donor origin. This finding implies that the donor cells were therefore the ARC, which are radiosensitive and that the AFC were of host origin and radio-resistant.

It was also demonstrated that in the case of most antigens, the bone marrow serves as the sole source of ARC in the rabbit. However, the sacculus rotundus cells and circulating leukocytes as well as bone marrow lymphocytes were capable of transferring immunocompetence to irradiated recipients with respect to one of the antigens used, sheep red blood cells. The interpretation of this latter finding has been discussed at length

(Chapter V.B). "Primed" bone marrow, in contrast to normal bone marrow, failed to transfer immunocompetence to irradiated recipients. A direct relationship was observed between the capacity of the irradiated recipient to respond with antibody formation and the reappearance of the ARC population in the bone marrow. Furthermore, the ARC activity could be initiated in vitro, by incubation of the cells with the antigen used in a concentration which could not induce an immune response if injected into the recipient rabbit. The ARC could be separated by passage of the normal bone marrow cells through an antigen-sensitized glass bead column, followed by elution of the cells from the glass beads. The ARC so obtained, which were all morphologically small lymphocytes, were found to be specific in their reactivity to the antigen originally adsorbed onto the glass beads and could not transfer immunocompetence to other antigens. However, the effluent cells which passed through the antigen-sensitized glass bead column could transfer immunocompetence to all the antigens tested except to that antigen adsorbed onto the column.

These findings, therefore, strongly suggest that the ARC in the normal rabbit bone marrow vacate the bone marrow following interaction with the antigen in vivo. An equally plausible, alternate theory is that the bone marrow ARC become immunologically tolerant following the intravenous administration of the antigen. The demonstration that the antigen-reactive cells in normal bone marrow interact with the antigen adsorbed onto glass beads or with a relatively high concentration of the antigen in vitro without being rendered tolerant but capable of expressing immunocompetence following their elution from the glass beads, support only the first concept presented above. One may then assume that the bone marrow of an immune rabbit but not any single cell in the marrow is, in fact, immunologically incompetent with respect to the specific immunizing antigen. Since the cells which vacate the bone marrow following antigenic stimulation appear to be directed to only a single antigen, it would appear that they all arise from a single precursor cell or clone of cells, thus leaving

the bone marrow deficient in cells capable of responding to this antigen. This concept implies the existence of population of bone marrow cells committed or programmed to interact with a site on the antigen molecule characterized by a unique molecular composition and configuration. How can this interpretation be reconciled with a pragmatic approach based on the deduction that there cannot exist more than just a small number of bone marrow antigen-reactive cells precommitted to react with any particular antigen, in view of the large number of antigens which are known to exist (microbial, synthetic, haptenic, drugs, inanimate protein antigens, viruses, etc.). The resolution of this dilemma rests on the probability that the small number of antigen-reactive cells in the resting state directed to a particular antigen are sufficient to mediate the immune response, in view of their capacity to undergo explosive proliferation following interaction with the antigen. This has been demonstrated for the thymic antigen-reactive cell in the mouse (70) and the bone marrow antigen-reactive cell in the rabbit

(46). This proliferative activity of the antigen-reactive cells in the rabbit takes place in an organ(s) other than the bone marrow, since the basal activity of the "primed" rabbit bone marrow cells in vitro is not higher than normal, resting levels (46). However, the target organ(s) for the bone marrow ARC and their further participation in the immune response remain to be elucidated.

The concept presented above begs the further assumption that, in the rabbit, the antibody-forming cells originate in lymphoid organs other than the bone marrow and that their final "resting" site, following the intravenous administration of the antigen, is the spleen (Figures 4 and 14). Where do the potential antibody-forming cells (AFC) originate and where do they reside in the absence of antigenic stimulation? In this study there are no experimental data to shed light on this question. Therefore, one can only speculate. Since antibody formation can be elicited in cells in the thymus (308), spleen (309) or draining lymph node (310, 311), depending on the

route of administration of the antigen (intrathymic, intravenous and foot-pad, respectively), it would appear that the potential antibody-forming cells are already residing in the lymphoid organs and that the presence of antigen at these sites constitutes one of the determining factors concerned with the initiation of the local immune response. Our results indicate that the committed antigen-reactive cells vacate the bone marrow following interaction with the antigen (activated antigen-reactive cells) and migrate to one or more of the lymphoid organs where they probably transfer activated antigen to the antibody-forming cells (Figures 12 and 13). Interaction of these cells with the activated antigen results in their transformation into memory cells, which are synonymous with Y cells (312) or antigen recognizing antibody-forming cells (261). These latter cells may, at this stage, be capable of forming, but not of releasing, humoral-type antibody, but they can be triggered to do so if stimulated by unprocessed or native antigen and are thereby transformed

into actual antibody-forming cells or Z cells (Figure 12) (312, 313). Depending on the type and nature of the immunization, these latter cells will masquerade as either plasma cells (310, 314-318) or lymphocytes (319-321).

It can also be argued that antigen-reactive cells must also, to a certain extent, be dispersed and cannot, if the above discussion has any validity, be localized solely to the bone marrow even in the resting unimmunized state. Moreover, it would be difficult to reconcile the concept of the bone marrow as the main source of antigen-reactive cells with the fact that primary immune responses have been achieved in vitro with rabbit lymph node and spleen fragments (231-233) and mouse spleen cells (91, 240, 322, 323). Actually, one may logically anticipate such a situation since the bone marrow is not a static assemblage of cells, but a fluid system with the cells free to enter the blood stream. It is therefore likely that some antigen-reactive cells are always vacating the bone marrow but that all the antigen-reactive cells of

the specific clone, and therefore of the same antigenic specificity, can be evicted from the bone marrow following the intentional injection of a massive dose of the antigen. The scheme of cellular interactions postulated in the induction of the primary immune response in the rabbit is presented in Figures 12 and 13.

The data presented in Chapter V.E indicate that the immunocompetent cell(s) affected in the induction of the immunologically-tolerant state in the neonate is probably identical to that affected in the suppression of the immune response by irradiation. In the former case, the antigen-reactive cell is made tolerant and therefore immuno-incompetent, and in the latter situation, the antigen-reactive cell is inactivated by the irradiation. The antigen-reactive cell, therefore, must be considered to have undergone some reaction(s) in the induction of tolerance since the bone marrow, following induction of the tolerant state, no longer possesses cells exhibiting antigen-reactive properties directed toward the tolerogenic antigen, similar to the "primed" bone marrow follow-

ing induction of an immune response. The "tolerant" marrow therefore simulates the "primed" marrow with respect to its immuno-incompetence in cell transfer experiments, although the mechanism whereby immuno-incompetence has been induced in the bone marrow in these two diametrically-opposed immune states is probably different. Interaction with antigen was postulated by Byers and Sercarz (313) to result in saturation of all intracellular complementary sites of the X cell. The question may therefore be asked whether the antigen-reactive cell actually becomes tolerant and remains in the bone marrow in an unresponsive state or whether it actually persists as an antigen-reactive cell in some other organ in the tolerant rabbit, as distinct from the antibody-forming cell characteristic of the primary response or the memory cell characteristic of the secondary immune response. Since it has been demonstrated that the antigen-reactive cells can interact with the antigen in vitro and not be rendered tolerant or immuno-incompetent

it is considered unlikely that interaction with antigen in vivo would have induced a tolerant state in this cell.

The concept that a multi-cellular system (ARC and AFC) exists to provide for the immune response is based on findings in two animal species, the rabbit and the mouse. The work in the rabbit is clear-cut. The bone marrow contains the cells which can interact with the antigen (ARC), the manifestation of the interaction consisting of blastogenesis and mitosis (proliferation), but not antibody formation. Although the organ of origin of the antibody-forming cell in the rabbit has not yet been established, it is definitely not the bone marrow (Chapter V.D). Therefore, in the rabbit, the terms ARC and AFC define exactly the functions intended - the interaction of the ARC with the antigen, leading to proliferation of the cells but not to antibody formation, and the interaction of the AFC with what is probably an activated or processed antigen moiety to form humoral-type antibodies. A similar situation apparently exists in the mouse except that the thymus, and not the

bone marrow, possesses the ARC. A number of investigators have reported that an immune response can be elicited in an irradiated mouse provided the mouse had been injected, intravenously, with isogeneic thymus and bone marrow cells (4, 5, 77).

It is of more than academic interest to note that the situation in the mouse appears to be quite different from that in the rabbit. In the normal adult mouse, it has been demonstrated that the thymus, and not the bone marrow, serves as a source of the ARC (4, 70) and that the bone marrow is the source of the AFC with respect to SRBC (5, 82). However, the situation is not as clear-cut as in the rabbit since ARC, as well as AFC, can also be detected among the spleen, lymph node and thoracic duct cells of the mouse depending upon the antigen used (211). Since cells of any of these latter organs can substitute for mouse thymus cells in the restoration of immunocompetence upon their transfer to irradiated or neonatally-thymectomized recipients (4, 5), they must be considered to manifest ARC activity.

Furthermore, lymph node, spleen or thymus cells can transfer immunocompetence to an immunologically-tolerant mouse (160-162). It has been demonstrated that the unresponsive cell in the tolerant rabbit is the ARC, and not the AFC. If it is also the ARC which is affected in the tolerant mouse, then these data favour the spleen and lymph node, in addition to the thymus, as sources of ARC in the mouse. The ARC in the mouse are therefore not as uniquely segregated, anatomically, as they are in the rabbit, where they can be found to reside only in the bone marrow.

Although the ARC, in their immunocompetent stage of life, are found in the bone marrow in the rabbit and in the thymus of the mouse, does it necessarily follow that these cells are endemic to these organs or could they be the progeny of stem cells derived from another organ. In this regard, the results of many investigations which have utilized the transfer of chromosomally-marked syngeneic cells into irradiated mice strongly suggest that the ARC is a bone-marrow derived cell (81, 82). However, these

investigations do not allude as to whether the bone marrow cells mature in the thymus to become competent ARC or whether they undergo the first stage of maturation in the thymus and become functionally active ARC only when they migrate into the peripheral lymphoid organs. Furthermore, it has been demonstrated that the potential ARC in the transferred bone marrow cell suspensions of mice need not physically penetrate into the thymus to mature to functionally-active ARC, but that they can mature in the spleen and lymph node of a thymectomized animal under the influence of a thymus graft placed in a semipermeable chamber, which is probably acting in an endocrine fashion (179). It would therefore appear that the bone marrow in the mouse serves as the source of the mature AFC and the immature ARC. Thus far, it has been demonstrated that the bone marrow in the rabbit serves as the source of mature ARC, and not of mature AFC. Whether it also functions as the source of immature AFC is not clear at the present time.

One aspect of the problem, however, remains to be clafified. What criteria should be used to distinguish the antigen-reactive cell? On the basis of the preceeding discussion, the term "antigen-reactive cell" or "antigen-sensitive cells" should be reserved for only those cells, at least in the rabbit and the mouse, which react with the antigen but do not subsequently form antibodies. However, some investigators concerned with elucidating methods for the demonstration, recognition and localization of ARC in the mouse, have greatly confused the situation, since they have utilized the term ARC to designate cells in the spleen capable of inducing "hemolytic foci" (273) or "bacterial immobilization in gel" (121), both activities necessitating the mediation of antibodies. Armstrong and Diener (121) state that "the method is based on the belief that when these ARC are injected into a lethally irradiated host, they embed in the spleen in predictable concentrations and respond to an antigenic stimulus by proliferating and differentiating into colonies of ARC." They, in fact, state

categorically that the success of this technique is dependent upon antibody secreted by the antigen-reactive cell. Kennedy et al (273) state that the interpretation of their results is dependent on two postulated properties of these cells: sensitivity to antigenic stimulation by sheep erythrocytes and ability to respond to this stimulation by proliferating to give rise to cells capable of hemolysin production. Playfair et al (75) arrived at a similar conclusion. Such an interpretation is, at the very least, inconsistent with the definition of the term antigen-reactive cell. In view of the fact that, in both the rabbit (46) and the mouse (4, 70), it has been demonstrated that ARC and AFC are independent cellular entities and do not differentiate one into the other, and since the mouse spleen has been shown to consist of a mixture of ARC and AFC (4, 5, 6, 91), the interpretations of Armstrong and Diener (121) and of Kennedy et al (273) rest on very tenuous grounds.

Another question which requires consideration is whether one can define the induction of the primary response in the rabbit as a result of a two-cell interaction,

the antigen-reactive and antibody-forming cells or whether it is necessary to postulate a third cell-type, the macrophage. The evidence favoring an eminent role for the macrophage in the induction of the immune response has been well documented and has accrued from both in vitro and in vivo investigations (108, 109). However, the exact stage in the immune response where the macrophage acts has hitherto not been precisely stated or defined. Evidence of a three-cell interaction has been presented by Mosier and Coppleson (107), Pribnow and Silverman (271) and Gallily and Feldman (115). Pribnow and Silverman (271) have demonstrated that the interaction of the antigen with macrophage is the initial cellular event in the induction of the primary immune response. They observed that irradiated rabbits that recieved non-immune lymph node cells and peritoneal macrophages which had been incubated with the antigen in vitro were unable to form antibody whereas normal recipients of these cells exhibited a typical primary immune response. The transfer of antigen-incubated lymph node cells, in the absence of macrophages, failed

to elicit an immune response in either normal or irradiated rabbits. Furthermore, macrophages which had been irradiated following their incubation with the antigen were unable to initiate an immune response when transferred to normal recipients.

On the basis of our results, these data imply that the antigen-reactive cell in the recipient, whose functions in the immune response commence following the macrophage-antigen interaction, is irradiation-sensitive to 800r but not to 550r in both the mouse and the rabbit. However, it would not appear to be as irradiation-sensitive as the macrophage which is inactivated by 550r irradiation (115). Therefore, the sequential transfer of immunologic information leading to the initiation of the immune response-macrophage to antigen-reactive cell to antibody-forming cell - is broken following irradiation.

The finding that ARC are found only in the bone marrow and that the bone marrow could be depleted of only one clone of cells reactive to the immunizing antigen but not to non-cross-reacting antigens reaffirms the

concept of Clonal Selection, first postulated by Jerne (326) and Burnet (307). It must be stressed, however, that insofar as the rabbit is concerned, the Clonal Selection Theory applies to the ARC only, and not the AFC. This is supported in our studies by the findings that clones of ARC can be separated from normal bone marrow cells by passage of the latter through antigen-sensitized glass bead columns. This would suggest that the bone marrow contains the different clones of lymphoid cells which either transform directly into or give rise, by mitosis and differentiation, to the pre-committed antigen-reactive cells. The concept of pre-commitment of the cell to interact with a particular antigen encountered at some future time (126, 238) is proven by our finding that the in vitro incubation of bone marrow cells with one antigen will stimulate only the groups of cells precommitted to react with that antigen. The in vitro stimulated marrow cells can still initiate an immune response against a non-cross-reacting antigen. This would indicate that the bone marrow ARC must possess antibody-like receptor group(s) on its surface, as has been sug-

gested by a number of investigators (111, 264) capable of interacting with the antigen and thus initiating the inter and/or intra-cellular chain of events which culminate in the humoral immune response. This interpretation of our data necessarily supports the side chain concept of Ehrlich presented almost a century ago (263). Other investigators (325-327) have also presented evidence in favor of the Clonal Selection Theory of antibody formation, although the organ site of origin of these cells has not previously been seriously entertained.

Based on our findings and those reported in the literature (3, 7, 18, 94), a partly speculative diagramatic representation is shown in Figures 15 and 16 describing the role of the rabbit bone marrow in the primary immune response. Following contact with an antigen for the first time, the rabbit bone marrow ARC(s) either migrates to the bursal homologue (appendix and sacculus rotundus) or to the thymus to undergo cellular maturation and mediate humoral and cellular immune responses respectively. These mature bone marrow-derived cells are then

capable of releasing the antigen in an activated form. Following exposure to the activated antigen, the antibody forming cells residing in the spleen and other organs (Figure 14) are then rendered capable of forming antibody or mediating the cellular immune reaction.

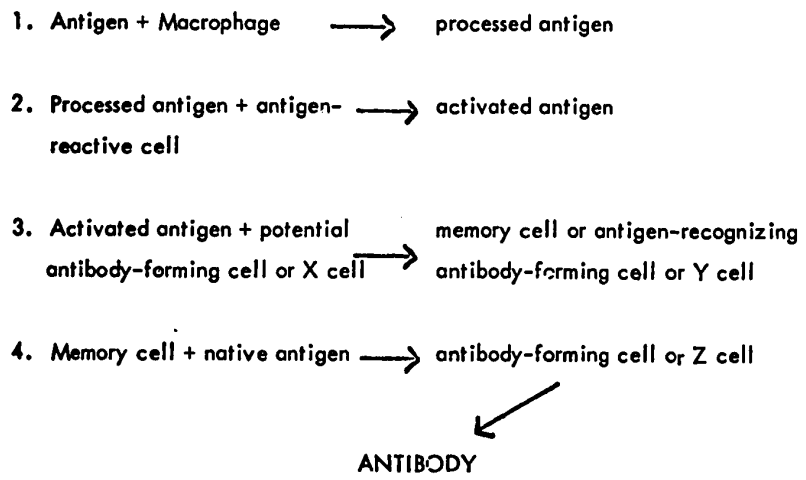

Figure 12. THE CELLULAR INTERACTIONS POSTULATED IN THE INDUCTION OF THE PRIMARY IMMUNE RESPONSE IN THE RABBIT

Figure 13. THE CELLULAR INTERACTIONS DURING THE IMMUNE RESPONSE IN THE RABBIT. A DIAGRAMATIC REPRESENTATION

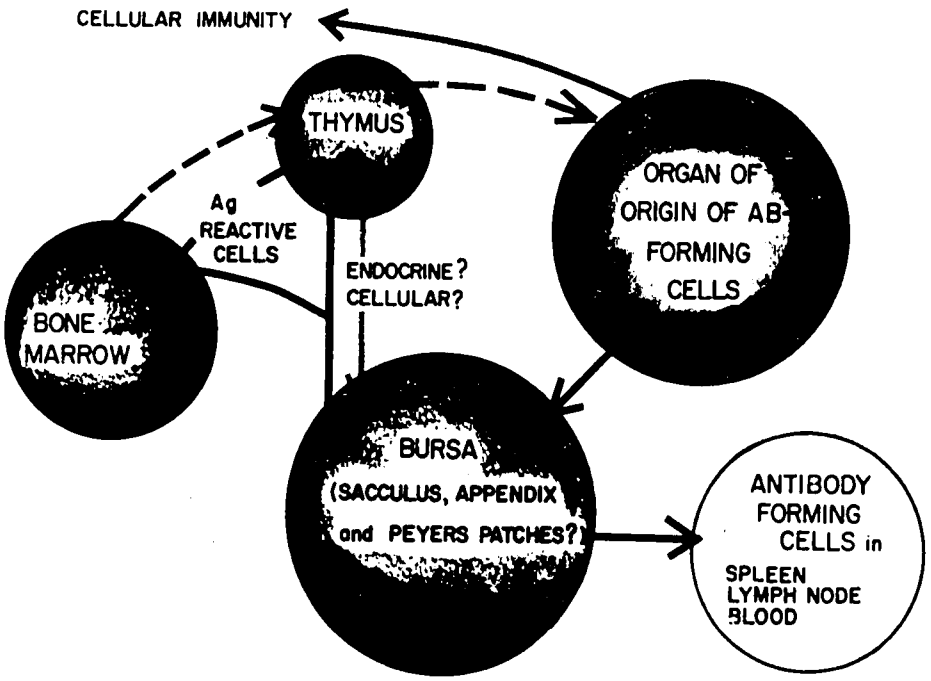

Figure 13. THE CELLULAR INTERACTIONS DURING THE IMMUNE RESPONSE IN THE RABBIT. A DIAGRAMATIC REPRESENTATION

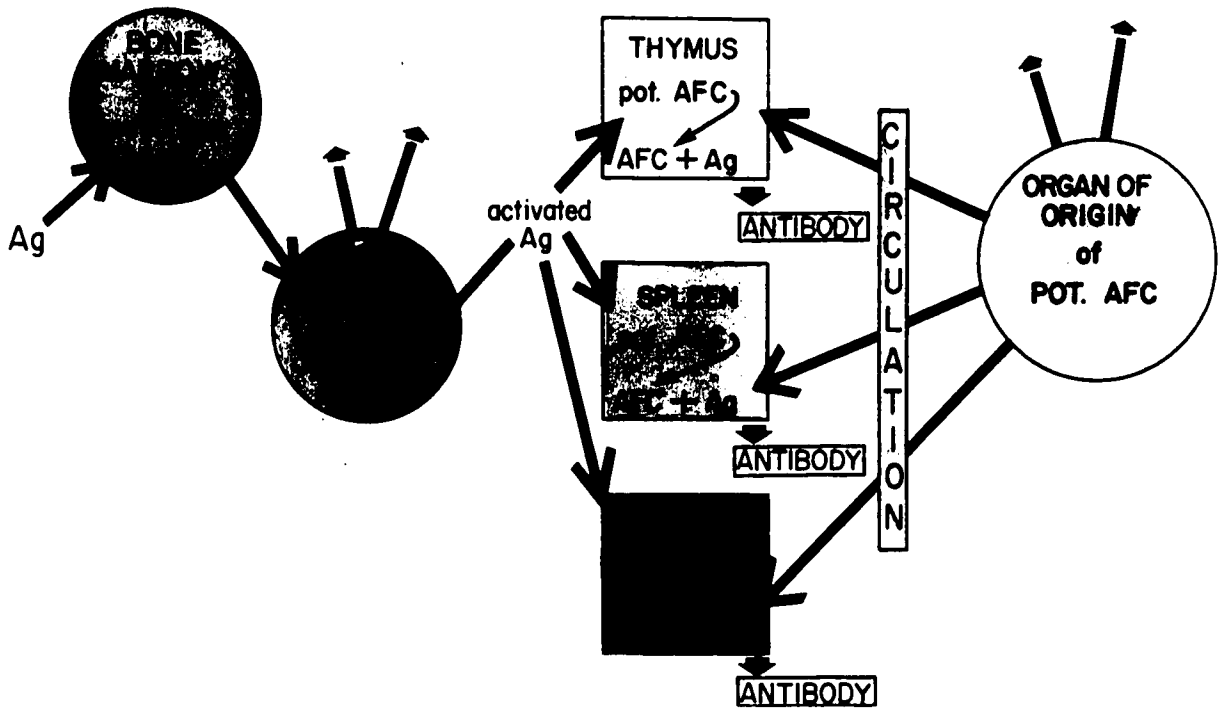

Figure 14. THE SOURCES AND DESTINATIONS OF ANTIGEN-REACTIVE (ARC) AND ANTIBODY-FORMING (AFC) CELLS IN THE RABBIT, FOLLOWING INTRAVENOUS ADMINISTRATIONS OF ANTIGEN

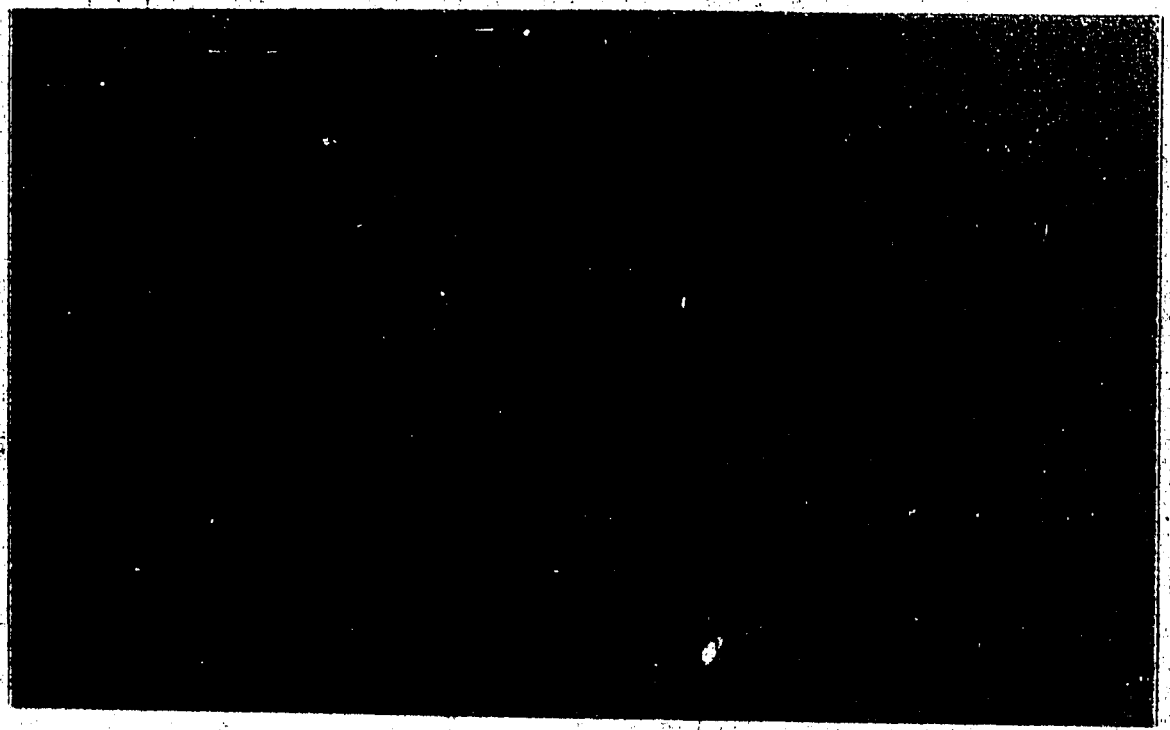

Figure 15. THE ROLE OF THE BONE MARROW CELL IN THE IMMUNE RESPONSE IN THE RABBIT

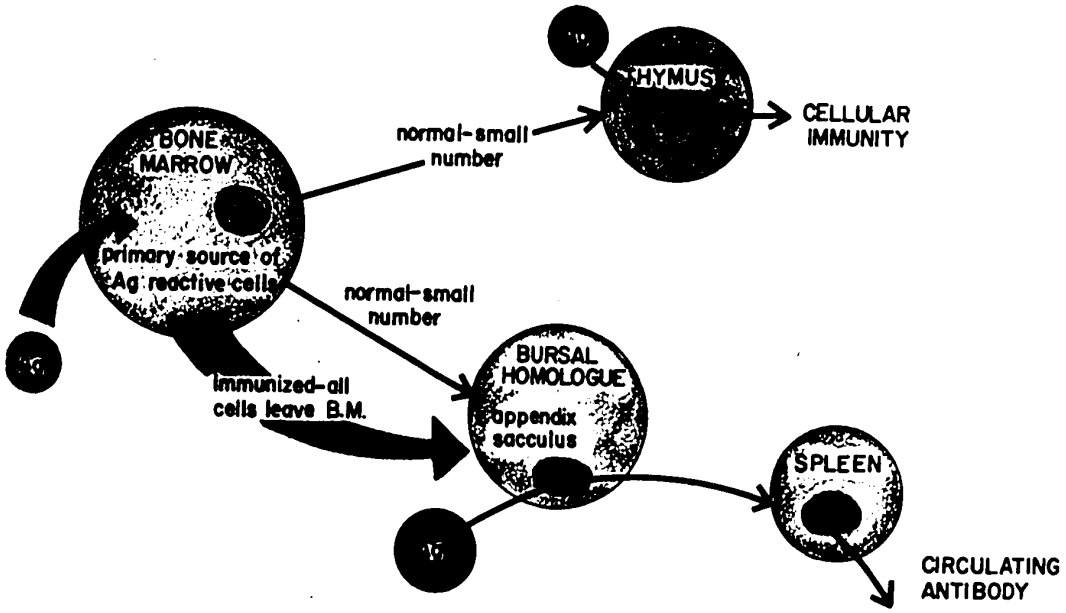

Figure 15. THE ROLE OF THE BONE MARROW CELL IN THE IMMUNE RESPONSE IN THE RABBIT

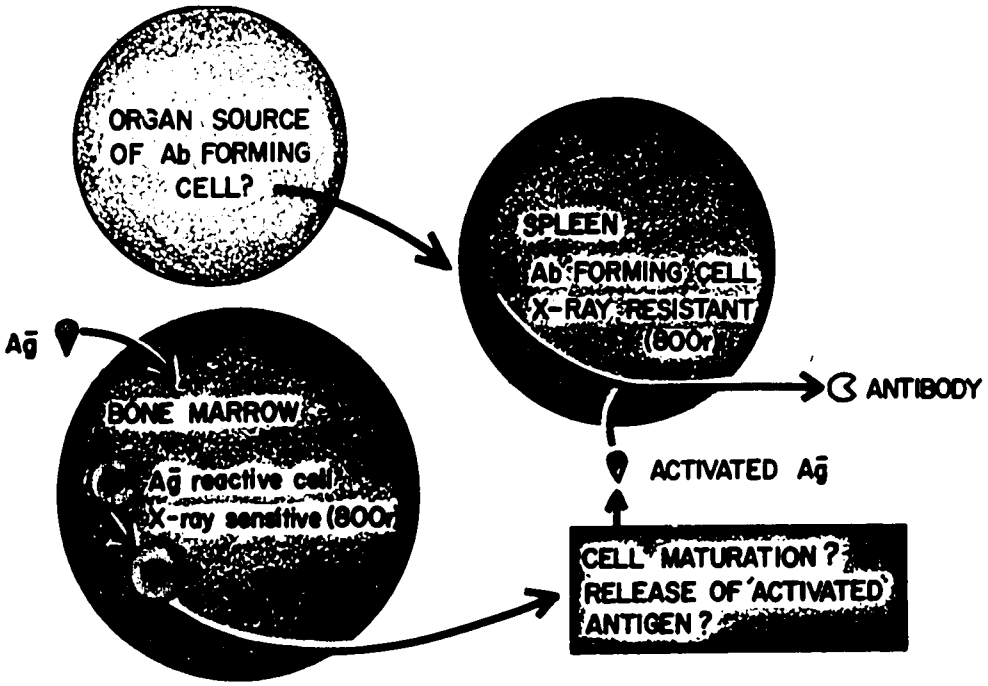

FIGURE 16 BONE MARROW SOURCE OF ANTIGEN REACTIVE CELLS IN THE RABBIT

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CHAPTER VII

SUMMARY AND CONCLUSIONS

1. Lymphoid cells of the various lymphoid organs of the normal rabbit were transferred into irradiated (800r) immunoincompetent allogeneic recipient rabbits. With respect to four of the five antigens tested, only the bone marrow cells could transfer antibody-forming capacity. In the case of the fifth antigen, sheep erythrocytes, the sacculus rotundus, mesenteric lymph node and circulating lymphoid cells, as well as the bone marrow cells, could transfer immunocompetence. This latter finding may represent a secondary response to the Forssman antigen present in sheep erythrocytes. It is concluded that the bone marrow constitutes the only organ source of the antigen-reactive cells in the normal unimmunized rabbit.

2. It was demonstrated that only viable normal bone marrow cells are capable of transferring antibody-forming

capacity to irradiated recipient rabbits. Neither sonicates nor heat-killed preparations of normal rabbit bone marrow cells possessed this capacity.

3. Irradiated rabbits given allogeneic bone marrow cells from normal adult donors responded to an injection of sheep red blood cells by forming circulating antibodies. Their spleen cells were also capable of forming many plaques using the hemolysis in gel technique and were also capable of undergoing blastogenesis and mitosis and of incorporating tritiated thymidine upon exposure to the specific antigen in vitro. However, irradiated rabbits injected with allogeneic bone marrow obtained from rabbits injected with sheep red blood cells 24 hours prior to sacrifice (primed donors) were incapable of mounting an immune response following stimulation with sheep red cells (Tables 59 and 60). This loss of reactivity by the bone marrow from primed donors is specific with respect to the immunizing antigen, since the immune response of the irradiated recipients to non-

cross-reacting antigens is unimpaired.

4. Treatment of the bone marrow donors with high-titered specific antiserum (passive immunization) to sheep red cells for 24 hours prior to sacrifice did not result in any diminished ability of their bone marrow cells to transfer antibody-forming capacity to sheep red blood cells. This finding suggests that a feedback mechanism does not operate in this situation.

TABLE 59

THE ROLE OF THE BONE MARROW IN THE RABBIT

IN THE INDUCTION OF THE IMMUNE RESPONSE

Types of cells tested	Response in Vitro Blastogenesis with respect to		Response in Vivo Transfer of Ab-forming capacity in irradiated host to	
	immunising Ag	unrelated Ag	immunising Ag	unrelated Ag
Normal bone marrow	+	+	+	+
Primed bone marrow*	-	+	-	+
Immune bone marrow**	-	+	+	+

* Obtained from rabbit 8-24 hours following immunisation, before immune response.

** Obtained from rabbit 8-20 days following immunisation.

TABLE 60

THE ROLE OF NORMAL AND PRIMED RABBIT BONE MARROW

IN THE MEDIATION OF IMMUNOLOGICAL REACTIVITY

Type of Response	Response Obtained with Normal Bone Marrow in Irradiated Recipient	Response Obtained with Primed Bone Marrow (i.e. S-rbc) in Irradiated Recipient
1. Response to Specific Antigen (i.e. S-rbc) in Vitro (Blastogenesis)	+	-
2. Response to Other Antigens in Vitro (Blastogenesis)	+	+
3. Transfer of Plaque-Forming Capacity to Specific Ag (S-rbc) in Irradiated Recipient Rabbits	+	-
4. Transfer of Plaque-Forming Capacity to Other Antigens in Irradiated Recipient Rabbits	+	+
5. Transfer of Antibody-Forming or Plaque-Forming Capacity to Immunologically-Tolerant Recipient Rabbits	+	-

5. Bone marrow cells obtained from rabbits of one allotype were injected into irradiated rabbits of a different allotype. The recipients were also injected with sheep red blood cells and their spleen cells were tested for plaque-forming capacity seven days later. Spleen cells of all recipients gave large numbers of plaques as did spleen cells incubated with antiserum directed toward donor allotype. However, incubation of the recipient spleen cells with antiserum directed toward recipient allotype completely suppressed plaque formation. These results demonstrate that antibody formation in irradiated recipients of transferred lymphoid cells is a property of the recipient animal and that the antibody-forming cell is relatively irradiation-resistant.

6. Rabbits were made immunologically tolerant to either HSA or BGG by the neonatal administration of the antigen. At ten weeks of age, they were challenged with the tolerogenic antigen and were found to be non-responsive. However, these tolerant rabbits could respond with

humoral antibody formation directed toward the tolerogenic antigen if they were treated with normal, allogeneic bone marrow or bone marrow obtained from a rabbit made tolerant toward a different antigen. They were incapable of responding if they were given bone marrow obtained from a rabbit previously made tolerant to the tolerogenic antigen. Irradiated rabbits were unable to respond if treated with tolerant bone marrow but could respond well if given normal bone marrow. Since it has previously been demonstrated that the antibody-forming cell, in an irradiated recipient of allogeneic bone marrow, is of recipient and not donor origin, the data presented strongly indicate that the unresponsive cell in the immunologically-tolerant rabbit is the antigen-reactive cell.

7. The antigen-reactive cells in normal rabbit bone marrow could be isolated from a suspension of marrow cells by passage of the cells through an antigen-sensitized

glass bead column. The cells which passed through the column were deficient in antigen-reactive cells directed to the antigen used to sensitize the glass beads whereas the cells eluted from the column could transfer antibody-forming capacity to irradiated recipients only with respect to the specific sensitizing antigen. The separation of the bone marrow antigen-reactive cells could not be achieved by passage of the cells through non-sensitized glass bead columns nor in the presence of excess free antigen in the column. Cells which were retained by, and later eluted from, the antigen-sensitized glass bead columns were mostly small mononuclear cells whereas cells which passed through the columns were morphologically similar to the original unfractionated bone marrow cell suspension. The data indicate the presence of an antibody or antibody-like structure, with defined immunologic specificity, on the surface of the normal bone marrow antigen-reactive cell.

8. By appropriate irradiation and cell transfer experiments, a direct correlation was observed between the presence of viable and immunologically-active antigen reactive cells in the bone marrow and the capacity of the rabbits to respond following immunization. Rabbits given 800r total body irradiation were unable to elicit a humoral immune response nor did they possess significant numbers of antigen-reactive cells. The ability to respond with humoral antibody formation did not reappear until antigen-reactive cells could be detected. These results strongly indicate that the presence of competent antigen-reactive cells is necessary for the successful induction of the humoral immune response in the rabbit.

9. Normal bone marrow antigen-reactive cells could be stimulated to commence the sequence of intracellular reactions characteristic of the afferent limb of the immune response by incubation with antigen in vitro in cell culture. Injection of these in vitro stimulated

bone marrow cells, along with antigen, into irradiated immuno-incompetent recipients resulted in a reconstitution of the host's immune competence characterized by plaque forming capacity by the spleen cells seven days later. Recipients of cells irradiated following in vitro incubation with the antigen gave normal immune responses whereas irradiation of the cells prior to in vitro incubation with the antigen completely suppressed the capacity of these cells to transfer immunocompetence. Bone marrow cells irradiated in vitro could exclude the dye but failed to undergo blastogenesis and mitosis when cultured in the presence of PHA.

10. Data in support of the clonal selection theory, with respect to the antigen reactive cells (ARC) present in the normal rabbit bone marrow, were also presented. Pre-commitment of the ARC to one antigen could not be subverted even if the cells were incubated for 24 hours in vitro in the presence of a second non-cross-reacting antigen in high concentration. Therefore, the ARC is unipotential with respect to antigen specificity.

CHAPTER VIII

BIBLIOGRAPHY

1. GOWANS, J.L. and McGREGOR, D.D.
   Progr. Allergy, 9: 1, 1965.

2. MEUWISSEN, H.J., STUTMAN, O. AND GOOD, R.A.
   Seminars in Hematology, 6: 28, 1969.

3. COOPER, M.D., PEREY, D.Y., GABRIELSEN, A.E., SUTHERLAND, D.E.R., McKNEALLY, M.F. and GOOD, R.A.
   Int. Arch. Allergy, 33: 65, 1968.

4. MILLER, J.F.A.P. and MITCHELL, G.F.
   J. Exp. Med., 128: 801, 1968.

5. MITCHELL, G.F. and MILLER, J.F.A.P.
   J. Exp. Med., 128: 821, 1968.

6. MOSIER, R.E.
   J. Exp. Med., 129: 351, 1969.

7. COOPER, M.D., PETERSON, R.D.A., SOUTH, M.A. and GOOD, R.A.
   J. Exp. Med., 123: 75, 1966.

8. GLICK, B.
   Poultry Sci., 37: 240, 1958.

9. MILLER, J.F.A.P., DOAK, S.M.A. and CROSS, A.M.
   Proc. Soc. Exp. Biol. Med., 112: 785, 1963.

10. GOOD, R.A., DALMASSO, A.P., MARTINEZ, C., ARCHER, O.K., PIERCE, J.C. and PAPERMASTER, B.W.
    J. Exp. Med., 116: 773, 1962.

11. AUERBACH, R.
    In The Thymus, Ciba Foundation Symposium, G.E.W. Wolstenholme and R. Porter (Eds.), p. 39, 1966.

12. PETERSON, R.D.A., COOPER, M.D. and GOOD, R.A.
    Amer. J. Med., 38: 579, 1965.

13. FINSTAD, J., PAPERMASTER, B.W. and GOOD, R.A.
Lab. Invest., 13: 490, 1964.

14. CAIN, W.A., COOPER, M.D. and GOOD, R.A.
Nature, 217: 87, 1968.

15. WARNER, N.L.
Aust. J. Exp. Biol. Med. Sci., 43: 439, 1965.

16. BILLINGHAM, R.E. and SILVERS, W.K.
In The Thymus, V. DEFENDI and D. METCALF (Eds.)
Wistar Inst. Press, Philadelphia, p. 41, 1964.

17. DENT, P.B. and GOOD, R.A.
Nature, 207: 491, 1965.

18. MEUWISSEN, H.J., KAPLAN, G.T., PEREY, D.Y. and GOOD, R.A.
Proc. Soc. Exp. Biol. Med., 130: 300, 1969.

19. EVERETT, N.B., RIEKE, W.O. and CAFFREY, R.W.
In The Thymus in Immunobiology, R.A. GOOD and A.E. GABRIELSEN (Eds.), Hoeber-Harper, New York, p. 291, 1964.

20. EVERETT, N.B., REINHARDT, W.O. and YOFFRY, J.M.
Blood, 15: 82, 1960.

21. BOAK, J.L., CHRISTIE, G.H., FORD, W.L. and HOWARD, J.G.
Proc. Roy. Soc., 169: 307, 1968.

22. HOWARD, J.G., BOAK, J.L. And CHRISTIE, G.H.
Ann. N.Y. Acad. Sci., 129: 327, 1966.

23. ELVES, M.W., GOUGH, J. and ISRAELS, M.C.G.
Exp. Cell. Res., 44: 624, 1966.

24. CUDKOWICZ, G., UPTON, A.C., SHEARER, G.M. and HUGUES, W.L.
Nature, 201: 165, 1964.

25. ELSON, L.A., GALTON, D.A.G. and TILL, M.
Brit. J. Haematol., 4: 355, 1958.

26. OSMOND, D.G. and EVERETT, N.B.
Blood, 23: 1, 1964.

27. GOWANS, J.L. and KNIGHT, E.J.
Proc. Roy. Soc., 159: 257, 1964.

28. CAFFREY, R.W., RIEKE, W.O. and EVERETT, N.B.
Acta Hemat., 28: 145, 1962.

29. CRADDOCK, C.G., WINKELSTEIN, M., MATSUYUKI, Y. and LAWRENCE, J.S.
J. Exp. Med., 125: 1149, 1967.

30. LITTLE, J.R., BRECHER, G., BRADLEY, T.R. and ROSE, S.
Blood, 19: 236, 1962.

31. OTTESEN, J.
Acta Physiol. Scand., 32: 75, 1954.

32. BUCKTON, K.E. and PIKE, M.C.
Nature, 202: 714, 1964.

33. NORMAN, A., SASAKI, M.S., OTTOMAN, R.E., AND FINGERHUT, A.G.
Science, 147: 745, 1965.

34. NOWELL, P.C.
Blood, 26: 798, 1965.

35. TURK, J.L.
Immunology, 5: 478, 1962.

36. TURK, J.L.
Brit. Med. Bull., 23: 3, 1967.

37. LUBAROFF, D.M. and WAKSMAN, B.H.
J. Exp. Med., 128: 1425, 1968.

38. LUBAROFF, D.M. and WAKSMAN, B.H.
J. Exp. Med., 128: 1437, 1968.

39. BLOOM, B.R. and BENNETT, B.
Fed. Proc., 27: 13, 1968.

40. DUMONDE, D.C.
Brit. Med. Bull., 23: 9, 1967.

41. NASPITZ, C.K. and RICHTER, M.
Progr. Allergy, 12: 1, 1968.

42. DUTTON, R.W.
Adv. Immunol., 6: 253, 1967.

43. BAIN, B., VAS, M.R. and LOWENSTEIN, L.
Blood, 23: 108, 1964.

44. BACH, F. and HIRSCHHORN, K.
Science, 143: 813, 1964.

45. SINGHAL, S.K. and RICHTER, M.
Int. Arch. Allergy, 33: 493, 1968.

46. SINGHAL, S.K. and RICHTER, M.
J. Exp. Med., 128: 1099, 1968.

47. SINGHAL, S.K., DAGUILLARD, F. and RICHTER, M.
Int. Arch. Allergy, 34: 119, 1968.

48. WEBER, W.T.
Immunology, 15: 173, 1968.

49. FORD, W.L. and GOWANS, J.L.
Seminars in Hematology, 6: 67, 1969.

50. FORD, C.E.
In The Thymus Experimental And Clinical Studies. G.E.W. WOLSTENHOLME and R. PORTER (Eds.), Ciba Symposium, Little Brown, Boston, p. 131, 1966.

51. PARROTT, D.M.V.
In Germinal Centres in Immune Responses. H. COTTIER (Ed.) Berlin, Springer-Verlag, P. 168, 1967.

52. GOLDSCHNEIDER, I. and McGREGOR, D.D.
Nature, 212: 1433, 1966.

53. LIDEN, S. and LINNA, T.J.
Int. Arch. Allergy, 35: 47, 1969.

54. WU, A.M., TILL, J.E., SIMINOVITCH, L. and McCULLOCH, E.A.
J. Exp. Med., 127: 455, 1968.

55. MICKLEM, H.S., FORD, C.E., EVANS, E.P. and GREY, J.
Proc. Roy. Soc., 165: 78, 1966.

56. MICKLEM, H.S., CLARKE, C.M., EVANS, E.P. and FORD, C.E.
Transpl., 6: 299, 1968.

57. MILLER, J.F.A.P., MARSHALL, A.H.E. and WHITE, R.G.
Adv. In Immunol., 2: 111, 1962.

58. SAINTE-MARIE, G. and LEBLOND, C.P.
Blood, 23: 275, 1964.

59. MURRAY, R.G. and WOODS, P.A.
Anat. Rec., 150: 113, 1964.

60. MATSUYAMA, M., WIADROWSKI, M.N. and METCALF, D.
J. Exp. Med., 123: 559, 1966.

61. HALL, J.G. and MORRIS, B.
J. Exp. Med., 121: 901, 1965.

62. AUSTIN, C.M.
Aust. J. Exp. Biol. Med. Sci., 46: 581, 1968.

63. WEISSMAN, I.L.
J. Exp. Med., 126: 291, 1967.

64. HARRIS, J.E. and FORD, C.E.
Nature, 201: 884, 1964.

65. CHAPERON, E.A., SELNER, J.C. and CLAMAN, H.N.
Immunology, 14: 553, 1968.

66. COCHRANE, C.G. and DIXON, F.J.
Adv. Immunol., 2: 205, 1962.

67. STROBER, S. and MANDEL, M.A.
Proc. Soc. Exp. Biol. Med., 130: 336, 1969.

68. CELADA, F.
J. Exp. Med., 124: 1, 1966.

69. NOSSAL, G.J.V., CUNNINGHAM, A., MITCHELL, G.F. and MILLER, J.F.A.P.
J. Exp. Med., 128: 839, 1968.

70. DAVIES, A.J.S., LEUCHARS, E., WALLIS, V. and KOLLER, P.C.
Transpl., 4: 438, 1966.

71. DAVIES, A.J.S., LEUCHARS, E., WALLIS, V., MARCHANT, R. and ELLIOTT, E.V.
Transpl., 5: 222, 1967.

72. LEUCHARS, E., CROSS, A.M. and DUKOR, P.
Transpl., 3: 28, 1965.

73. JERNE, N.K. and NORDIN, A.A.
Science, 140: 405, 1963.

74. JERNE, N.K., NORDIN, A.A. and HENRY, C.
In Cell Bound Antibodies, B. AMOS and H. KOPROWSKI (Eds.), Wistar Inst. Press, p. 109, 1963.

75. PLAYFAIR, J.H.L., PAPERMASTER, B.W. and COLE, L.J.
Science, 149: 998, 1965.

76. CLAMAN, H.N., CHAPERON, E.A. and TRIPLETT, R.F.
J. Immunol., 97: 828, 1966.

77. CLAMAN, H.N., CHAPERON, E.A. and TRIPLETT, R.F.
Proc. Soc. Exp. Biol. Med., 122: 1167,1966.

78. CLAMAN, H.N., CHAPERON, E.A. and SELNER, J.C.
Proc. Soc. Exp. Biol. Med., 127: 462, 1968.

79. TAYLOR, R.B.
Nature, 220: 611, 1968.

80. MANY, A. and SCHWARTZ, R.S.
Clin. Res., 17: 356, 1969 (Abst)

81. DORIA, G. and AGAROSSI, G.
Proc. Natl. Acad. Sci., 58: 1366, 1967.

82. DORIA, G. and AGAROSSI, G.
Nature, 221: 871, 1969.

83. FORD, C.E. and MICKLEM, H.S.
Lancet I: 359, 1963.

84. RADOVICH, J., HEMINGSEN, H. and TALMAGE, D.W.
J. Immunol., 109: 756, 1968.

85. GLOBERSON, A. and AUERBACH, R.
J. Exp. Med., 126: 223, 1967.

86. UMIEL, T., GLOBERSON, A. and AUERBACH, R.
Proc. Soc. Exp. Biol. Med., 129: 598, 1968.

87. GOLDSCHNEIDER, I. and McGREGOR, D.D.
J. Exp. Med., 127: 155, 1968.

88. STUTMAN, O. and GOOD, R.A.
Proc. Soc. Exp. Biol. Med., 130: 848, 1969.

89. ELLIS, S.T., GOWANS, J.L. and HOWARD, J.C.
In Cold Spring Harbor Symposium on Antibodies, 32: 395, 1967.

90. McGREGOR, D.D., McCULLOCH, P.J. and GOWANS, J.L.
Proc. Roy. Soc., 168: 229, 1967.

91. DUTTON, R.W. and MISHELL, R.I.
J. Exp. Med., 126: 443, 1967.

92. RAIDT, D.J., MISHELL, R.I. and DUTTON, R.W.
J. Exp. Med., 128: 681, 1968.

93. COIN, W.A., WEIDANZ, W.P. and COOPER, M.D.
Fed. Proc., 26: 571, 1967.

94. PEREY, D.Y., COOPER, M.D. and GOOD, R.A.
Science, 161: 261, 1968.

95. HULLINGER, L. and SORKIN, E.
Immunology, 9: 391, 1965.

96. SORKIN, E. and LANDY, M.
Experientia, 21: 677, 1965.

97. HUMMELER, K., HARRIS, T.N., TOMASSINI, N., HECHTEL, M. and FARBER, M.B.
J. Exp. Med., 124: 255, 1966.

98. HIRSCHHORN, K., BACH, F., KOLODNY, R.L., FIRSCHEIN, L. and HASHEM, N.
Science, 142: 1185, 1963.

99. ROSEMAN, J.M., LESERMAN, L.D., FITCH, F.W. and ROWLEY, D.A.
J. Immunol., 102: 1002, 1969.

100. ARMSTRONG, W.D., DIENER, E. and SHELIAM, G.R.
J. Exp. Med., 129: 393, 1969.

101. STROBER, S.
Nature, 219: 649, 1968.

102. WILLARD, H.G. and SMITH, L.G.
Transpl., 4: 56, 1966.

103. MAKINODAN, T. and ALBRIGHT, J.F.
Progr. Allergy, 10: 1, 1966.

104. PAPERMASTER, B.W.
In Cold Spring Harbor Symposium on Antibodies, 32: 447, 1967.

105. SERCARZ, E. and COONS, A.H.
In Mechanisms of Immunological Tolerance, M. HASEK (Ed.), p. 73, 1962.

106. SHEARER, G.M. and CUDKOWICZ, G.
J. Exp. Med., 129: 935, 1969.

107. MOSIER, D.E. and COPPLESON, L.W.
Proc. Natl. Acad. Sci., 61: 541, 1968.

108. ADLER, F.L,, FISHMAN, M. and DRAY, S.
J. Immunol., 97: 554, 1966.

109. FISHMAN, M.
J. Exp. Med., 114: 837, 1961.

110. FELDMAN, M. and GALLILY, R.
In Cold Spring Harbor Symposium on Antibodies, 32: 415, 1967.

111. MITCHISON, N.A.
In Cold Spring Harbor Symposium on Antibodies, 32: 431, 1967.

112. MITCHISON, N.A.
Immunology, 16: 1, 1969.

113. SIMONS, M.J. and FITZGERALD, M.G.
Clin. Exp. Immunol., 4: 55, 1969.

114. HERSH, E.M. and HARRIS, J.E.
J. Immunol., 100: 1184, 1968.

115. GALLILY, R. and FELDMAN, M.
Immunology, 12: 197, 1967.

116. TYAN, M.L. and HERZENBERG, L.A.
J. Immunol., 101: 446, 1968.

117. MAKINODAN, T., GENGOZIAN, N. and CONGDON, C.C.
J. Immunol., 77: 250, 1956.

118. KENNEDY, J.C., SIMINOVITCH, L., TILL, J.E. and McCULLOCH, E.A.
Proc. Soc. Exp. Biol. Med., 120: 868, 1965.

119. TILL, J.E., McCULLOCH, E.A. and SIMINOVITCH, L.
Proc. Natl. Acad. Sci., 51: 29, 1964.

120. BECKER, A.J., McCULLOCH, E.A. and TILL, J.E.
Nature, 197: 452, 1963.

121. ARMSTRONG, W.D. and DIENER, E.
J. Exp. Med., 129: 371, 1969.

122. SYEKLOCHA, D., SIMINOVITCH, L., TILL, J.E. and McCULLOCH, E.A.
J. Immunol., 96: 472, 1966.

123. METCALF, D.
In Cold Spring Harbor Symposium on Antibodies, 32: 583, 1967.

110. FELDMAN, M. and GALLILY, R.
In Cold Spring Harbor Symposium on Antibodies, 32: 415, 1967.

111. MITCHISON, N.A.
In Cold Spring Harbor Symposium on Antibodies, 32: 431, 1967.

112. MITCHISON, N.A.
Immunology, 16: 1, 1969.

113. SIMONS, M.J. and FITZGERALD, M.G.
Clin. Exp. Immunol., 4: 55, 1969.

114. HERSH, E.M. and HARRIS, J.E.
J. Immunol., 100: 1184, 1968.

115. GALLILY, R. and FELDMAN, M.
Immunology, 12: 197, 1967.

116. TYAN, M.L. and HERZENBERG, L.A.
J. Immunol., 101: 446, 1968.

117. MAKINODAN, T., GENGOZIAN, N. and CONGDON, C.C.
J. Immunol., 77: 250, 1956.

118. KENNEDY, J.C., SIMINOVITCH, L., TILL, J.E. and McCULLOCH, E.A.
Proc. Soc. Exp. Biol. Med., 120: 868, 1965.

119. TILL, J.E., McCULLOCH, E.A. and SIMINOVITCH, L.
Proc. Natl. Acad. Sci., 51: 29, 1964.

120. BECKER, A.J., McCULLOCH, E.A. and TILL, J.E.
Nature, 197: 452, 1963.

121. ARMSTRONG, W.D. and DIENER, E.
J. Exp. Med., 129: 371, 1969.

122. SYEKLOCHA, D., SIMINOVITCH, L., TILL, J.E. and McCULLOCH, E.A.
J. Immunol., 96: 472, 1966.

123. METCALF, D.
In Cold Spring Harbor Symposium on Antibodies, 32: 583, 1967.

124. MITCHISON, N.A.
In Regulation of the Antibody Response, B. CINADER (Ed.), p. 54, 1968.

125. NOSSAL, G.J.V., SHORTMAN, K.D., MILLER, J.F.A.P., MITCHELL, G.F. and HASKILL, J.S.
In Cold Spring Harbor Symposium on Antibodies, 32: 369, 1967.

126. SHEARER, G.M., CUDKOWICZ, G., CONNELL, M.S.J. and PRIORE, R.L.
J. Exp. Med., 128: 437, 1968.

127. SHEARER, G.M., CUDKOWICZ, G. and PRIORE, R.L.
J. Exp. Med., 129: 185, 1969.

128. TREMTIN, J.N., WOLF, N., CHENG, V., FAHLBERG, W., WEISS, D. and BONHAG, R.
J. Immunol., 98: 1326, 1967.

129. SHARP, J.A., BURWELL, R.G.
Nature, 189: 474, 1960.

130. SCHOENBERG, M.D., MUMAW, V.R., MOORE, R.D., and WEISBERGER, A.S.
Science, 143: 964, 1964.

131. McFARLAND, W., HEILMAN, D.H. and MOOREHEAD, J.F.
J. Exp. Med., 124: 851, 1966.

132. SHORTMAN, K.
Aust. J. Exp. Biol. Med. Sci., 44: 271, 1966.

133. RABINOWITZ, Y.
Blood, 23: 811, 1964.

134. PLOTZ, P.H. and TALAL, N.
J. Immunol., 99: 1236, 1967.

135. OPPENHEIM, J.J., LEVENTHAL, B.G. and HERSH, E.M.
J. Immunol., 101: 262, 1968.

136. SUTHERLAN, G.B. and CAMPBELL, D.H.
J. Immunol., 80: 294, 1958.

137. WIGZELL, H. and ANDERSON, B.
J. Exp. Med., 129: 23, 1969.

138. HASKILL, J.S.
Nature, 216: 1229, 1967.

139. HASKILL, J.S., LEGGE, D.G. and SHORTMAN, K.
J. Immunol., 102: 703, 1969.

140. MAGE, M.G., EVANS, W.H. and PETERSON, E.A.
Proc. Soc. Exp. Biol. Med., 127: 478, 1967.

141. SHORTMAN, K. and SZENBERG, A.
Aust. J. Exp. Biol. Med. Sci., 47: 1, 1969.

142. DICKE, K.A., VAN HOOFT, J.I.M. and VAN BEKKUM, D.W.
Transpl., 6: 526, 1968.

143. MORRISON, J.H. and TOEPFER, J.R.
Am. J. Physiol., 213: 923, 1967.

144. SINGHAL, S.K., RICHTER, M. and OSMOND, D.G.
Int. Arch. Allergy, 34: 224, 1968.

145. BRUNETTE, D.M., McCULLOCH, E.A. and TILL, J.E.
Cell Tissue Kinet., 1: 319, 1968.

146. MEL, H.C. and JULLIEN, P.
Biophys. J., 8: 110, 1968.

147. SUNDARAM, K., PHONDKE, G.P. and SUNDARESAN, P.
Immunology, 13: 433, 1967.

148. LANCE, E.M. and TAUB, R.N.
Nature, 221; 841, 1969.

149. CINADER, B.
In Regulation of the Antibody Response, B. CINADER (Ed.), p. 3, 1968.

150. LENNOX, E.S. and COHN, M.
Ann. Rev. Biochem., 36: 365, 1967.

151. VAN FURTH, R.
Seminars in Hematology, 6: 84, 1969.

152. SELL, S. and GELL, P.G.H.
J. Exp. Med., 122: 423, 1965.

153. MERLER, E. and JANEWAY, C.A.
Proc. Natl. Acad. Sci., 59: 393, 1968.

154. BERT, G., MASSARO, A.L. and MAJA, M.
Nature, 218: 1078, 1968.

155. DRESSER, D.W. and MITCHISON, N.A.
Adv. Immunol., 8: 129, 1968.

156. BILLINGHAM, R.E., BRENT, L. and MEDAWAR, P.B.
Phil. Trans. Roy. Soc. London B., 239: 357, 1956.

157. BATTISTO, J.R. and CHASE, M.W.
J. Exp. Med., 118: 1021, 1963.

158. FRIEDMAN, H.
J. Immunol., 89: 257, 1962.

159. WEIGLE, W.O. and DIXON, F.J.
J. Immunol., 82: 516, 1959.

160. BROOKE, J.S. and KARNOVSKY, M.J.
J. Immunol., 87: 205, 1961.

161. MARTINEZ, C. and GOOD, R.A.
In Conceptual Advances in Immunology and Oncology, Harper and Row, N.Y. (Publ.), p. 417, 1963.

162. MITCHISON, N.A.
In Mechanisms of Immunological Tolerance, M. HASEK, A. LENGEROVA and M. VOJTISKOVA (Eds.), Academic Press, New York, p. 245, 1962.

163. McCULLOCH, P.J. and GOWANS, J.L.
In The Lymphocyte in Immunology and Haemopoiesis, J.M. YOFFEY (Ed.), p. 234, 1967.

164. HARRIS, G.
Immunol., 12: 159, 1967.

165. COHEN, M.W. and THORBECKE, G.J.
Proc. Soc. Exp. Biol. Med., 112: 10, 1963.

166. FREI, F.C., BENACERRAF, B. and THORBECKE, G.J.
Proc. Natl. Acad. Sci., 53: 20, 1965.

167. BIRO, C.E. and GARCIA, G.
Immunology, 8: 411, 1965.

168. GOLUB, E.S. and WEIGLE, W.O.
J. Immunol., 99: 624, 1967.

169. TYAN, M.L. and COLE, L.J.
Nature, 208: 1223, 1965.

170. KEISER, G., COTTIER, H., BRYANT, B.J. and BOND, V.P.
In The Lymphocyte in Immunology and Haemopoiesis, J.M. YOFFEY, Ed.), p. 149, 1966.

171. EVERETT, N.B. and CAFFREY, R.W.
In The Lymphocyte in Immunology and Haemopoiesis, J.M. YOFFEY (Ed.), p. 108, 1967.

172. HUDSON, G. and YOFFEY, J.M.
In The Lymphocyte in Immunology and Haemopoiesis, J.M. YOFFEY (Ed.), p. 131, 1967.

173. BIERRING, F. and GRUNNET, I.
Acta Anat., 59: 182, 1964.

174. BRYANT, B.J. and COLE, L.J.
In The Lymphocyte in Immunology and Haemopoiesis, J.M. YOFFEY (Ed.), p. 170, 1967.

175. BENNETT, M. and CUDKOWICZ, G.
In The Lymphocyte in Immunology and Haemopoiesis, J.M. YOFFEY (Ed.), p. 183, 1967.

176. GLOBERSON, A. and FELDMAN, M.
Transpl., 2: 212, 1964.

177. FELDMAN, M. and GLOBERSON, A.
Ann. N. Y. Acad. Sci., 120: 182, 1964.

178. HARRIS, P.F., HARRIS, R.S. and KUGLER, J.H.
Brit. J. Haematol., 12: 419, 1966.

179. OSOBA, D.
Proc. Soc. Exp. Biol. Med., 127: 418, 1968.

180. AGAROSSI, G. and DORIA, G.
Transpl., 6: 419, 1968.

181. WU, A.M., SIMINOVITCH, L., TILL, J.E. and McCULLOCH, E.A.
Proc. Natl. Acad. Sci., 59: 1209, 1968.

182. VOLKMAN, A. and GOWANS, J.L.
Brit. J. Exp. Path., 46: 62, 1965.

183. SPECTOR, W.G., WALTERS, N.I. and WILLOUGHBY, D.A.
J. Path. Bact., 90: 181, 1965.

184. VIROLAINEN, M.
J. Exp. Med., 127: 943, 1968.

185. VOLKMAN, A.
J. Exp. Med., 124: 241, 1966.

186. BALNER, H.
Transpl., 1: 217, 1963.

187. GOODMAN, J.W.
Blood, 23: 18, 1964.

188. PINKETT, M.O., COWDREY, C.R. and NOWELL, P.C.
Am. J. Path., 48: 859, 1966.

189. CHAPMAN, N.D., PARKHOUSE, R.M.E. and DUTTON, R.W.
Proc. Soc. Exp. Biol. Med., 117: 708, 1964.

190. LOMBOS, O., HUTAS, Z., SZONYI, L. and GOFMAN, L.
Ann. Pediat., 201: 86, 1963.

191. VAN FURTH, R., SCHUIT, H.R.E. and HIJMANS, W.
Immunology, 11: 19, 1966.

192. VAN FURTH, R. and DEN DULK, M.D.
J. Immunol., 96: 920, 1966.

193. VAN FURTH, R., SCHUIT, H.R.E. and HIJAMNS, W.
Brit, J. Hemato., 12: 202, 1966.

194. STOLOFF, I.L.
J. Immunol., 85: 304, 1960.

195. HOBSON, D., PORTER, K.A. and WHITBY, J.L.
Brit. J. Exp. Path., 40: 15, 1959.

196. GENGOZIAN, N., MAKINODAN, T. and SHEKARCHI, I.C.
J. Immunol., 86: 113, 1961.

197. STONER, R.D. and BOND, V.P.
J. Immunol., 91: 185, 1963.

198. DORIA, G., GOODMAN, J.W., GENGOZIAN, N. and CONGDON, C.C.
J. Immunol., 88: 20, 1962.

199. UHR, J.W. and FINKELSTEIN, M.S.
Progr. Allergy, 10: 37, 1967.

200. UHR, J.W. and MOLLER, G.
Adv. Immunol., 8: 81, 1968.

201. McCLUSKEY, R.T., BENACERRAF, B. and McCLUSKEY, J.W.
J. Immunol., 90: 466, 1963.

202. SPECTOR, W.G.
Brit. Med. Bull., 23: 35, 1967.

203. KOSUNEN, T.U., WAKSMAN, B.H., FLAX, M.H. and TIHEN, W.S.
Immunology, 6: 276, 1963.

204. KOSUNEN, T.U., WAKSMAN, B.H. and SAMUELSON, I.K.
J. Neuropath. Exp. Neurol., 22: 367, 1963.

205. PRENDERGAST, R.A.
J. Exp. Med., 119: 377, 1964.

206. BURSTEIN, N.A. and WAKSMAN, B.H.
Yale J. Biol. Med., 37: 195, 1964.

207. McGREGOR, D.D.
J. Exp. Med., 127: 953, 1968.

208. SIMMONS, R.L., WOLF, S.M., CHANDLER, J.G. and NASTUK, W.L.
Proc. Soc. Exp. Biol. Med., 120: 81, 1965.

209. FIELD, E.O. and GIBBS, J.E.
Transpl., 3: 634, 1965.

210. STUTMAN, O., YUNIS, E.J., TEAGUE, P.O. and GOOD, R.A.
Transpl., 6: 514, 1968.

211. DALMASSO, A.P., MARTINEZ, C., SJODIN, K. and GOOD, R.A.
J. Exp. Med., 118: 1089, 1963.

212. PLAYFAIR, J.H.L.
Nature, 222: 882, 1969.

213. ARGYRIS, B.F.
J. Immunol., 100: 1255, 1968.

214. UPHOFF, D.E.
Transpl. Proc., 1: 39, 1969.

215. UPHOFF, D.E.
J. Nat. Cancer Inst., 42: 243, 1969.

216. MURPHY, J.B.
J. Am. Med. Assoc., 62: 1459, 1914.

217. JACOBSON, L.O., SIMMONS, E.L., MARKS, E.K. and ELDREDGE, J.
Science, 113: 510, 1951.

218. REKERS, P.E., COULTIER, M.P. and WARREN, S.L.
Arch. Surg., 60: 635, 1950.

219. LORENZ, E., CONGDON, C.C. and UPHOFF, D.
Radiology, 58: 863, 1952.

220. LINDSLEY, D.L., ODELL, T.T. and TAUSCHE, F.G.
Proc. Soc. Biol. Med., 90: 512, 1955.

221. NOWELL, P.C., COLE, L.J., HABERMEYER, J.G. and ROAN, P.L.
Cancer Research, 16: 258, 1956.

222. MAKINODAN, T.
Proc. Soc. Exp. Biol. Med., 92: 174, 1956.

223. FORD, C.E., HAMERTON, J.L., BARNES, D.W.H., LOUTIT, J.E.
Nature, 177: 452, 1956.

224. SELLER, M.J. and POLANI, P.E.
Lancet, I: 18, 1969.

225. GATTI, R., MEUWISSEN, H.J., ALLEN, H.D., HONG, R. and GOOD, R.A.
Lancet, II: 1366, 1968.

226. BACH, F.H., ALBERTINI, R.J., JOO, P., ANDERSON, J.L., and BORTIN, M.M.
Lancet, II: 1364, 1968.

227. CHOU, C.T., CINADER, B. and DUBISKI, S.
Int. Arch. Allergy, 32: 583, 1967.

228. BOYDEN, S.V.
J. Exp. Med., 93: 107, 1951.

229. BOYD, W.C.
Fundamentals of Immunology, Interscience Publishers, Inc., N.Y., 1956.

230. ARMSTRONG, W.D., DIENER, E. and SHELLAM, G.R.
J. Exp. Med., 129: 393, 1969.

231. McARTHUR, W.P., HUTCHINSON, A.J. and FREEMAN, M.J.
Nature, 222: 83, 1969.

232. TAO, T.W.
J. Immunol., 101: 1253, 1968.

233. THEIS, G.A. and THORBECKE, G.J.
Fed. Proc., 28: 427, 1969 (Abst.).

234. FORD, W.L.
Immunology, 15: 609, 1968.

235. WIGZELL, H.
J. Exp. Med., 124: 953, 1966.

236. DIXON, F.J., JACOT-GUILLARMOD, H. and McCONAHEY, P.J.
J. Exp. Med., 125: 1119, 1967.

237. FINKELSTEIN, M.S. and UHR, J.
Science, 146: 67, 1964.

238. HENRY, C. and JERNE, N.K.
J. Exp. Med., 128: 133, 1968.

239. WEILER, E., MELLETZ, E.W. and BREUNINGER-PECK, E.
Proc. Natl. Acad. Sci., 54: 1310, 1965.

240. ROBINSON, W.A., MARBROOK, J. and DIENER, E.
J. Exp. Med., 126: 347, 1967.

241. WALSH, P., MAURER, P. and EGAN, M.
J. Immunol., 98: 344, 1967.

242. SELL, S.
Unpublished Observations.

243. OUDIN, J.
J. Exp. Med., 112: 125, 1960.

244. DRAY, S. and YOUNG, G.O.
J. Immunol., 81: 142, 1958.

245. SELL, S.
Science, 153: 641, 1966.

246. DUBISKI, S., DUDZIAK, Z., SKALBA, D. and DUBISKA, A.
Immunology, 2: 84, 1959.

247. CHOU, C.T., DUBISKI, S. and CINADER, B.
J. Exp. Med., 126: 305, 1967.

248. KELUS, A.S. and GELL, P.G.H.
Progr. Allergy, 11: 141, 1967.

249. HARRIS, T.N., HARRIS, S. and FARBER, M.B.
J. Exp. Med., 158: 21, 1958.

250. HARRIS, T.N., HARRIS, S. and FARBER, M.B.
Immunology, 99: 438, 1967.

251. HARRIS, S., HARRIS, T.N. and OGBURN, C.A.
J. Immunol., 99: 447, 1967.

252. HARRIS, T.N., DRAY, S., ELLSWORTH, B. and HARRIS, S.
Immunology, 6: 169, 1963.

253. HARRIS, T.N., HARRIS, S., BEALE, H.D. and SMITH, J.J.
J. Exp. Med., 100: 289, 1954.

254. ABDOU, N.I. and McKENNA, J.M.
Int. Arch. Allergy, 34: 589, 1968.

255. GALLILY, R. and FELDMAN, M.
Immunology, 12: 197, 1967.

256. ARGYRIS, B.F.
J. Immunol., 99: 744, 1967.

257. ARGYRIS, B.F.
J. Exp. Med., 128: 459, 1968.

258. ANANUE, E.R. and ASKONAS, B.A.
Immunology, 15: 287, 1968.

259. REBUCK, J.W., WHITEHOUSE, F.W. and NOONAN, S.M.
Biochem. Pharm., 16: Suppl., 159, 1967.

260. NACHTIGAL, D., GREENBERG, E. and FELDMAN, M.
Immunology, 15: 343, 1968.

261. RICHTER, M.
Proc. Natl. Acad. Sci. (Submitted for Publication).

262. SERCARZ, E.E. and BYERS, V.S.
J. Immunol., 98: 836, 1967.

263. EHRLICH, P.
By M. MARQUART, William Heinemann Medical Books, Ltd., London, p.p. 65-76, 1949.

264. GELL, P.G.H.
In Cold Spring Harbor Symposium on Antibodies, 32: 441, 1967.

265. FIDALGO, B.V. and NAJJAR, V.A.
Proc. Natl. Acad. Sci., 57: 957, 1967.

266. DAGUILLARD, F. and RICHTER, M.
Unpublished Observations.

267. PAUL, W.E., SISKIND, G.W. and BENACERRAF, B.
J. Exp. Med., 127: 25, 1968.

268. TALIAFERRO, W.H., TALIAFERRO, L.G. and JAROSLOW, B.N.
In Radiation and Immune Mechanisms, Academic Press, New York, Publishers, 1964.

269. LEONE, C.A.
In The Effects of Ionizing Radiations on Immune Processes, C.A. LEONE (Ed.), Gordon and Breach, Publishers, New York, 1962.

270. HARRIS, J.W. and NOONAN, T.R.
Int. J. Radiat. Biol., 13: 183, 1967.

271. PRIBNOW, J.F. and SILVERMAN, M.S.
J. Immunol., 98: 225, 1967.

272. HEGE, J.S. and COLE, L.J.
J. Immunol., 99: 61, 1967.

273. KENNEDY, J.C., TILL, J.E., SIMINOVITCH, L. and McCULLOCH, E.A.
J. Immunol., 94: 715, 1965.

274. SVEHAG, S. and MANDEL, B.
J. Exp. Med., 119: 21, 1964.

275. TALIAFERRO, W.H. and TALIAFERRO, L.G.
J. Infect. Dis., 95: 117, 1954.

276. COONS, A.H.
Harvey Lectures, 53: 113, 1959.

277. STENDER, H.S., STRAUCH, D. and WINTER, H.
In The Effects of Ionizing Radiations on Immune Processes. C.A. LEONE (Ed.), Gordon and Breach, Publishers, New York, p. 315, 1962.

278. NOSSAL, G.J.V.
Aust. J. Exp. Biol., 37: 499, 1959.

279. DIXON, F.J. and McCONAHEY, P.J.
J. Exp. Med., 117: 833, 1963.

280. GENGOZIAN, N. and MAKINODAN, T.
J. Immunol., 80: 189, 1958.

281. MORGAN, P., SHERWOOD, N.P., WERDER, A.A. and YOUNGSTROM, K.
J. Immunol., 84: 324, 1960.

282. VIAHOVIC, S. and STANKOVIC, V.
Acta Allergol., 16: 329, 1961.

283. SILVERMAN, M.S. and CHIN, P.H.
J. Immunol., 75: 321, 1955.

284. FITCH, F.W., WISSLER, R.W., LAVIA, M. and BARKER, P.
J. Immunol., 76: 151, 1956.

285. LOEWI, G., TEMPLE, A., NIND, A.P.P. and AXELRAD, M.
Immunology, 16: 99, 1969.

286. HORIUCHI, A. and WAKSMAN, B.H.
J. Immunol., 100: 974, 1968.

287. DUKOR, P. and DIETRICH, F.M.
Int. Arch. Allergy, 32: 521, 1967.

288. NETTESHEIM, P. and HAMMONS, A.S.
Nature, 220: 297, 1968.

289. MILLER, J.J.
J. Immunol., 92: 673, 1964.

290. HOWARD, A.
In Effects of Ionizing Radiations on Immune Processes. C.A. LEONE (Ed.), Gordon and Breach, Publishers, New York, p. 163, 1962.

291. JAROSLOW, B.N.
J. Infect. Dis., 107: 56, 1960.

292. MICKLEM, H.S. and BROWN, J.A.H.
Immunology, 4: 318, 1961.

293. BERLIN, B.S.
J. Immunol., 93: 315, 1964.

294. DIXON, F.J., TALMAGE, D.W. and MAURER, P.H.
J. Immunol., 68: 693, 1952.

295. UYEKI, E.M. and KLASSEN, R.S.
J. Immunol., 101: 973, 1968.

296. VANN, D.C. and MAKINODAN, T.
J. Immunol., 102: 442, 1969.

297. HARRIS, T.N., HARRIS,S. and TULSKY, E.
J. Immunol., 82: 26, 1959.

298. FISHMAN, M. and SHECKMEISTER, I.L.
J. Exp. Med., 101: 275, 1955.

299. KASAKURA, S. and LOWENSTEIN, L.
J. Immunol., 101: 12, 1968.

300. PETROV, R.V., SESLAVINA, L.S. and PANTELEJEV, E.I.
Nature, 217: 558, 1968.

301. PERKINS, E.H. and MAKINODAN, T.
J. Immunol., 92: 192, 1964.

302. LOUWAGIE, A.C., SCHOFIELD, R. and LAJTHA, L.C.
Nature, 216; 370, 1967.

303. KINDRED, J.E.
Am. J. Anat., 67: 99, 1942.

304. FELDMAN, M. and MEKORI, T.
Immunology, 10: 149, 1966.

305. LIACOPOULOS, P. and HERLEM, S.G.
Int. Arch. Allergy, 34: 95, 1968.

306. LIACOPOULOS, P.
Texas Reports on Biol. and Med., 23: 63, 1965.

307. BURNET, F.M.
The Clonal Selection Theory of Acquired Immunity. Vanderbilt University Press, Nashville, Tenn., 1958.

308. MARSHALL, A.H.E. and WHITE, R.G.
Brit. J. Exp. Path., 42: 379, 1961.

309. CAMPBELL, P.A. and LA VIA, M.F.
Proc. Soc. Exp. Biol. Med., 124: 571, 1967.

310. EIDINGER, D.
Immunology, 15: 357, 1968.

311. HORNE, C.H.W. and WHITE, R.G.
Immunology, 15: 65, 1968.

312. BYFIELD, P. and SERCARZ, E.E.
J. Exp. Med., 129: 897, 1969.

313. BYERS, V.S. and SERCARZ, E.E.
J. Exp. Med., 127: 307, 1968.

314. LANGEVOORT, H.L.
Lab. Invest., 12: 106, 1963.

315. HALL, J.G., MORRIS, B., MORENO, G.D. and BESSIS, M.C.
J. Exp. Med., 125: 91, 1967.

316. CUNNINGHAM, A.J.
Aust. J. Exp. Biol. Med. Sci., 46: 141, 1968.

317. LEDUC, E.H., AVRAMEAS, S. and BOUTEILLE, M.
J. Exp. Med., 127: 109, 1968.

318. NOSSAL, J.G.V.
Brit. J. Exp. Path., 40: 301, 1959.

319. GOWANS, J.L. and UHR, J.W.
J. Exp. Med., 129: 1017, 1966.

320. CANNON, D.C.
Arch. Path., 83: 188, 1967.

321. ATTARDI, G., COHN, M., HORIBATA, K. and LENNOX, E.S.
J. Immunol., 92: 346, 1964.

322. MARBROOK, J.
Lancet, II: 1279, 1967.

323. MARBROOK, J.
Clin. Exp. Immunol., 3: 367, 1968.

324. JERNE, N.K.
Proc. Natl. Acad. Sci., 41: 849, 1955.

325. TALMAGE, D.
Ann. Rev. Med., 8: 239, 1957.

326. LEDERBERG, J.
Science, 129: 1649, 1959.

327. ROWLEY, D.A., FITCH, F.W., MOSIER, D.E., SOLLIDAY, S., COPPLESON, L.W. and BROWN, B.W.
J. Exp. Med., 127: 983, 1968.