CELLS INVOLVED IN CELLULAR IMMUNE REACTIONS IN VITRO

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THE ORGAN SOURCE(S) OF THE CELLS MEDIATING A CELLULAR IMMUNE REACTION IN VITRO

ABSTRACT

The objective of this investigation was to establish an in vitro counterpart to the in vivo graft-versus-host reaction. The generation in vitro of plaque-forming cells from spleen of rabbits previously immunized with sheep red blood cells could be completely suppressed by the simultaneous incubation of the memory cells with allogeneic circulating lymphocytes, sacculus rotundus, appendix or Peyer's patches cells (collectively referred to as SAPP effector cells). Spleen and lymph node cells were less effective in inhibiting the in vitro immune response whereas thymus and bone marrow exhibited no inhibitory activity. It was demonstrated that the inhibition of the generation of plaque-forming cells is not mediated by the release of cytotoxic antibodies directed to the memory spleen cells by the allogeneic lymphocytes.

Following immunization with the appropriate antigen, allogeneic lymphocytes or SRBC so as to induce cell mediated and humoral responses respectively, the effector cells were shown to migrate to the thymus, whereas antibody forming cells were shown to migrate initially to the Peyer's patches.

These results support the conclusion that the reaction represents an in vitro counterpart of the graft-versus-host reaction and imply a SAPP organ origin for the cells mediating this reaction in the rabbit.

THE ORGAN SOURCE(S) OF THE CELLS MEDIATING A CELLULAR IMMUNE REACTION IN VITRO

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CHAPTER ONE

ABBREVIATIONS USED IN THE TEXT

Ab	: antibody
AFC	: antibody-forming cell
Ag	: antigen
ARC	: antigen reactive cell
BGG	: bovine gamma globulin
BSA	: bovine serum albumin
CMI	: cell mediated immunity
GALT	: gut associated lymphoid tissue
GVHR	: graft-versus-host reaction
HRBC	: horse red blood cells
Ig	: immunoglobulin
Med. PS.	: medium containing penicillin and streptomycin
Memory rabbits	: rabbits immunized 3-6 months previously
NRS	: normal rabbit serum
PFC	: plaque-forming cells
SAPP organs	: sacculus rotundus, appendix and Peyer's patches
SAPP cells	: cells from SAPP organs
SRBC	: sheep red blood cells

CHAPTER TWO

HISTORICAL REVIEW

I THE LYMPHOID SYSTEM AS A SOURCE OF THE IMMUNOCOMPETENT CELL.

A. General Considerations.

The fundamental function of the immune response is to serve as a means of defense against infection by invading organisms. This is usually accomplished by the secretion of specific antibody into the circulation directed against the invasive agent. In addition, it is now generally accepted that rejection of foreign tissue grafts, defense against certain viral infections and the prevention of aberrent cell differentiation are functions that can be directly attributed to the immunological system.

The immune response itself is a complex phenomenon involving the differentiation of cells in at least two directions-- one of these leads to the production of cells which specialize in the

synthesis and secretion of humoral antibodies of the various immunoglobulin classes while the other leads to the production of specifically sensitized cells which are responsible for initiating the events generally referred to as cell-mediated immunity.

Considerable evidence has accumulated which unequivocally implicates the lymphocyte, to the exclusion of all other cells, in the mediation of the immune response.

In this chapter, the role played by the lymphoid system in the immune response is reviewed with particular reference to the heterogeneity of the system, the pluripotentiality of the lymphoid cells and the interactions which occur between the cells of the different lymphoid organs following antigenic stimulation.

B. Central and Peripheral Lymphoid Systems.

A central lymphoid organ has been defined by Richter and Algom (18) and Parrot and de Soussa (11) as an organ the extripation of which leads to histological changes in one or more of the other lymphoid

organs. These histological changes usually consist of the depletion of lymphocytes from well demarcated areas in the other lymphoid organs such as the lymph nodes, spleen and appendix (19). From an immunologic point of view, a central lymphoid organ has been defined as one which seeds the peripheral organs with immunocompetent cells or the precursors of these cells. Glick observed that the bursa of Fabricius is essential for the development of lymphoid cells capable of antibody production (1) a fact that was further confirmed by other studies (2,3). Similarly, the studies of Miller et al (4) and of Good et al (5) have shown that the thymus, like the bursa, behaves as a central lymphoid organ mainly engaged in cellular immunity. By definition, these two organs are central lymphoid organs since they contain lymphoid cells which are in the process of differentiation but do not as yet exercise the immunological functions attributable to fully differentiated immunocompetent cells. This has been demonstrated by the lack of capacity of cells from the thymus to exercise graft-versus-host reactions (6) and of bursal cells to produce antibodies (7). Accordingly, the generally accepted view is that an

undifferentiated lymphoid bone marrow stem cell will, for example, enter the thymus where it will be subjected to the influence of that organ, and proliferate into a population of cells capable of expressing graft rejection, delayed hypersensitivity, and graft-versus-host reactions, the so called thymus dependent functions, following their expulsion from the thymus into the circulation (5,8).

Similarly, a stem cell entering the bursa of birds, or the gut-associated lymphoid organs of certain mammals will, under the influence of that organ, differentiate into a population of lymphoid cells capable of producing antibodies in the appropriate peripheral lymphoid tissues (9). Any direct immunological function which the central lymphoid organs may possess can be attributed to the presence of mature lymphocytes in these organs that either have not yet migrated to the periphery or which constitute a peripheral lymphoid component within the central lymphoid organ (10).

Peripheral lymphoid organs, on the other hand, are defined as organs the extripation of which does not lead to any detectable histological changes

in any of the other organs (18). In the peripheral lymphoid organs immunocompetent lymphocytes show two distinctive distribution patterns. The lymphocytes which occupy the deep cortical areas of the lymph nodes and perivascular areas of splenic follicles are responsible for delayed hypersensitivity reactions, allograft rejection, and graft-versus-host reactions. These cells are dependent on the thymus for their maintenance and function (19). The other population of immunocompetent lymphocytes constitute the inner and outer mantle areas of primary follicles in the lymph nodes as well as those of the spleen. These cells are bursa dependent and give rise to antibody forming cells or their precursors (3, 11).

Since the cells to which immunological functions have been attributed constitute cells of thymic (T-cells) origin and bone marrow (B-cells) origin, it is necessary at this point to discuss these cells in a more detailed manner.

C. Bone Marrow Dependent or Bone Marrow Derived Cells (B-Cells).

Cells originating from bone marrow cells

are generally referred to as B-cells. It has been shown that immunocompetent cells are derived from bone marrow or fetal liver cells during embryonic life (12). Experiments by Trentin et al (13) have shown that, upon transfer of syngeneic marrow derived hematopoietic spleen colonies to irradiated mice, a large population of immunocompetent cells of donor origin, as shown by T_6 chromosome marker, is established in the spleen, mesenteric nodes and bone marrow of recipient animals (13). However, Claman et al (15) have demonstrated the necessity for transferring both thymus and marrow cells into heavily irradiated recipients in order to obtain a plaque forming cell (PFC) response to SRBC.

Further support of the bone marrow origin of the cell responsible for antibody production was shown conclusively by Wu et al (16, 17). They infused syngeneic chromosomally-marked bone marrow cells into lethally irradiated recipients. The same chromosome marker was found in hemopoietic colonies of the spleen, thymus and the popliteal lymph nodes. Following the foot pad injection of SRBC, more than 50% 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 (16, 17).

D. Thymus Dependent or Thymus Derived Cells (T-Cells).

Although stem cells for all immunocompetent cells exist in the bone marrow, whether these cells will exhibit humoral or cellular immunity is probably determined by whether they come under the influence of the thymus or the bursa.

The demonstration by Miller (20) that neonatal thymectomy in mice prevented the proper development of all cell mediated and some antibody responses revealed the thymus as a tissue of unique importance. Removal of the thymus in adult life had no apparent immediate consequences on the capacity of the thymectomized animal to mount an immune response (20). Neonatal thymectomy, however, resulted in the depletion of cells in the periarteriolar regions of the spleen, the paracortical areas in lymph

nodes, the majority of thoracic duct lymphocytes and about 50% of the circulating lymphocytes (21). From a functional standpoint the lack of such cells could be correlated with a deficiency in the ability to reject grafts, to induce graft-versus-host reactions and to make antibodies to some protein antigens, bacteria and erythrocytes. There was, however, little or no impairment in the antibody response to other antigens such as pneumococcal polysaccharides and haemocyanin (22).

In other studies by Miller (23) and Miller, Doak and Cross (4), it was shown that adult thymectomy had only a slow waning effect on immunocompetence, while if thymectomy was followed by lethal irradiation and bone marrow replacement, immunocompetence was impaired to the same extent as that which follows neonatal thymectomy. Evidence for the relationship between the thymus and the pool of recirculating small lymphocytes was shown by Weissman (24). The thymus cells of a newborn or young rat were labeled in situ by the direct intrathymic inoculation of tritiated thymidine. Labelled thymocytes then appeared exclusively in the paracortical areas of the lymph nodes and the periarteriolar lymphocyte sheaths of the spleen, making up to 10 to 32% of all the cells

in these sites in the neonatal rat. Conversely, neonatally thymectomized rats have shown a decrease in the density of the cells in the above mentioned areas of the lymph nodes and spleen (25). Experiments using thoracic duct cannulation have shown that in normal CBA mice six weeks of age the average total number of lymphocytes that could be mobilized within 48 hours of drainage was 86 million cells. This figure dropped to 2 to 3 million in neonatally thymectomized mice of the same age (26). Mice which were thymectomized at the age of two to three months showed no significant change in the number of lymphocytes drained within 48 hours during the first few weeks following thymectomy. However, twelve months after thymectomy the total number of lymphocytes drained within 48 hours fell to 36% of control values obtained from sham thymectomized mice (26). In irradiated mice thymectomized one month before irradiation, the number of cells in the pool did not increase much beyond its post irradiation value of about 5 million cells (27). Thus, mice thymectomized at birth or thymectomized and irradiated in adult life

suffer from a reduced number of circulating lymphocytes which can readily explain the immunological defects associated with thymectomy.

In still further experiments to identify the origin of immunocompetent lymphocytes, Mitchell and Miller (31) have shown that repeated injections of genetically marked thymus cells into neonatally thymectomized recipients led to some increase in the output of thoracic duct cells and the majority of these cells had the genetic marker of the injected donor thymus cells. This again shows that recirculating small lymphocytes may be thymus-derived.

Although, as mentioned above, humoral antibody responses are in general much less affected then cellular responses by thymectomy, Jeejeebhoy and others (23, 28 and 29) have found that a new antigen administered nine or more months after adult thymectomy will elicit a diminished humoral response. Taken together, these results imply that the thymus in the adult must continue to influence the development of an adequate population of long lived recirculating immunologically competent small lymphocytes. As a result of the limited life span of these cells the circulating pool is eventually

depleted and immune incapacity becomes apparent.

Thumus-dependent lymphocytes are morphologically similar to other lymphocytes yet distinguishable by virtue of certain surface properties. Dowenhoff et al (32) have shown that these cells could be transformed into blast cells by stimulation with plant mitogens such as phytohaemagglutinin (PHA) and be inactivated by a heterogenous antilymphocyte serum assumed to be specific to thymus dependent cells. Raff and Wortis (33) have established a system of iso-antigens limited to thymus dependent lymphoid cells. Using this system, approximately 40% of spleen cells, and 65-85 per cent of lymph node and thoracic duct cells were found to carry this antigen (33). Furthermore, it seems established that there exists at least two populations of T-cells which can be distinguished both serologically and functionally. One population of cells contains the T L antigen while the other seems to lack it (T L negative) (34). The latter population is the one that resides in the medullary area of the thymus and which is competent to perform cell mediated reactions (34). Both populations however are characterized by their content of the antigen which is found only on

thymus and thymus derived cells (35). Another property associated with thymus dependent lymphocytes is the secretion of soluble factors following interaction with antigen. Among these factors are macrophage-immobilizing, mitogenic, cytotoxic and other factors which are now recognized (36).

E. Gastro-Intestinal Associated Lymphoid Tissues.

Since the discovery by Glick and his coworkers of the importance of the bursa of Fabricius of the chicken in confering the capacity to give a humoral response (1, 2), investigators have tried to establish the existence of an equivalent organ or organs with a similar function in the mammal. The results of neonatal thymectomy in rodents suggested the existence of a separate line of lymphoid cells responsible for immunoglobulin production and plasma cell development, since thymectomy greatly depresses the ability to mount a cell-mediated response but produces neither depression of immunoglobulins nor impairment of the antibody response to many antigens (20, 21, 22).

The observation of immunological deficiency diseases in man suggested the existence of two lines of lymphoid cells as defined by experiments in the chicken (37). Patients with the Bruton form of sex-linked recessive agammaglobulinemia are extremely deficient in plasma cells and lack germinal center organization in their lymphoid organs (38). Consequently they have a severe deficiency of circulating immunoglobulins but have relatively normal numbers of lymphocytes in their circulation and can exercise all of the functions linked with cell-mediated immunologic responses (39). In contrast, patients with the Di George syndrome have either a poorly differentiated thymus or no thymus at all and seem to lack any capacity for cellular immunity, yet these patients possess germinal centers, plasma cells and produce all of the immunoglobulins in relatively normal amounts (40, 41). Seen in the perspective provided by the studies in chickens, it seems that mammals also are in possession of a bursal system of cells.

The first insight as to the identity of this second or non-thymic central lymphoid tissue in mammals came from the recognition of the morph-

ologic similarity between the appendiceal lymphoid tissue of the rabbit and the bursa of Fabricius in chickens (43, 44). It was found that removal of the appendix in neonatal rabbits interfered with their capacity to form antibodies to bovine gamma-globulin (45). Konda and Harris (46) have also shown that following appendectomy and whole body x-irradiation of 3 week old rabbits, there is a significant reduction in antibody responsiveness. Cornes (47) showed that the morphologic maturation of the Peyer's patches like that of the thymus is largely independent of antigenic stimulation and occurs in many species long before birth.

Cooper et al (48) observed that the removal of the gastrointestinal associated lymphoid tissues in young adult rabbits, coupled with high doses of total body irradiation, had a deleterious effect on their immunologic capacity similar to that of bursectomy and x-irradiation in chickens (315). In these experiments, antibody responsiveness was impaired whereas the subsequent ability of such rabbits to express cell-mediated immunity was intact (48).

Further support for the gut-associated

lymphoid tissues of the rabbit being the functional equivalent of the bursa of Fabricius in the chicken came from the experiments of Cooper et al (9) in which the neonatal removal of the sacculus rotundus, appendix and Peyer's patches reduced significantly the levels of the circulating immunoglobulins and the circulating lymphocytes, and led to a marked increase in the death rate of these animals. These observations relate well with earlier ones by Jacobson and co-workers who found that shielding of the appendix in rabbits exposed to total body irradiation resulted in retention of antibody forming capacity, whereas control animals whose appendices were not shielded showed marked interference with these functions (49). Similar findings were reported by Hanaoka et al (53). From an ontogenic point of view the gut-associated lymphoid tissues in the rabbit develop after the thymus but before the spleen and lymph nodes (47). Like the thymus, these tissues derive from epithelial mesenchymal interaction and maintain a close lympho-epithelial relationship throughout life (61). Repopulation studies by Evans et al (26) showed the gut-associated lymphoid tissues to be different from those seen in the thymus, spleen and lymph nodes. When lethally ir-

radiated mice were injected with 10^5 bone marrow cells and 10^7 lymphoid cells carrying distinct marker chromosomes, it was observed that the thymus was almost entirely repopulated by myeloid cells whereas the Peyer's patches were repopulated by lymphoid elements (62). Furthermore, the Peyer's patches of rabbits differ from the thymus in being poorly developed at birth (47, 63). More direct evidence which implicates the gut-associated lymphoid organs in the mediation of the humoral immune response has come from the work of several investigators (50, 51, 52, 53, 54 and 55). Richter et al (50) have demonstrated that, 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 SRBC. Their results suggest that the bone marrow serves as the prime source of ARC but that the sacculus rotundus and mesenteric node may also possess committed AFCs directed toward certain particulate antigens. Similar findings were also reported by Armstrong et al (51) who demonstrated, using Salmonella adelaide polymerized flagellin in mice, that not only the bone marrow but also mesenteric lymph

nodes and Peyer's patches contain committed AFCs. Henry et al (52) have been successful in obtaining a primary immune response to SRBC with cultures of normal rabbit Peyer's patches cells. They have also shown that cultures prepared from the Peyer's patches of rabbits which have received one or two injections of SRBC contain both IgM and IgG memory cells which have apparently migrated from the spleen (52). They have thus concluded that the anatomic relationship within the rabbits Peyer's patches does not favour the interaction of antigen with immunocompetent cells, but that this organ must normally serve as a source of reactive cells which migrate to other sites where they can be stimulated. These findings however tend to support the view that the Peyer's patches are in the category of primary lymphoid organs. In agreement with this view is the hypothesis proposed by Ozer and Waksman (55) who suggest that the appendix, sacculus rotundus and Peyer's patches in the rabbit act as a source of a cell essential to gamma-M antibody formation (54). They maintain that this cell is not immunocompetent in itself but normally must migrate to peripheral lymphoid organs such as the spleen where it can

interact with antigen in cooperation with a marrow-derived cell (54). In another report (53), the authors have shown that antigen given directly into the appendiceal artery of an irradiated rabbit is highly immunogenic, thus implying that cells of the appendix possess reactivity for antigen before they enter the peripheral pool. It may be that the appendix in the rabbit is comparable with the bursa of Fabricius of the chicken. Similarly, the appendix appears to control gamma-M formation and corresponding memory but fails to produce antibodies itself (54).

On the other hand, it has been shown that Peyer's patches contain cells which are antibody forming (58) and seed antibody forming and memory cells to the draining lymph nodes and the spleen (58, 59). Indeed, Richter et al (50) have shown that immunologic reactivity to SRBC could be restored to irradiated (1200R) rabbits by cells of the sacculus rotundus, which resembles a large Peyer's patch, but not by appendiceal cells. Abdou and Richter (60) have also provided indirect evidence that one of the cells of the sacculus rotundus

may in fact be a precursor of the antibody forming cells in their system. It appears however that one or all of the gut-associated lymphoid tissues either function to provide the peripheral lymphoid organs with a steady stream of cells primed against various antigens found in the gastrointestinal tract, or alternatively, that they provide cells non-specifically stimulated which participate elsewhere in the gamma-M response to any antigen. In the latter case it would appear that these gut organs share properties of both central and peripheral lymphoid organs.

F. Cellular Cooperation.

(i) Humoral immunity.

It is clear that the humoral immune response involves a number of distinct phases beginning with the introduction of the antigen and ending with the production of specific antibodies. The terminal events, such as the production of antibodies, their structure and specificity etc., have been well established (127). Earlier events however remain somewhat obscure. The phase dealing with the cells involved in the immune response has been the subject

of a vast number of investigations (see preceding pages). The most intriguing question is whether more than one cell system exists for the mediation of the humoral immune response, one being concerned with the response to antigen and another responsible for the production of antibodies. Investigations along this line were reported by Miller and Mitchell (67) who showed that thymectomized mice failed to respond with haemolysin plaque formation to sheep erythrocyte but could do so if they were reconstituted with viable thymus or thoracic duct lymphocytes. Spleen cells from reconstituted mice were exposed to anti-H_2 sera directed against either the donor of the thymus cells used for reconstitution or against the neonatally thymectomized host. Only the isoantisera directed against the host could significantly reduce the number of hemolysin forming cells present in the spleen cell suspension, showing that they were of host origin. The authors concluded that there are cell types in the thymus or thoracic duct lymph with capacities to react specifically with antigen and to induce the differentiation to antibody-forming cells of precursor cells derived from a separate

cell line present in the neonatally thymectomized hosts (67). Other evidence for the cooperation of these two cell lines has come from the experiments of Davies et al (65). Irradiated recipient mice were grafted with thymic tissue and given injections of bone marrow cells from donors carrying different chromosome markers, thus individual cells in the recipient were "typed". Employing this approach, recipient mice were immunized and their spleen cells were transferred to further irradiated mice, some of which were pre-treated so as to reject only thymus derived or only bone marrow derived cells. When the thymus derived cells were rejected the antibody response in the second host was diminished, but when the marrow derived cells were rejected the immune response was abolished all together. It was thus concluded that marrow-derived and not thymus-derived cells synthesize antibody, but that both together act synergistically. However, despite their failure to form antibody, thymus-derived cells could be shown to respond in vivo to SRBC and other antigens by mitosis and blastogenesis (66). Mitchell

and Miller (30) carried out basically similar experiments with neonatally thymectomized mice restored with semi-syngenic thymus cells; iso-antisera against those transplantation antigens contained only in the thymus cells did not inhibit the PFC response against SRBC, while antisera against the host did.

An elegant demonstration of the need of two cell types in the antibody response was presented by Claman and his co-workers (15). Irradiated mice injected with both syngeneic thymus and bone marrow cells responded with an almost normal PFC response. On the other hand, those mice injected with syngeneic thymus cells alone or marrow cells alone responded very poorly.

In the rabbit, Richter and Abdou (68) have presented evidence for the cooperation of two functionally distinct but morphologically similar lymphocytes. The antibody response of 800R irradiated rabbits to SRBC was abolished but could be restored by reconstitution with allogeneic bone marrow cells. However, the PFCs obtained were of host type as judged by anti-allotype inhibition of PFC formation (68). When the rabbits were given

a whole body irradiation dose of 1200R, reconstitution with allogenic bone marrow cells failed to induce PFC formation in the host (69). The authors postulated that a radio-sensitive marrow-derived cell had acted as an antigen reactive cell (ARC) and had cooperated with a host-derived relatively more radio-resistant cell, which they considered to be a precursor of the PFC.

(ii) Cell mediated immunity.

In cell mediated immunity it has been claimed that a typical response, such as the graft-versus-host and reaction also exhibits thymus-marrow cooperation (70). Cooperation has been demonstrated between macrophages and immune spleen cells in inducing cytotoxicity against tumour cells in vitro (71). The apparent need for more than a single determinant for the induction of delayed skin responses (72) has been cited as evidence that true cooperation among immunocompetent thymus-derived cells can occur.

II IN VITRO DEMONSTRATION OF THE CYTOTOXIC EFFECTS OF LYMPHOID CELLS.

A. General Considerations.

The typical histopathological picture of the tissue lesion (196) as seen in the autoimmune diseases either spontaneously occurring in man or experimentally induced in animals, delayed hypersensitivity, tumor rejection and allograft rejection, has served as a basis for the recognition of the tissue-damaging immune functions of lymphoid cells. Lymphocyte-mediated tissue injury is believed to be of prime importance in these instances since tissue-destructive immune reactions can in fact be provoked in non-sensitized recipients by transfer of lymphoid cells from sensitized donors rather than by the transfer of immune serum (195).

One of the first successful demonstrations of the cytotoxic effect of lymphoid cells in vitro was reported by Govaerts (73). Immune thoracic duct cells from a dog which had rejected a kidney graft four days earlier were used as effector cells. Target cells were obtained from the remaining kidney of the "donor" dog. The immune cells were found to

adhere to a monolayer of the target cells which then showed morphological signs of cell damage leading to cell death and lysis over a period of twenty-four to forty-eight hours. This effect was not observed when cells from a dog grafted with a kidney from an unrelated donor were used as effector cells.

Roseneau and Moon (74) demonstrated a similar effect using L-strain tumor cells as target cells and spleen cells of BALB/c mice as effector cells. The latter were sensitized by the intraperitoneal and intrasplenic injections of L-strain cell suspensions. The authors noted that the addition of complement was not required during the incubation to bring about the cytotoxic effect and no mouse serum proteins could be detected on the target cells by immunoflourescent antibody techniques. It was thus concluded that the effect was not mediated by antibodies (74).

The cell that initiates the in vitro cytotoxic reaction is assumed to be a sensitized lymphocyte equipped with its own recognition sites for antigens on the cell it destroys. However, it

seems clear that normal lymphoid cells are also capable of mediating this reaction through a variety of pathways (71). This non-specific cytotoxicity *in vitro* provides the corner-stone for an understanding of the principles underlying the role of effector cells in cell-destructive reactions *in vivo*.

B. Methods.

In order to throw more light on the mechanism of these tissue-damaging reactions, a number of direct *in vitro* models have recently been described. In principle, excess lymphoid cells are added to target cells in culture. After a certain interval of time, which varies depending on the particular system under study, destruction of the target cell takes place and can be recorded. In the following sections the different cells, various *in vitro* model systems, and some methodological aspects will be reviewed.

(i) Target cell(s).

Cell lines of normal tissues or tumors established in tissue culture are most frequently

used as target cells (75, 76). These cells have the advantage of being adapted to tissue culture conditions and are easy to culture in monolayers or in suspensions. Lundgren et al (77) have shown that cells grown in monodisperse suspensions are more suitable target cells than cells attached to glass, being more readily available for contact with effector cells from all sides and thus increasing the sensitivity of the method. These cells are also not influenced by enzymes or other factors which may detach cells growing in monolayers on glass (77). Brondz (78) has reported the use of macrophages cultured on glass as target cells for lymphoid cells from animals sensitized to the transplantation antigens of the macrophage donor.

Chicken erythrocytes have also been employed as target cells (79). When these erythrocytes are passively coated with antigen, they act as targets for lymphoid cells from donors sensitized to that antigen. Using a somewhat different system of assaying cell death, Friedman (80) reported the use of spleen cells obtained from mice immunized with SRBC. These cells are

capable, when incubated in agar gel containing SRBC and complement, of forming plaques. Inhibition of plaque formation, by prior incubation with effector cells obtained from mice of another strain previously sensitized to the target cells, was taken as a measure of cell death and was found to be quantitatively related to the number of effector cells used.

(ii) Effector cell(s).

In the majority of experiments, effector cells have consisted of a mixture of lymphocytes of various sizes, monocytes and macrophages. In many cases, however, these cell suspensions have contained predominantly small lymphocytes which were seen adhering to the target cells. A number of authors (81, 82, 83) have, however, described cytotoxic effects mediated by peritoneal exudate cells, the majority of which displayed the morphologic characteristics of macrophages. Old et al (84) claimed that macrophages were observed to phagocytize the target cell and the effect was almost certainly mediated by the opsonic effect of humoral antibody. It was later reported that

some cytotoxic activity of the peritoneal cells was retained even after removal of the antibody (85).

(iii) Assay systems.

1. Destruction of monolayers:

The disruption of target cell monolayers after incubation with lymphoid cells and/or the detachment of the cells from the glass is taken as a measure of the degree of cytotoxic activity of the effector cells. Various modifications have been introduced. A common one is to photograph a selected part of the cell monolayer before and after incubation with effector cells and to subsequently evaluate the degree of cell damage (73, 86 and 87). Granger and Weiser (81) applied drops of effector cells in suspension onto target monolayers in a well defined area. The reduction of target cell density or plaque formation was scored, and gave a semi-quantitative evaluation of cell damage. In this method the number of effector cells could be controlled and the results are easy to read; however, long incubation periods are often

required for development of the plaques (88).

A drawback of all monolayer techniques is the frequently observed non-specific detachment of the cells. Certain enzymes released from dying cells may contribute to the detachment of undamaged target cells (77). In other instances, the target cell will normally not survive the experiment and its detachment will lead to errors in the evaluation of the results (165).

2. Release of radio-active isotope.

The principle of this technique is that isotopically-labelled target cells are incubated with effector cells and the release of isotope, usually expressed as a percentage of total radioactivity present in the target cells, represents a cumulative measure of cell damage. DNA-synthesizing target cells are incubated with ^{3}H or ^{14}C-thymidine, thus incorporating the label into the DNA of the cell. Completely damaged and disintegrated cells will release DNA which can be detected and quantitated by appropriate methods (89).

Hirata (91) has demonstrated that only damaged cells are susceptible to proteolytic enzymes. On the basis of this finding, Klein and

Perlmann (90) have treated labelled target cells with trypsin and measured the released isotope.

Addition of cold thymidine will prevent the re-utilization of released isotope from damaged cells by DNA-synthesizing cells (92). Using this method, DNA labelled cells can be used for long-term incubations with the effector cells.

^{51}Cr labelling techniques used in red cell survival studies have been adapted by Wigzel (94) and Sanderson (95) for quantitation of antibody-induced lysis of nucleated cells in vitro. Holm and Perlmann (93) have used ^{51}Cr labelling for the determination of cell-mediated lysis of cells growing in suspension or in monolayers. Other authors have since used this method (96, 97, 98). Methods utilizing ^{3}H or ^{14}C-thymidine are less sensitive than the one which utilizes ^{51}Cr-labelled cells as target cells (93). The latter cells when exposed to effector cells were found to release ^{51}Cr within three hours as compared to at least 18 hours of incubation required for the release of thymidine ^{14}C from labelled target cells (93). Chromium-51 is non-covalently bound to proteins and other cell con-

stituents. Furthermore, chromate is reduced during binding and the isotope is therefore not reutilized by the cells (93). Wigzel (94) reported the release of from 80 to 95 per cent of the radio-activity (Cr^{51}) from damaged cells while only a very small percentage (about 15 per cent) of the label was found to be spontaneously released during 24 hours of incubation at 37°C. This normal release of Cr^{51} occurred mainly from cells that were damaged or were dying at the onset of the experiment (99).

3. Inhibition of metabolism.

Granger and Williams (100) have reported that lymphoid cells coming into contact with culture cells will alter the metabolism of the latter, a change that can be assayed by the degree of inhibition of incorporation of radioactive amino acids which have been added to the culture by the target cells (101). This technique measures cytotoxicity as reflected primarily by inhibition of growth. A drawback of this method is that both the effector cells and target cells may synthesize proteins and the inhibition of incorporation of radioactive amino acids by target cells may be masked by their incorporation by the effector cells.

4. Cell counting techniques.

Effector cells are added to a known number of target cells in the form of a monolayer which has been seeded in flat bottomed tubes or petri dishes. After incubation for a specified period, the supernatant is discarded and the remaining target cells are removed from the glass, either mechanically or by trypsinization. The cells are then stained with vital stain and the number of unstained cells is enumerated (78, 102, 103). Alternatively, target cell nuclei are counted after digestion of the cytoplasm with citric acid (76). Counting methods are relatively laborious and highly subjective and are accompanied by large methodological errors. Recently, however, modifications which take into account many of the drawbacks of this method have been introduced (104, 105).

Criteria for an ideal method to determine cell-mediated cytotoxicity are difficult to assess. It could be said, however, that a method encompassing a) a high degree of detection of cell damage, b) detection of small differences in the number of dead cells, c) quantitive estimation and kinetic measurement of cell damage and d) low background (spontaneous cell death) would be a method of choice. Un-

fortunately, none of the methods available at present fulfills all of these criteria.

(iv) General culture conditions.

1. Tissue processing.

The processing of freshly isolated cells or tissues depends considerably on the type of metabolic activity or biological function to be investigated. In cell suspensions, the original organization of the tissue is destroyed and the resulting cell suspension is subjected to a uniform environment. Conversely, in organ culture the original architectural structure of the tissue is retained and the whole system is maintained under as nearly physiological conditions as possible (131, 132). The centers of small tissue fragments rapidly become necrotic; nevertheless, unstimulated preparations retain their ability to respond to antigen for up to 8 days (133). In cell suspensions, viability may fall to 50 per cent within 24 to 48 hours, after which cells appear to die less rapidly. In spite of a seemingly high cell mortality, the unstimulated suspensions retain the ability to proliferate in response to antigen (134, 135, 198) and

PHA (199, 200). It is thus clear that loss of viability measured in the whole heterogeneous cell population does not reflect upon the small number of cells which constitute the sub-populations of cells involved in the immunological response.

2. Chemically defined media.

Until recently the nutritive media employed for cultivation of cells in vitro consisted almost exclusively of blood plasma or serum body fluids, exudates and extracts of tissues and organs. The first attempts to devise chemically defined media were made in 1911 and 1912 by Lewis and Lewis (136) who found that chick-embryo tissues survived in Locke's solution supplemented with amino acids and polypeptides.. They also reported that the addition of glucose to balanced salt solutions improved their cell-sustaining capacities (137). Eagle (153) has shown that Na^+, K^+, Mg^{++}, Ca^{++}, Cl^- and $H_2PO_4^-$ are essential for the survival of Hela cells in culture. Vogelaar and Erlichman (138) reported that the addition of hemin, cystine, insulin, thyroxine and glucose to Tyrode's solution enabled them to maintain strains of human thyroid fibroblasts in an active state of

proliferation for 3 months. Progress continued in this manner with improvement of the existing synthetic media through the addition of various ingredients such as amino acids (140), vitamins (139), co-enzymes (149) etc.

Amino acids were found to be key compounds in cell metabolism and indispensable to growing cells (140, 141, 142, 143). The relative amounts in which they were added to the media corresponded to their concentrations as they were found in biological solutions (140). However, these were not necessarily the optimal proportions in which they should have been included in the nutrient media (144). The minimum number of amino acids which will support growth of certain strains of cells appears to be twelve, namely, arginine, cysteine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine (141, 142).

As the vitamins were identified and their importance in nutrition recognized, they were incorporated into media for cell culture and although their mode of action was at first obscure their effects

on cell nutrition could be easily demonstrated. Baker (139) included in his media the then known vitamins C, A, D, B_1 and B_2. Most media today contain thiamine, riboflavin, the folic acid group, pyrodoxine, nicotinic acid, pantothenate, choline and inositol (145, 146). The use of purines and pyrimidines has been investigated by Parker (147). There is however no firm conclusion as to their essentiality for cell nutrition but they may be significantly stimulatory (148).

In preliminary studies on the effect of co-enzymes, Armour's porcine-liver co-enzyme concentrate in various doses was found to yield a six fold increase in the population of L-strain cell cultures in seven days (149). When the active enzymes were tested individually they had no effect (150). Three other co-enzymes -- co-carboxylase, flavin adenine dinucleotide and uridine-triphosphate, were also found to be useful in nutritional media (150).

3. Gaseous phase.

Interesting studies have also been conducted of the gaseous requirements of cells <u>in vitro</u>.

Harris (151) found that CO_2 is beneficial for the outgrowth of cells from explanted chick-embryo tissues. Swim and Parker (152) also reported that CO_2 was essential for the propagation of six established strains of mammalian fibroblasts. Many mammalian cells do not require added CO_2 (as $NaHCO_3$) as sufficient CO_2 is produced metabolically to satisfy their nutritional needs (154). However, most investigators have used media buffered with bicarbonate and equilibrated it with a gaseous phase of 5 per cent CO_2 and 95 per cent O_2. The high concentration of oxygen, although toxic to cells, (132) is required solely to ensure that a reasonable oxygen tension exists at the level of the cells (159).

4. Serum.

In spite of the effectiveness of chemically defined media, many investigators include in their systems varying amounts of the homologous normal serum, the final concentration of which is usually 10-20 per cent. It is assumed that the beneficial effects of the serum can be attributed to a whole range of complex low and high molecular weight ingredients. Using rabbit spleen fragments cultured in Eagle's medium, Mountain (155) showed that the ad-

dition of a wide range of purines, pyrimidines, vitamins and essential amino acids in low concentrations normally required for cell proliferation could be replaced by the addition of normal rabbit serum. Similar observations were made by Vaughan et al (134). Parkhouse and Dutton (156) have, on the other hand, shown that serum could be replaced by a serum dialyzate. Ambrose (157) found that lymph node fragments synthesized more antibody when serum was replaced by 0.01 to 1 M of hydrocortisone, provided the medium was supplemented with serine. The replacement of serum with hydrocortisone was also reported by Halliday and Garvey (158).

C. Different Model Systems.

(i) Cytotoxic reactions of lymphocytes obtained from sensitized animals.

Lymphocytes from specifically-immunized animals or humans can destroy, in vitro, target cells carrying the antigens to which the lymphocyte donors had been immunized. The first experimental approach along this line was that of Govaerts in 1960 (73) described earlier (see Chapter Two, I, A).. A large number of similar investigations have since been performed

in many laboratories.

The main microscopic observation in such a reaction is the aggregation of effector cells to target cells. Roseneau and Moon (74), Koprowski and Fernandes (86), Taylor and Culling (102), Wilson (106) and Brondz (78) have reported that cells from normal donors or from donors sensitized to unrelated target cells can be washed off whereas effectors cells obtained from donors sensitized to these target cells cannot be easily removed by washing. Aggregation is a feature that usually precedes morphological changes and cell death (74, 86).

A common feature of target cells is their possession of antigens to which donor effector cells can be sensitized. These antigens can be naturally occurring constituents of the target cell surface or, in certain instances, artificially attached (107). Möller (103) and Möller and Möller (88) suggest that in vitro destruction of lymphoid cells by sensitized effector cells constitutes a non-immunological phenomenon in which cells may be killed through an unknown mechanism when coming into close contact with cells of a different genetic and therefore antigenic origin (108). Thus, although effector cells come into contact with target cells through the interaction

of specific receptor sites on the two cells it has been postulated that the actual killing is due to confrontation of the target cells with foreign histocompatibility antigens on the effector cells (88, 103).

It has been shown that experimentally induced tumors in mice can be destroyed by lymphoid cells obtained from tumor-sensitized syngeneic animals (109). Tumor cells with non-cross reacting tumor antigens are not affected (109, 112).

In several human systems, a similar result was obtained (110, 111). Destruction of certain tumors in vitro was demonstrated with the patients' own lymphoid cells and with those from other patients carrying a tumor of the same type. These lymphoid cells however did not affect healthy cells of the same individuals or cells from patients carrying unrelated tumors (110, 111).

In experiments with the type of cell-mediated cytotoxicity described above, heat-inactivated serum is usually included in the incubation mixtures; however, the addition of complement has had no effect (78, 103). Arguments for these reactions being the expression of a cell-mediated activity independent of humoral antibodies are derived from observations

which demonstrate the capacity of antibodies to inhibit the cytotoxic effects of the effector cells. Thus, treatment of mouse target cells with heat inactivated allo-antibodies directed against their H_2 antigen before or during exposure to effector cells obtained from sensitized allogeneic mice strongly reduced the cell-mediated cytotoxic effect of the latter cells (103). The inhibition was found to be immunologically specific. This observation is presumably due to the competition of antibody with sensitized effector cells for the same antigenic sites, although a contributing effect of steric hindrance cannot be excluded owing to the fact that incomplete covering of antigenic sites by iso-antibody raised in genetically different donors was also found to be inhibitory (98, 113).

Lymphoid cells of spleen, lymph node or thoracic duct origin or circulating WBC of sensitized animals exhibit cytotoxic activity to varying degrees, the variation probably being a reflection of the proportion of effector cells in these organs rather than varying degrees of cytotoxicity of the effector cells per se (103, 115, 116). However, thymus effector cells from immunized animals were found to be only

slightly active or inactive.

From data published thus far, it cannot be concluded which cells within the functionally-heterogeneous population of lymphocytes participate in the in vitro cytotoxic reaction. The assumption may be made that the lymphocytes that trigger the reaction in vivo or some of their descendants may function as cytotoxic cells in vitro (25, 31). It has been shown, however, that the reaction of a small number of sensitized lymphocytes with antigen will lead to profound changes in the activity of the lymphocytes which respond with the secretion of a number of biologically-active soluble factor(s) such as MIF, lymphotoxin and mitogenic factor (116, 117, 118). Conversely, the reaction of a few macrophages with antigen seems to be capable of inducing morphological transformation and DNA synthesis in lymphocytes (119). Interactions between effector cells and other cell types could therefore be a contributing factor in the cytotoxic reaction. Cellular interactions have recently been observed in the induction of humoral antibodies (see previous chapter).

The mechanisms by which target cell death is invoked is not well defined; however, a characteristic feature of the reaction is the antigen-specific aggreg-

ation of the effector cell to the target cell. When contact between the cells is prevented, damage of the target cells does not take place (115, 120). Microscopic observations and time lapse cinematography with target cells in monolayers show that some effector cells, after random movements, will become temporarily attached to the target cells which finally seem to undergo osmotic lysis. In these studies it was not shown whether or not lysis was actually produced by the attached cells (120).

A number of authors have shown that effector cells have to be alive to be capable of inducing their specific cytotoxic effect. No such activity has been obtained with dead cells or with extracts or eluates of sensitized effector cells (78, 115, 121). Available evidence suggests that effector cells do not themselves die during the reaction (106). Brunner et al (116) have shown that the cytotoxic potential of a suspension of effector cells remained unchanged when these cells were added to a fresh batch of target cells after having killed the first one. This finding could in fact mean that reaction with antigen, in this case target cells, may enhance the cytotoxic

potential of the effector cell population by their activation or by further recruitment of other cells.

The demonstration that unsensitized lymphoid cells could be made cytotoxic by exposing them to RNA or ribosomes from sensitized cells points to a possible mechanism of recruitment (75, 115, 122, 123, 124).

Wilson (125) has shown that effector cells from sensitized animals will aggregate to target cells at low temperatures but under these conditions will fail to destroy them. Also, it was found that pretreatment of effector cells with antimycin A (a suppressor of electron transport in the respiratory chain) impairs their cytotoxic activity when incubated at concentrations that inhibit respiration without being toxic to the cells during the experimental period (126). Taken together, these two observations reflect the fact that metabolic processes are necessary for the cytotoxic reaction.

Furthermore, RNA and protein synthesis inhibitors such as imuran, actinomycin-D and cyclohexamide have been found to inhibit the cytotoxicity of effector cells when incubated at concentrations that block their metabolic activities but are too low to actually kill the cells (114, 115).

In short term experiments, treatment of effector cells with trypsin in low concentration has been observed to abolish their activity (128). Although this inhibition was temporary and reversible, cytotoxicity of the effector cells was strongly and irreversibly suppressed when treatment with trypsin was followed by cyclohexamide. It was suggested that the removal of protein and the prevention of further synthesis may have disrupted the cytotoxic mechanism through the removal of the antibody-like receptors necessary for the lytic reaction which precedes cell death (128).

Mauel et al (128) reported the suppression of cytotoxic activity by ethylenediamine tetracetic acid (EDTA). However addition of Ca^{++} and Mg^{++} fully restored their activity.

X-irradiation at doses of 15,000R seems to be inhibitory probably because it affects the viability of the effector cells (129).

Wilson (115) has reported on the kinetics of the target cell destruction reactions. Detectable destruction was first noted after 20 hours of incubation but was virtually complete within 50 hours. There

was a direct relationship between target cell death and the number of effector cells added. It was suggested that one effector cell would suffice to affect adversely one target cell, and, by extrapolating these results, that only 1 to 2% of the total number of cells in the effector cell suspension added were immunologically active.

Similar results were obtained by Brunner et al (75) using another system. These authors demonstrated cell death after only 3 hours of incubation and the reaction was complete i.e. total target cell death after 12 hours (114). Complete target cell destruction could be achieved in one hour when the ratio of effector cells used to target cells was 100: 1 (128).

In summary, it could be said that in vitro cytotoxicity mediated by effector cells from sensitized animals involves at least two steps. The first is an immunologically-specific step in which contact between effector cell and target cell is established. This aggregation of cells probably requires as a prerequisite the presence of antibody-like receptors on the effector cells. In the second step target cell lysis ensues. This latter reaction requires the participation of viable and metabolically-active

cells. Although most evidence to date suggests that cell-to-cell proximity is a principal requirement for the lytic step, the possibility that cytotoxic factors may be released by the effector cells has not been ruled out.

(ii) Cytotoxic reactions of effector cells, obtained from unimmunized animals, triggered by antibodies to antigens on target cells.

In the studies to be discussed below, chromium^{51} labelled chicken erythrocytes or Chang liver cells coated with various protein antigens such as PPD or guinea pig thyroglobulins have been used as target cells. These cells were treated with heat-inactivated antisera to antigens on the target cells raised in guinea pigs. The cells were then incubated with lymphoid cells obtained from a number of different organs of normal guinea pigs, usually spleen or blood lymphocytes (79, 126, 161). Target cell damage is generally observed within one to two hours after the addition of the effector cells, and is determined by the degree of ^{51}Cr liberated into the supernatant solutions. Effector cells were observed

to adhere to the antibody-treated target cells prior to the damage of the latter (165). Target cells were not lysed by complement in the absence of effector cells (79, 126).

MacLennan and his co-workers (161, 162) analyzed lymphoid cells obtained from the blood, lymph node, and spleen of humans, rats and rabbits for their capacity to induce cytotoxicity to chromium^{51}-labelled Chang liver cells, originally derived from a human source. They showed that the cytotoxic reaction could occur between the effector and target cells irrespective whether they are autologous, syngeneic, allogeneic or xenogeneic with respect to each other. Malignant lymphocytes from patients with chronic lymphatic leukemia or Burkitt's lymphoma, as well as thymic lymphocytes and non-lymphoid cells were found to be more or less inactive as effector cells (169).

Evidence for the lytic mechanisms being non complement independent comes from experiments by Holm and Perlmann (166) in which they showed that extremely high dilutions of antiserum (up to 10^{-6}), far beyond those active in conventional complement-dependent lytic systems, were effective in rendering normal lymphocytes cytotoxic. This activity by the

antiserum did not correlate with its lytic or agglutinating titers (166). Similar findings were made by MacLennan et al (162) who correlated the lytic activity to a predominantly 7 S-type antibody and by Lo Buglio et al (167), who found that certain lymphocytes bind to the Fc portion of an IgG molecule attached to red cells. This binding leads to damage of the latter cell in the absence of C' (167). These findings infer that different mechanisms are operative in conventional antibody-mediated complement-dependent lysis and antibody-dependent cell-mediated complement independent cytotoxicity. It is assumed that the antibodies involved in the latter instance are of a special class or sub-class which are incapable of lysing the target cells in the presence of C' in the absence of the effector cells (162).

Evidence has been presented (165) which demonstrates that cell-to-cell contact through the antigen-antibody complex formed on the surface of the target cell is essential for the cytotoxic reaction to take place. Chromium^{51}-labelled chicken erythrocytes coated with either PPD or thyroglobulin were mixed in equal proportion and exposed to effector

cells in the presence of antiserum against only one of the antigens. It was observed that the cells on which the antigen-antibody complexes were formed were the only ones to be damaged as detected by the release of ^{51}Cr into the supernatant (165).

Pre-treatment of the effector cells with antimycin A inhibits their cytotoxic activity (126). The reaction is also inhibited by pre-treatment of the effector cells with heterologous antilymphocyte serum (ALS) (168). Light and electron microscopic investigations suggest that the ALS suppresses the reaction by changing the surface properties of the effector cells (168).

(iii) Cytotoxic reactions of lymphocytes stimulated by soluble mitogenic agents or allogeneic lymphoid cells.

It has been observed by a number of investigators (92, 99, 103, 173) that non-specific stimulation of lymphocytes by mitogenic agents renders them destructive to many types of target cells including cells originating from the donor. In these investigations, lymphocytes from human blood or from the blood, thoracic duct or lymphoid organs of guinea pigs,

rats, mice or chicken were stimulated with PHA, streptolysin and staphylococcal filtrate. These cells were then added to chicken red blood cells or tumour cells acting as target cells either in suspension or in monolayers. A cytotoxic reaction ensued which culminated in cell death (92, 99, 103, 173). Incubation of normal human circulating lymphoid cells with allogeneic circulating lymphoid cells in culture also leads to the activation of an effector cell population. Subsequent incubation of these cells with unrelated target cells leads to destruction of the latter (177). Stimulation of lymphoid cells by PHA has been shown to induce blast-cell transformation and DNA synthesis which correlates well with the cytotoxicity of these cells. However, DNA synthesis is not required per se to render the lymphocytes cytotoxic since the cells exhibit optimal cytotoxic activity at a time when DNA synthesis has hardly been initiated (99, 177). It has been observed (92, 98, 178) that cells obtained from lymphoid and non-lymphoid organs which are relatively unsusceptible to stimulation by PHA are only weakly cytotoxic. These cells include normal thymus cells and lymphocytes from patients with chronic lymphatic leukemia, Burkitt's

lymphoma and Hodgkin's disease.

X-irradiation and pre-treatment of the effector cells with hydrocortisone (181) or with inhibitors of protein synthesis does not suppress their PHA-induced cytotoxic effect on target cells (182). In contrast, pre-treatment of the lymphoid cells with ALS (77, 166, 168) inhibits their cytotoxicity. Taken together, these data suggest that the PHA-induced cytotoxic reaction of lymphocytes involves an early step of activation probably leading to changes in their surface properties. The final transformation to a blast cell does not appear to be necessary for the cytotoxic activity to be manifest.

(iv) Cytotoxic reactions of lymphocytes on target cells carrying complement components.

^{51}Cr-labelled chicken red blood cells were treated with very low subthreshold concentrations of heat inactivated rabbit anti-Forssman serum containing primarily 19S antibodies. Under these conditions no cytotoxic reaction appeared when lymphocytes from

human peripheral blood were added. The red blood cells were then treated sequentially with purified components of human complement C'1, C'2 and C'4 (107). No lysis was observed within 24 hours after addition of the lymphocytes (107, 183). However, when C'3 was also bound to the target cells, 70-80% of the cells were lysed within 20 hours of incubation with the effector cells. This cytotoxic reaction was found to be mediated only by viable effector cells and could be inhibited by pre-treatment of the cells with antimycin A (107, 183).

Müller-Eberhard et al (107) have shown that this cytotoxic reaction by the effector cells was lost after fractionation of the effector cell populations on glass bead columns. They obtained a fraction of pure lymphocytes which seemed to lack any cytotoxic activity. In contrast, a second fraction isolated from the glass bead column containing 20% lymphocytes and 80% monocytes was found to be strongly cytotoxic (107). Although this suggests that the effector cells in their system are monocytes, a lymphocyte-monocyte interaction cannot be ruled out (107).

In other experiments, the target cells treated with the first four complement components were brought to the C'7 stage by the addition of

C'5, C'6 and C'7. These cells were rapidly and completely lysed upon addition of effector cells (107, 183). Unlike the previous experiments, glass-bead purified lymphocytes and monocyte enriched fractions of effector cells were cytotoxic. This reaction also required viable cells and was inhibited by antimycin A (107, 183, 184). The authors concluded (107, 183) that some effector cells carry or are able to produce C'8 which is required for the cell-mediated cytotoxic reaction of target cells carrying C'1 to C'7 (107), whereas the cell-mediated destruction of target cells carrying C'1 to C'4 involves phagocytosis following a step of immune adherance (107, 183).

III RELEVANCE OF CELL-MEDIATED CYTOTOXIC REACTIONS TO DISEASES WITH IMMUNOLOGIC ETIOLOGY.

A. General Considerations.

The availability of *in vitro* techniques which detect and measure specific cell-mediated immunity has permitted the elucidation of the role of cell-mediated immune responses in a variety of diseases and pathologic conditions. It is now generally recogn-

ized that the cell-mediated immune response constitutes the mechanism responsible for the rejection of an allograft, tumor rejection, graft-versus-host reactivity, delayed cutaneous hypersensitivity, autoimmunity and resistance to certain pathogens. In all of these situations, cytolytic mechanisms similar to those seen *in vitro* play a role in the chain of events leading to tissue destruction *in vivo*. In this chapter, the possible correlations between the various *in vitro* models described earlier and their respective *in vivo* counterparts will be outlined.

B. Rejection of Allografts or Host-Versus-Graft Reactions.

The initial indication of the rejection of a solid allograft by a non-sensitized recipient consists of the accumulation of large pyroninophilic cells within the paracortical areas of the regional lymph nodes (201). Gowans et al (185) have reported that these cells arise from small lymphocytes which have been stimulated by foreign antigens released from the graft. They have also observed that these cells constantly recirculate from the blood into the lymph nodes via transmigration through the post capillary venule and back through the thoracid duct.

Upon stimulation with certain antigens, these cells may transform rapidly to become large blast cells with typical clusters of polyribosomes in their cytoplasm (180). These activated cells are characteristically present in the grafted organ in direct contact with the foreign cells of the graft at the time of early acute rejection (187). Their high degree of specificity is probably imparted by antibody bound closely to the cell membrane (188). It was demonstrated (189) that C'8 could be involved in this reaction although a number of other factors including MIF, lymphotoxin, transfer factor and interferon have been shown to be released by activated lymphocytes (190). These factors may confer cytotoxicity onto non-sensitized lymphocytes, thus recruiting a larger number of cells and invoking their participation in the rejection process (190). The mechanism by which recipient cells recognize the presence of foreign antigenic determinants is thought to be through the release of histocompatibility antigens from the surfaces of grafted cells into intercellular spaces and thence into the lymphatics and blood vessels (191, 192). Evidence has been advanced

that antigenic material can be recovered from the renal-vein plasma within hours of kidney transplantation in dogs (191). Strober and Gowans (192), from their experiments involving the perfusion of kidneys with labelled foreign thoracic-duct lymphocytes, concluded that these latter cells are antigenically stimulated as they come into contact with the endothelium of the transplant (192). The route by which transport of information to the effector cells in the lymphoid organs occurs has been elegantly demonstrated by the experiments of Barker and Billingham (193), who showed that a flap of skin deprived of lymphatic drainage but retaining a narrow vascular pedicle can support the long-term survival of inlay skin allografts. When lymphatic connections were re-established, the foreign graft was rejected. Similar observations were made by Cronkhite et al (194), who clearly demonstrated the importance of lymphatic channels as afferent conductors in the development of the immune response to tissue allografts.

The role of immune serum in allograft rejection has been investigated. Hasek et al (195) have shown that tolerant ducks could be induced to

reject allografts by transfer of large volumes of immune serum or isolated immune gammaglobulin directed to the allograft. Similar results have been reported by Spong et al (196) who showed that immune serum from Lewis rats grafted with a kidney from a Norwegian brown donor rat undergoing a first set renal rejection, produced perivascular mononuclear infiltrates in the kidney of Norwegian brown rats following parenteral administration (196). The role of humoral antibodies in the rejection of solid allografts was also demonstrated by Clark et al (197). In their experiments, a dog kidney was placed, for a few hours, into an allogeneic recipient who was then heavily irradiated. The kidney was then retransplanted into the donor and it was rapidly rejected (197). These results suggest a synergistic interaction between humoral antibodies and lymphoid cells. This is analogous with in vitro models for cytotoxicity in which cell destruction may be induced by small amounts of antibody to antigens on the target cell surface. When produced within the graft, these antibodies may be absorbed locally but may be sufficient to provoke cell-mediated lytic reactions similar to

those observed in vitro (165). (Section II, C (ii).

C. Delayed Hypersensitivity.

As early as 1890, Koch (202) observed that the subcutaneous injection of protein derived from M. tuberculosis into a tuberculous animal resulted in a generalyzed fever as well as local induration and erythema at the injection site. That this reaction was associated with cells of the lymphoid series was shown by Landsteiner and Chase (203), who were able to passively transfer delayed type hypersensitivity with peritoneal exudate cells. Histologically, skin reactions begin much earlier than the gross signs (204, 205). Initially, the skin test site is infiltrated with polymorphonuclear leukocytes followed by mononuclear cells, both lymphocytes and macrophages, about 8 to 12 hours after injection. The cells appear to collect in perivascular islands in the dermis, and infiltration reaches a peak at 24 to 48 hours (205-209). Passive transfer of delayed hypersensitivity has been accomplished with lymphoid cells taken from many sources of the sensitized animal including spleen, lymph node, thymus and peripheral

blood cells but not serum (205, 206, 207, 208). Although there are occasional reports of transfer of delayed hypersensitivity in animals with cell-free extracts (210, 211), most investigators have found that live cells are required (203, 206, 208, 209). Cummings (211) has shown that, in recipients depleted of lymphoid cells by x-irradiation, delayed hypersensitivity could not be evoked by transfer of cells from sensitized donors. Experiments in which isotopically labeled cells were transferred have shown that only 1-2 per cent of the cells at the test site are of donor origin. The majority of the infiltrating mononuclear cells were of host origin (212, 213, 214).

Although Uhr et al (215), Gell (216) and others (212) have shown that delayed hypersensitivity can be elicited when circulating antibodies cannot be demonstrated, Karush and Eisen (218) have claimed that the reaction is mediated through the action of minute amounts of "high-affinity" antibodies.

Asherson and Loewi (225) have shown that synergism between humoral antibodies and lymphoid cells in delayed hypersensitivity can occur. When

either serum or peritoneal exudate cells from guinea pig donors immunized with BSA or BGG was passively transferred to non-sensitized recipients, a delayed skin reaction was observed within 24 hours after injection of the specific antigen (225). The histology of the cutaneous lesions after transfer of antiserum alone was characterized by infiltration with polymorphonuclear leukocytes. These were absent after 48 hours. When both serum and cells from sensitized donors were transferred, polymorphonuclear cells predominated during the first hours and histiocytes and lymphocytes at 48 hours (226). It was concluded that potentiation of delayed hypersentivity by immune serum was in part due to provocation of a local inflammatory reaction and in part due to local retention of antigen at the site of the skin reaction (226).

It is also postulated (117, 118, 224) that the factors which inhibit macrophage migration in vitro may be of importance in the retention of macrophages and perhaps other leukocytes at the delayed hypersensitivity test site in vivo. These findings suggest that soluble factors are released from a few sensitized cells which may activate or limit non-

sensitized lymphocytes and provoke inflammation by increasing vascular permeability (222, 223).

It could be said that in a delayed hypersensitivity reaction, antigen is retained at the test site where it is injected; this triggers a sequence of reactions which closely resemble those seen in vitro and which have been described in the previous chapter. The sensitized lymphocytes may have a direct cytotoxic effect on the antigen-coated parenchymal cells (See section ii, C, (i)) or they may become activated by interaction with antigen leading to non-discriminatory injury of surrounding tissues (See section II, C, (iii)). Conversely, if humoral antibodies are involved in the delayed hypersensitivity reaction, antigen-antibody complexes formed on the surface of macrophages may induce a cytotoxic reaction by non-sensitized lymphocytes, a mode of action similar to that described in vitro (See section II, C, (ii)).

D. Graft-Versus-Host Reaction(s).

The graft-versus-host reaction occurs when lymphocytes of immunologically mature donors are in-

jected into allogeneic immunologically-immature recipients. The first such reaction was described by Dempster (227) and Simonsen (228) who noted the presence of pyroninophillic cells of the plasma cell series in the cortices of canine renal homografts 3 to 4 days following transplantation and suggested that these cells were of donor origin and might be engaged in an immunologic reaction against the antigens of the host. Billingham and Brent (229) subsequently noticed that attempts to procure tolerance of homografts by inoculating both bird embryos and young rodents with blood or lymphoid cells from allogeneic adult donors frequently resulted in a fatal wasting syndrome which exacted a heavy toll among the recipients. This condition was characterized by a failure of normal development, hepatomegaly, splenomegaly, hypertrophy and then atrophy of the lymphoid tissues and frequent skin abnormalities. They concluded that these pathologic effects were the outcome of a graft-versus-host (GVH) reaction (230). These reactions are dependent on the existence of major histocompatibility differences between the donor and recipient animals (231, 232, 233).

The inability of the host to prevent immunological assault by donor cells occurs if the host is physiologically immature, is genetically tolerant to donor cells, or has a generalized depression of his immunologic capacities as a result of x-irradiation, antimetabolite administration or neonatal thymectomy (231, 234, 235).

Graft-versus-host reactions induced in immunologically mature recipients are usually self limiting and transferred cells are probably eliminated by host-versus-graft reactions (236).

Elkins (237) has described the events which occur following the inoculation of lymphoid cells from parental strain rats beneath the renal capsules of F_1-hybrid hosts. A local self-limiting invasive destructive reaction develops which can destroy 25 per cent of the renal parenchyma leaving other organs and tissues unaffected. This reaction was also regarded as a local graft-versus-host reaction. There is evidence that the destructive reaction does not involve only simple confrontation of immunologically competent donor cells and antigen-bearing cells of the host renal parenchyma. Thus, prior irradiation

of the host to the extent that the level of circulating host mononuclear leukocytes is severely depressed inhibits the development of the renal lesion following implantation of the donor cells. Elkins interpreted this finding to indicate that transplantation antigens of the kidney were apparently not involved (237, 238). Kidney destruction was also brought about by lymphoid cells from parental strain donors, injected into parental strain kidney that had been grafted into F_1-hybrid host (239). In this situation the F_1-hybrid lymphocytes stimulate the donor cells which may then become nonspecifically cytotoxic to the autologous kidney tissue. (See section II, C, (iii)).

Brent and Medawar (241, 242) described a tuberculin-like cutaneous reaction in guinea pigs induced by the intracutaneous inoculation of immunocompetent lymphocytes from normal donors to allogeneic irradiated hosts. This reaction is known as the normal lymphocyte transfer reaction (NLTR) and reflects the reactivity of donor cells to host isoantigens prior to the development of the host response. The intensity of the reaction is related

to the degree of immunogenetic and therefore antigenic disparity between the donor and the host (240, 241). Extensive studies by Brent and Medawar (241, 242) of the events which occur in the NLTR in immunologically incompetent guinea pigs have shown that following transfer of the cells an initial inflammatory episode occurs which reaches its peak after 24 hours. This was considered by the authors to be the outcome of an immunologic recognition event not involving proliferation by the donor cells since this phase of the reaction was not inhibited by antiproliferative agents. On the third to fourth day a "flare up" phase takes place which leads to a second and more violent inflammatory episode. In contrast to the first phase, the flare up phase is abolished by treatment with antimitotic agents. The flare up is regarded as a manifestation of sensitized donor cells which have arisen after contact with host cellular antigens. These observations are analogous to the nonspecific cytotoxicity in vitro of lymphoid cells activated by contact with allogeneic cells. Stimulation of donor cells in the flare up phase may have been brought about in a manner similar to that observed in vitro where heavily irradiated lymphocytes

which are unable to proliferate are known to stimulate allogeneic lymphocytes in mixed cultures *in vitro* (See section II, C, (iii)).

CHAPTER THREE

AIMS AND OBJECTIVES OF THE WORK

In the previous chapter evidence was presented favouring a two-cell model for the induction of the humoral immune response in the mouse and in the rabbit. The evidence presented was based on both *in vivo* and *in vitro* studies using immunologically mature animals. It was demonstrated that in the mouse the thymus and the bone marrow supply the antigen reactive cell(s) (ARC) and antibody forming cell(s) (AFC), respectively. In the rabbit, however, it is the bone marrow which supplies the ARC and the source of the AFC has not yet been determined.

Investigations have also been carried out with respect to the ontogenic source of the virgin immunocompetent cells or their precursors by extirpating organs during the early neonatal period in the hope of preventing dissemination of information-bearing cells or immunocompetent cells from these organs. Recent investigations have implicated the gastro-intestinal associated lymphoid organs in the humoral response in the rabbit. Extirpation of

these organs -- sacculus rotundus, appendix and Peyer's patches -- in the neonatal state or in the adult state followed by whole body irradiation resulted in a marked inhibition of antibody formation when the rabbits were immunized at a later date. Evidence was also presented demonstrating that immunologic reactivity could be restored to heavily irradiated rabbits by the transfer of allogeneic sacculus rotundus cells. It has also been shown that the gastro-intestinal lymphoid organs are sources of cells which synthesize gamma-M antibodies.

However, an aspect of the field which has been neglected is the study of the in vivo migration pathway(s) of immunocompetent cells. Such a study could be beneficial not only in terms of it shedding light upon the roles of the various organs involved in the mediation of the humoral immune response as providers of the immunocompetent cells, and as organs of transit or sites of maturation for the immunocompetent cells but should furthermore alert investigators as to the long term effects on the

immune response of extirpation of lymphoid organs such as the thymus, spleen, appendix, etc., which are usually surgically removed without considering sufficiently the immunological implications involved.

Very few studies have been carried out with the intention of determining the organ sources of the cells mediating the cellular immune response. It has been established, however, that neonatal thymectomy or adult thymectomy followed by irradiation does result in the emergence of a state characterized by immunodeficiency with respect to cell-mediated immunity. Thus, an allograft will be tolerated for a longer period of time if implanted onto such thymectomized recipients. In view of the extensive studies which have been carried out to delineate the interelationships of various lymphoid organs with respect to the humoral immune response, the paucity of such studies with respect to cell mediated immunity is striking.

No systematic investigation has been carried out with the aim of analysing the relationship of lymphoid organs in the mediation of cell-

mediated immunity, the ontogenic sources of the cells involved and their in vivo migration pathway(s) following antigenic stimulation.

This investigation is concerned with the elucidation of the organ source(s) and the in vivo migration pathway(s) of the cells involved in cell mediated immunity. The migration pathway(s) of cells mediating humoral immunity was also investigated in order to relate the two types of immunity with respect to this parameter.

CHAPTER FOUR

MATERIALS AND METHODS

I MATERIALS.

Animals and Immunization Procedures:

Adult 4 to 6 pounds white or black outbred NewZealand rabbits were purchased from local breeders. The rabbits were maintained in well-ventillated temperature-controlled animal houses and were fed and watered at regular intervals of time. Rabbits were immunized by the intravenous injection of 1 ml of a 10 per cent suspension of SRBC or HRBC. They were not sacrificed until three to six months later. These rabbits are referred to as "Memory Rabbits".

Antigens:

Red Blood Cells: sheep red blood cells (SRBC) and horse red blood cells (HRBC) were obtained from the laboratories of the Institut de Microbiologie (Montreal) as sterile suspensions in Alsever's solution and were stored at 4°C. The red cells were washed several times in sterile saline (0.9 per cent sodium

chloride) before use.

Antisera:

Anti-SRBC and anti-HRBC: Antisera to SRBC and HRBC were prepared in rabbits by the intravenous administration of one ml of a ten per cent 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 were obtained following centrifugation of the clotted bloods and were stored at -10°C until used.

Complement:

Commercially-available dried guinea pig serum (Hyland Laboratories, Los Angeles, Calif.) was used as the source of complement throughout this study. It was dissolved and diluted in saline (0.9 per cent sodium chloride) prior to use.

Dextran Solution: (6 per cent solution)

Sixty grams of Dextran (mol. wt. 200,000-275,000, British Drug House) were dissolved in one

liter of saline (0.9 per cent sodium chloride) at 40-45°C. The solution was then divided into 100 ml vials and sterilized. Heparin was added to the Dextran solution by means of a syringe to a final concentration of 150 units of heparin per ml of the Dextran solution.

Media:

1. CMRL 1066: (Microbiological Associates, Bethesda, Maryland.)
2. Eagle's Medium, (Microbiological Associates)
3. Fisher's Medium, (Microbiological Associates)
4. Ham's Medium, (Microbiological Associates)
5. Joklick's Medium, (Microbiological Associates)
6. McCoy's Medium, (Microbiological Associates)
7. Medium-199, (Microbiological Associates)
8. Mishel and Dutton: This was prepared as described by the authors (243) to contain the

the following:

Eagle's minimal essential medium	100 ml
Glutamine (200 MM / ml)	1 ml
Minimal essential medium (100 x conc.)	1 ml
Sodium pyruvate (100 MM/ml)	1 ml

"Nutritional Cocktail Medium":

This was prepared to have the following composition:

Eagle's minimal essential medium without $NaHCO_3$ with non essential amino acids (Microbiological Associates)	35.0 ml
Essential amino acids, 50 times concentrated, (Microbiological Associates)	5.0 ml
Glutamine, 200 MM per ml, (Microbiological Associates)	2.5 ml
Dextrose, 500 mg per ml	1.0 ml
$NaHCO_3$, 7.5 per cent solution (Microbiological Associates)	7.5 ml

Penicillin-Streptomycin Solution:

Stock solution (Microbiological Associates) containing 5000 units penicillin and 5000 µg streptomycin per ml was added to all media used to a final concentration of 200 units of penicillin, and 200 µgm streptomycin per ml of culture medium, or Nutritional medium.

Plastic Ware:

Tubes: Capped, disposable, sterile plastic tubes, 17 x 100 mm (Falcon Plastics, Los Angeles, Calif., Cat. #2004).

Petri-Dishes: Disposable, sterile 35 x 10 mm. (Falcon Plastics, Los Angeles, Calif. Cat. #1008).

Rabbit Serum:

Normal rabbit serum (NRS), obtained from Microbiological Associates, Bethesda, Md., was added to the culture medium to a final concentration of 20 per cent. The complement in the NRS was inactivated by heating at 56°C for 30 minutes before use.

II METHODS.

1) Preparation of Cell Suspensions.

Rabbits were sacrificed by the intravenous injection of nembutal (50 mg per kg body weight) and the spleen, popliteal lymph nodes, thymus, appendix, sacculus rotundus and Peyer's patches were extirpated and placed into disposal sterile plastic tubes containing chilled medium 199. After washing the organs several times with sterile saline, the tissues were cut into small fragments and the cells were expressed by the application of pressure onto the fragments on a sterile wire mesh (50 mesh). The cells were collected into sterile medium 199 fortified with 200 units penicillin and 200 µg streptomycin per ml (Med - PS). The cells were centrifuged once more and resuspended in 2 to 5 ml of Med-PS to give a final concentration of 2×10^7 to 6×10^7 cells per ml.

Bone marrow cells were obtained by splitting the femur and tibia with a bone cutter, collecting the bone marrow into Med-PS and shaking the bone marrow vigorously for about a minute to

free the cells from the stroma and fat. The cells were washed twice with Med-PS in the same manner as described above for the other lymphoid cells.

Circulating leukocytes were obtained by collecting 35 ml of blood from the heart into a sterile 50 ml syringe containing 17 ml of a 6 per cent heparinized Dextran solution. The final ratio of blood to Dextran solution was 2:1 and the heparin concentration was 50 units per ml of blood drawn. The syringe contents were dispersed into sterile plastic tubes which were then placed in a vertical position in a 37°C incubator. When the red cells had sedimented sufficiently, the plasma layer containing the white cells was decanted and centrifuged at 2,000 rpm for 10 minutes. The cells were suspended in Med-PS and re-centrifuged at 1,000 rpm for 10 minutes. They were then resuspended in 1 to 2 ml of Med-PS.

2) Culture Techniques.

A- Induction of plaque-forming cell (PFC) response in vitro.

One tenth ml aliquots of the suspension of

cells to be cultured (either spleen cells of memory rabbits or cells of any of the lymphoid organs of normal rabbits) containing 2-4 x 10^7 mononuclear cells were pipetted into sterile plastic Petri dishes. Washed SRBC, 0.1 ml containing the appropriate number of cells, were added to the Petri dish along with 0.8 ml of the appropriate medium. All media used with the exception of the Mishell-Dutton medium were made up to contain normal rabbit serum (NRS) to a final concentration of 20 per cent. The complement in NRS was inactivated by heating at 56°C for 30 minutes before use. The Petri dishes were placed into a specially constructed air tight plexiglass chamber (10 in. x 9 in. x 6 in.) equipped with inlet and outlet tubes through which various gas mixtures could be infused. Gasing was usually carried out for 15-20 minutes, at a flow rate of 5 liters per minute, at daily intervals unless otherwise stipulated. Following gasing, the inlet and outlet tubes were sealed and the chamber was then placed on a rocker platform (Bellco Glass Incorp., Vineland, N.J.) in a 37°C incubator and subjected to horizontal excursions at the rate of 6-7 complete cycles per minute.

Each day, beginning at the commencement of culture, the Petri dishes were fed with 0.1 ml of the "Nutritional Cocktail Medium".

At varying times following the initiation of the culture, the Petri dishes were removed from the chamber, the cells were gently freed from the plastic surface of the Petri dish to which they had adhered by means of a rubber policeman and they were transferred into sterile Falcon plastic tubes. The cells were centrifuged at 800 rpm for 10 minutes. They were then suspended in medium 199 to a cell concentration of 1 - 4 x 10^7 cells/ml and were analyzed for the number of hemolytic plaque-forming cells (PFC).

B- The inhibition of a plaque-forming cell response by incubation with allogeneic lymphoid cells (allogeneic inhibition).

The procedure followed was essentially that described above except that 1 to 10 x 10^7 lymphoid cells obtained from a normal allogeneic black rabbit (if the donor of the potential plaque-forming cells was a white rabbit and vice versa) were added to the culture plate at the beginning of the culture

or at intervals of time thereafter.

3) Hemolytic Plaque Assay.

The technique used is that described by Jerne and Nordin (244) with slight modifications. Agarose was used in place of agar as described in the original procedure.

Lymphoid cells (0.1 ml), washed sheep or horse red cells (0.1 ml of a 10 per cent suspension) and agarose (0.1 ml of a 0.5 per cent solution) were mixed thoroughly in a 46°C water bath and layered into Petri dishes (2 inches in diameter) containing a thin basal layer (2cc) of a 1.5 per cent agar solution that had been allowed to solidify. The plates were allowed to stand for 2 hours. One ml of commercial guinea pig serum diluted ten fold was then added. The plates were left at 37°C for a further two hours. Plaques were counted with the aid of a magnifying lens and the results were expressed as the number of plaque-forming cells (PFCs) per 10^6 lymphoid cells plated. All assays were carried out in duplicate and the variation in the number of plaques observed

was consistently less then ±10 per cent from the mean.

4) 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 out put 6.97R per minute.

5) Irradiation of Cells in Vitro.

Lymphoid cells to be irradiated were suspended in medium 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; 280Kv peak at 18 ma, half value layer 1.2 cm copper, and focal surface distance 50 cm.

6) Dye Exclusion Test.

The viability of various cells was determined by the dye exclusion test using 0.1 per

cent trypan blue. A drop of the dye was added to one ml of the cell suspension and the latter was then analysed in a hemocytometer. Cells that took up the dye were considered to be dead cells. The viability of the cells, on the basis of 200 cells counted, was recorded as per cent of dead cells.

7) Sonication of Cells.

Cell sonicates were prepared by placing 4 ml of the cell suspension into a plastic tube and subjecting it to 15,000 cycles per second for 30 seconds using a Fisher Ultrasonic Probe (Fisher Scientific Co.).

8) In Vitro Determination of the Cytotoxicity of Supernatants from Cell Cultures.

For the analysis of the cytotoxic activity of the supernatants from cell culture, 0.1 ml of the lymphoid cell suspension (2×10^6) was mixed with 0.1 ml of the supernatant tested and 0.1 ml of undiluted guinea pig serum (a source of complement)

in a plastic tube. The tubes were incubated for 60 minutes at 37°C at which point 0.6 ml Medium 199 and 0.3 ml of a 0.2 per cent solution of trypan blue were added (281). The cells were then analyzed microscopically to determine the number of viable cells, defined as cells which excluded the dye.

9) In Vitro Determination of the Antibody Content of Supernatants from Cell Cultures.

A- Anti-SRBC antibody titres.

Serial two fold dilutions of the culture supernatant were prepared in 1 ml volume to which 0.1 ml of a 2.5 per cent suspension of sheep red blood cells was added. Undiluted guinea pig complement, 0.1 ml, was added to each tube. The tubes were shaken until the cells were evenly dispersed, then placed in a 37°C water bath for 30 minutes and the degree of hemolysis was recorded. In control tubes saline was substituted for the solution to be tested. The titre is expressed as the reciprocal of the highest dilution of solution with which hemolysis was observed.

B- Anti-lymphocyte antibody-content.

The culture supernatants were analyzed for the presence of antibodies directed to the cultured spleen lymphocytes by the fluorescent antibody (sandwich) technique, originally described by Coons et al (245). Smears of spleen memory cells used for the cultures were air-dried on microscope slides and fixed with ethanol for 10 minutes. The slides were stored at 4°C until used. The slides were exposed to the potential antibody-containing supernatants for 30 minutes at room temperature, following which they were washed with buffered saline and exposed to fluorescein-conjugated horse anti-rabbit gamma-globulin for 30 minutes. The slides were then washed with buffered saline, mounted in buffered glycerol and analyzed under a Reichert fluorescence microscope (Zetopan, binolux).

CHAPTER FIVE

EXPERIMENTAL PROTOCOLS, RESULTS AND DISCUSSIONS

I THE OPTIMAL CONDITIONS FOR THE INDUCTION OF THE IMMUNE RESPONSES IN VITRO.

A. The Induction of the Secondary Immune Responses in Vitro.

Experimental procedures:

The initial sequence of experiments was carried out to determine the optimal conditions for the induction of a secondary immune response in vitro. Rabbits were immunized three to six months prior to sacrifice by the intravenous injection of 10^9 SRBC (memory rabbits). The spleen, bone marrow, thymus, popliteal lymph nodes, circulating WBC, sacculus rotundus, appendix and Peyer's patches were extirpated and the cell suspensions of the organs were prepared as described in MATERIALS AND METHODS. The cells were then cultured with 10^7 SRBC as described in MATERIALS AND METHODS. The following parameters were varied individually with the aim of determining

the optimal conditions for culture.

1- Optimal gaseous phase:

Two mixtures of gases, 10 per cent CO_2, 7 per cent O_2 and 80 per cent N_2 and 5 per cent CO_2, 18 per cent O_2 and 77 per cent N_2, were used for gasing the incubation chamber at the initiation of the culture and on subsequent days. Gasing was continued for 15-20 minutes each time after which the chamber was sealed as described in MATERIALS AND METHODS.

2- Optimal culture medium:

Commercially available culture media were used. Eight different media were assayed for their capacity to facilitate the optimal generation of PFC's from the lymphoid cells obtained from memory rabbits.

3- Effect of rocking of culture plates:

The culture chambers were either maintained in a stationary position or placed on a rocker platform (Bellco Glass Inc., Vineland, N.J.) and subjected to horizontal excursions at the rate of 6-7 complete cycles per minute for the duration of

culture.

4- Optimal concentration of memory lymphoid cells:

Varying numbers of memory lymphoid cells -- 2-4 x 10^6, 2-4 x 10^7 or 2-4 x 10^8 were placed in each culture plate. In each instance a constant number of SRBC was added as stimulating antigen.

5- Optimal dose of stimulating antigen:

Varying numbers of SRBC 10^5, 10^6, 10^7 or 10^8, were added to a constant number of memory lymphoid cells per culture plate.

6- Optimal time for culture:

The cells were harvested at varying intervals of time following the initiation of culture and the content of plaque-forming cells (PFCs) were determined as described in MATERIALS AND METHODS.

The specificity of the secondary response to the sensitizing antigen in vitro was investigated by culturing SRBC memory lymphoid cells with SRBC and a non-cross-reacting antigen, HRBC. Conversely, lymphoid cells obtained from memory rabbits immunized

to HRBC were cultured with SRBC as stimulating antigen. Also, memory cells of rabbits immunized with SRBC were cultured with the stroma or sonicate prepared from 10^7 SRBC.

The effect of various additives on the induction of secondary immune response to SRBC in vitro was also investigated. Control culture plates containing 2-4 x 10^7 memory cells and 10^7 SRBC were prepared as described in MATERIALS AND METHODS. The culture medium was CMRL-1066 containing 20 per cent of normal rabbit serum and 200 units of penicillin and streptomycin per ml.

Other culture plates contained 10 μg/ml of hydrocortisone or 20 μg/ml of L-asparagine or an additional 200 units of penicillin and streptomycin per ml. The gaseous phase was 5 per cent CO_2, 18 per cent O_2 and 77 per cent N_2. The cells were harvested and assayed for their PFC content on various days following initiation of culture.

Results:

The initial conditions and medium used for culturing the cells were those described by

Mishell and Dutton (243) (Table I). Under these conditions, only the spleen cells from memory rabbits (rabbits which had been given a primary injection of 10^9 SRBC intravenously 3-6 months prior to sacrifice) could give hemolytic plaque-forming cells (PFC) following *in vitro* stimulation with SRBC. The optimal response was observed at 6-8 days following the initiation of culture (Table I). Different gaseous phases (Table II) and media (Table III) were then used in order to determine the optimal conditions for the induction of the secondary PFC response. The gaseous phase which facilitated optimal generation of PFCs was 5 per cent CO_2, 18 per cent O_2 and 77 per cent N_2 (Table II). The two media which consistently facilitated maximum immune responsiveness *in vitro* were CMRL-1066 and Joklick, with the optimal response occurring between days 4 and 8 (Table III). Only the spleen cells of the memory rabbits could give a PFC response *in vitro* irrespective of the medium used. A very low or no response was obtained unless the cultures were maintained on the rocker

platform and rocked at 6-7 complete cycles per minute for the duration of culture (Table IV). It was also observed that the optimal PFC response was attained when 2-4 x 10^7 splenic lymphoid cells were incubated with 10^7 SRBC (Table V). However, the sensitivity of the system is not as affected by varying the concentration of the antigen, SRBC, as responses of similar magnitude were obtained when 2 x 10^7 splenic lymphoid cells were incubated with either 10^5 or 10^6 or 10^7 SRBC (Table VI).

As shown in Table VII, the PFC response is specific since spleen cells obtained from SRBC memory rabbits and cultured with HRBC, a non-cross-reacting antigen, did not give rise to PFC's for SRBC, nor did they give rise to PFC's for HRBC if cultured with SRBC. Furthermore, memory spleen cells obtained from an HRBC immunized rabbit did not give a PFC response to SRBC if incubated in culture with SRBC (Table VII).

It was observed that SRBC sonicates and stroma could not induce the secondary immune response <u>in vitro</u> (Table VII).

As can be seen from Table VIII the addition of hydrocortisone, L-asparagine or a further

200 units of penicillin and streptomycin inhibited the PFC response of memory spleen cells to a considerable degree.

Discussion:

The system used initially in these experiments was essentially that described by Mishell and Dutton (243) for the induction of the primary response with mouse spleen cells, in the expectation that a similar result might be obtained with rabbit cells. In order to insure that the conditions could, in fact, sustain rabbit lymphoid cells and facilitate the various cellular interactions and transformations as a result of stimulation by antigen in vitro, attempts were first made to induce the secondary response in vitro.

Previous investigations involving the transfer of lymphoid cells from immunized memory rabbits to irradiated immuno-incompetent rabbits disclosed that only the spleen cells of all the lymphoid organs in the memory rabbit possessed the capacity to confer immune responsiveness. Using

allotype markers it was shown that the PFC in the spleen of the recipient rabbit is, in fact, derived from the donor animal (Richter and Abdou, unpublished results). Incubation of recipient spleen cells at the time of PFC analysis with anti-allotype serum directed toward the donor, but not the recipient, of the memory spleen cells could inhibit the PFC response in vitro. It was therefore anticipated that the memory spleen cells would be the only lymphoid cells capable of giving rise to PFCs in culture when stimulated with antigen. In fact, memory spleen cell cultures invariably gave rise to large numbers of PFCs following 4-8 days in culture whereas at no time did cultures of lymphoid cells of any of the other lymphoid organs tested exhibit this property (Table I). It made no difference whether the cells of a lymphoid organ were cultured individually or in combinations with those of any other non-responding lymphoid organ. The best conditions for the induction of the secondary response with rabbit splenic cells did not parallel those described by Mishell and Dutton (243) for the normal mouse cell cultures. The best media proved to be CMRL-1066 and Joklick, with the gaseous

phase being 5 per cent CO_2, 18 per cent O_2 and 77 per cent N_2. As with the mouse cells (243), continuous rocking of the rabbit cultures was essential for the response to ensue. This in vitro secondary response was rarely noted before day 4 of culture and usually was maximal at days 6 to 8 of culture.

The observed inhibitory effect on the number of plaque-forming cells by the addition of hydrocortisone (10 μg/ml) or penicillin-streptomycin (400 units per ml) of culture media is consistent with other observations on the effect of antibiotics and steriods (reviewed by Gabrielsen and Good) (261). Their effect can be manifested either at the inductive phase of the immune response which involves the initial processing of the antigen by lymphoid cells or at the effector phase which comprises the expressions of the immune response such as antibody production or cell mediated injury to target cells.

Studies on the in vitro effect of certain antibiotics by Smiley et al (262) and Ambrose and Coons (263) have shown that low doses of puromycin were effective in inhibiting antibody production usually without killing the cells. In other ex-

periments involving secondary stimulation of lymph nodes fragments from sensitized rabbits with diphtheria toxoid and BSA, Ambrose (264) showed that small amounts of cortisone (0.01 - 10 µg per ml) will permit or even enhance antibody production. However, when 100 µg or 1000 µg were incorporated in the culture media, antibody production was suppressed.

A similar in vitro result was reported by Strauder (265) who used the Jerne plaque technique for assessing levels of 19S hemolytic antibody against sheep red cells. Puromycin and actinomycin reduced the number of plaques, as did hydrocortisone after 3 hours of exposure to 100 or 1000 µg per ml of culture media.

Cohen et al (266), using an in vitro culture system to assess the action of glucocorticoids on the cytolytic effect of lymphocytes, concluded that hydrocortisone has a selective action on antigen reactive lymphocytes rather than the antibody forming cells.

TABLE I

INDUCTION OF THE SECONDARY IMMUNE RESPONSE TO SRBC IN VITRO WITH VARIOUS RABBIT LYMPHOID TISSUE OBTAINED 3-6 MONTHS FOLLOWING IN VIVO PRIMING.

Type of Lymphoid Tissue Cultured	No. of Cells Cultured	No. of S-rbc added to culture	Culture Medium	Gaseous Phase	PFC per 10^6 recovered mononuclear cells assayed on the following days after initiation of culture			
					Day 4	Day 6	Day 8	Day 10
Spleen	3.0×10^7	10^7 cells	Mishell & Dutton	10% CO_2 7% O_2 83% N_2	130	220	200	100
Bone marrow	2.6×10^7	"	"	"	0	0	0	0
Thymus	3.0×10^7	"	"	"	2	1	0	0
Lymph node (Popliteal)	1.8×10^7	"	"	"	0	0	0	0
Circulating WBC	2.0×10^7	"	"	"	3	2	0	0
Sacculus rotundus	4.0×10^7	"	"	"	0	0	0	0
Appendix	4.0×10^7	"	"	"	0	0	0	0
Peyer's patches	2.8×10^7	"	"	"	0	0	0	0

TABLE II

INDUCTION OF THE SECONDARY IMMUNE RESPONSE TO SRBC IN VITRO: THE EFFECT OF VARYING THE GASEOUS PHASE.

Type of Lymphoid Tissue Cultured	No. of Cells Cultured	No. of S-rbc added to culture	Culture Medium	PFC per 10^6 recovered mononuclear cells assayed on the following days after initiation of culture					
				Day 4		Day 6		Day 8	
				10* 7 83	5** 18 77	10 7 83	5 18 77	10 7 83	5 18 77
Spleen	$2\text{-}3\times10^7$ cells	10^7 cells	Mishell & Dutton	130	200	220	320	200	410
Bone marrow	"	"	"	0	0	0	0	0	0
Thymus	"	"	"	2	4	1	0	0	0
Lymph node (Popliteal)	"	"	"	0	1	0	0	0	0
Circulating WBC	"	"	"	3	2	2	0	0	0
Sacculus rotundus	"	"	"	0	0	0	0	0	0
Appendix	"	"	"	0	1	0	0	0	0
Peyer's patches	"	"	"	0	2	0	0	0	0

*10% CO_2, 7% O_2 and 83% N_2
**5% CO_2, 18% O_2 and 77% N_2

TABLE III

INDUCTION OF THE SECONDARY IMMUNE RESPONSE WITH RABBIT SPLEEN CELLS IN VITRO: EFFECT OF DIFFERENT CULTURE MEDIA ON THE IMMUNE RESPONSE TO SRBC ON VARIOUS DAYS FOLLOWING INITIATION OF CULTURE.

Medium used	PFC/10^6 recovered spleen cells assayed on the following days after initiation of culture*									
	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 14
Mishel & Dutton	0	0	200	230	320	400	410	N.D.	160	8
Ham's +20%NRS	N.D**	0	240	360	380	440	360	N.D.	N.D.	N.D.
Joklick+20% NRS	0	0	400	410	500	610	480	N.D.	N.D.	N.D.
CMRL-1066 + 20% NRS	0	4	520	600	600	520	400	N.D.	N.D.	N.D.
McCoy's + 20% NRS	N.D.	0	N.D.	200	N.D.	100	N.D.	N.D.	N.D.	N.D.
Fisher + 20% NRS	N.D.	N.D.	N.D.	120	N.D.	70	N.D.	N.D.	N.D.	N.D.
Medium 199 + 20% NRS	N.D.	N.D.	N.D.	80	N.D.	70	N.D.	N.D.	N.D.	N.D.
Eagle's + 20% NRS	N.D.	N.D.	N.D.	80	N.D.	80	N.D.	N.D.	N.D.	N.D.

* All culture plates contained 2-4 x 10^7 spleen cells and 10^7 SRBC/ml of culture medium. Gaseous phase was 5%, 18% & 77% for CO_2, O_2 & N_2 respectively.

** N.D. = Not done

TABLE IV

INDUCTION OF THE SECONDARY IMMUNE RESPONSE WITH RABBIT SPLEEN CELLS IN VITRO: EFFECT OF ROCKING OF CULTURE PLATES.

Conditions of Incubation*	PFC/10^6 recovered mononuclear cells assayed six days after initiation of culture	
	Stimulated Spleen Cells (SRBC added)	Unstimulated Spleen Cells (No SRBC added)
Rocked Horizontally Six times per min.	300	8
Stationary	46	1

* The culture medium was Joklick plus 20% NRS. The gaseous phase was 5% CO_2, 18% O_2 and 77% N_2

TABLE V

INDUCTION OF THE SECONDARY IMMUNE RESPONSE WITH RABBIT SPLEEN CELLS IN VITRO: EFFECT OF VARYING CONCENTRATIONS OF SPLEEN CELLS ON THE RESPONSE TO STIMULATION WITH SRBC.

Concentration of Memory Spleen Cells at Initiation of Culture*	No. of PFC/10^6 recovered mononuclear cells assayed 6 days after initiation of culture	
	Spleen Cells stimulated with 10^7 SRBC	Spleen Cells unstimulated (No SRBC added to culture)
$2-4 \times 10^6$/ml	145	0
$2-4 \times 10^7$/ml	390	2
$2-4 \times 10^8$/ml	70	0

* Culture medium was Joklick + 20% NRS.
Gaseous phase was 5% CO_2, 18% O_2 and 77% N_2.

TABLE VI

INDUCTION OF THE SECONDARY IMMUNE RESPONSE WITH RABBIT SPLEEN CELLS IN VITRO: EFFECT OF VARYING THE DOSE OF SRBC USED FOR STIMULATION AND THE MAINTENANCE OF CULTURE IN DIFFERENT GASEOUS PHASES.

No. of S-RBC added to each culture plate containing 2×10^7 spleen cells/ml of medium*	No. of PFC/10^6 recovered mononuclear cells assayed 6 days after initiation of culture and maintained under the following gaseous phases:		
	AIR**	10% CO_2 7% O_2 83% N_2	5% CO_2 18% O_2 77% N_2
1×10^5	0	328	N.D.
1×10^6	0	390	90
1×10^7	0	320	430
1×10^8	0	130	160

* Culture medium was Joklick + 20% NRS.
** 20% O_2, 80% N_2, less than 0.1% CO_2.

TABLE VII

INDUCTION OF THE SECONDARY IMMUNE RESPONSE WITH RABBIT SPLEEN CELLS IN VITRO: THE SPECIFICITY OF THE SECONDARY RESPONSE TO THE SENSITIZING ANTIGEN.

Memory rabbit immunized with	2×10^7 splenic "memory" lymphoid cells cultured under following conditions*	PFC/10^6 recovered mononuclear cells assayes on the following days after initiation of culture:			
		Day 4	Day 6	Day 8	Day 10
S-RBC	Medium CMRL-1066 + 20% NRS + Nil	0	0	0	N.D.
S-RBC	Medium CMRL-1066 + 20% NRS + 10^7 SRBC	390	410	300	140
S-RBC	Medium CMRL-1066 + 20% NRS + 10^7 HRBC	0	0	0	N.D.
S-RBC	Medium CMRL-1066 + 20% NRS + SRBC Stroma (equivalent to 10^7 S-rbc)	8	12	2	N.D.
S-RBC	Medium CMRL-1066 + 20% NRS + SRBC Sonnicate (equivalent to 10^7 S-rbc)	0	4	0	N.D.
H-RBC	Medium CMRL-1066 + 20% NRS + 10^7 S-rbc	0	0	0	0

* All culture plates contained $2 - 4 \times 10^7$ memory spleen cells. Gaseous phase was 5% CO_2, 18% O_2 and 77% N_2.

TABLE VIII

INDUCTION OF THE SECONDARY IMMUNE RESPONSE WITH RABBIT SPLEEN CELLS IN VITRO: EFFECT OF VARIOUS ADDITIVES ON THE IMMUNE RESPONSE TO SRBC.

Following materials added to culture*	PFC/10^6 recovered spleen cells assayed on the following days after initiation of culture:			
	Day 4	Day 6	Day 8	Day 10
Control (no S-RBC)	0	0	0	N.D.
Control	70	333	375	160
Hydrocortisone (10 ug/ml)	5	72	115	100
Penicillin - Streptomycin (200 units/ml) (Total 400 units/ml)	2	54	185	150
L-asparagine (20 ug/ml)	N.D.	80	70	N.D.

* All culture plates contained 2 - 4 x 10^7 memory spleen cells and 10^7 SRBC. Medium is CMRL-1066 + 20% NRS + 200 units of Penicillin and Streptomycin. Gaseous phase was 5% CO_2, 18% O_2 and 77% N_2

B. Failure of Individual Rabbit Lymphoid Cells to Induce a Primary Immune Response in Vitro.

Experimental procedures:

Lymphoid cell suspensions were prepared from the different lymphoid organs of normal adult unimmunized rabbits as described in MATERIALS AND METHODS. Spleen cells were cultured alone using different culture media and gaseous phases. In other experiments, cell suspensions from each of the lymphoid organs were cultured alone in medium CMRL-1066 or in combination with other lymphoid cells from the same rabbit. The number of lymphoid cells cultured was usually between 2-4 x 10^7 cells incubated with 10^7 SRBC as stimulating antigen. When combinations of cell types were cultured 2 x 10^7 lymphoid cells of each organ were cultured with a similar number of cells of the other organs.

Results:

Attempts were carried out to induce a primary response with the different lymphoid cells,

using a variety of media. Only six out of sixteen experiments were successful in this regard (Table IX). In three cases, spleen cells, but not any of the other organ cell preparations, elicited a low grade PFC response, and this only when the cells were cultured in Ham's medium. In three other experiments, although no single cell preparation gave a PFC response, mixed cultures of spleen and appendix or spleen and bone marrow cells gave very strong PFC responses after four days in culture (Table IX). A somewhat lesser response was obtained with a mixed culture of spleen and thymus cells. Mixed cultures of spleen with either Peyer's patches cells, sacculus rotundus cells or circulating leukocytes did not respond with plaque-forming cells in vitro. Neither did mixed cell cultures of bone marrow and thymus cells.

Discussion:

Although the optimal conditions required for inducing a secondary immune response were achieved, their application in attempts to induce a primary immune response with lymphoid cells from unimmuniz-

ed rabbits were essentially non-productive. It is noteworthy, however, that the only successes attained were with spleen cells cultured in Ham's medium, not in CMRL-1066 or Joklick which were the media which facilitated the optimal secondary immune response (see above). However, these results were somewhat discouraging in view of the small number of PFCs stimulated and by the fact that none of the preparations of lymphoid cells of any of the other normal lymphoid organs investigated gave a PFC response in vitro. An unexpected finding, however, was the marked PFC response attained with a mixed culture of normal spleen and either normal appendix or bone marrow cells and a somewhat lesser response with a mixture of spleen and thymus cells. However, this response proved to be very inconsistent as only 3 out of 10 experiments attempted gave the results referred to above (Table IX). It would appear that, under appropriate conditions attained with the cells of a minority of rabbits, a marked humoral immune response could be obtained only with mixed cell cultures and not with individual organ cultures. Since previous investigations have demonstrated that the primary immune response in the rabbit requires the mediation of the antigen-

reactive cell (ARC) and the antibody-forming cell (AFC) (247, 248) and that the immunocompetent cell residing in the bone marrow is the ARC (247), the successful immune response obtained *in vitro* with a combination of bone marrow and spleen cells would suggest that the spleen constitutes a source of the AFC. However, this interpretation does not provide an understanding as to the nature of the immunocompetent cells which mediated the immune response with a mixture of spleen and appendix cells. It may be that the spleen has both ARCs and AFCs, much like the thoracic duct cells in the mouse (26; 249), but they are both present in insufficient numbers to provide for optimal interactions resulting in antibody formation. The addition of bone marrow cells rich in ARCs facilitates the maximum stimulation of the AFCs in the spleen cell preparation whereas the addition of appendix cells, which may be a major source of the AFC (46, 55, 250, 251), may provide sufficient AFCs to allow the immune responses to ensue. However, it must be stressed that the primary immune responses obtained *in vitro* were so infrequently observed that it is early to theorize at this time, as to the types of cells involved.

Notwithstanding the results described here, other investigators have apparently been more successful at inducing a primary response *in vitro* with rabbit lymphoid cell cultures. Henry et al (52) obtained a primary PFC response to SRBC with cultures of normal rabbit Peyer's patches cells and spleen cells and Theis and Thorbecke (246) observed a primary plaque-forming cell response to SRBC with normal rabbit spleen cells *in vitro*. However, in both instances, the responses observed with cells of normal previously unimmunized rabbits were consistently much lower than those observed with spleen cells of previously immunized rabbits. Whether these responses represent primary responses in the true sense of the word may be seriously questioned in view of the fact that the *in vitro* responses have been obtained with the cells of a single normal lymphoid organ whereas the primary response *in vivo*, in both the mouse and the rabbit, is mediated by the interaction of at least two organ specific, functionally - distinct lymphocytes, the virgin antigen-reactive cell (ARC) and the virgin antibody-forming cell (AFC) (15, 30, 247, 248). In the mouse, the virgin ARC is localized in the thymus

(30, 252-255) and the virgin AFC in the bone marrow (30, 252). In the normal rabbit, the virgin ARC is localized in the bone marrow (50, 64, 248, 256) and the source of the virgin AFC is not yet definitely known although one source may be the appendix (55). However, in both of these animal species, cells of two distinct lymphoid organs must interact in order for humoral antibody formation to be induced.

TABLE IX

INDUCTION OF THE PRIMARY IMMUNE RESPONSE IN VITRO WITH RABBIT LYMPHOID CELLS.

Cells Cultured*	Medium Used	Gaseous Phase	PFC per 10^6 recovered mononuclear cells harvested at following day of culture			
			3	4	5	6
Spleen	Mishell & Dutton** (0/4)	10:7:83-CO_2:O_2:N_2	0	0	0	0
Spleen	Ham (3/6)	5:17:78-CO_2:O_2:N_2	0	10	30	15
			0	15	40	18
			0	12	67	38
Spleen	Joklick or McCoy or Fisher or CMRL-1066 or Eagle (0/15)	5:17:78-CO_2:O_2:N_2	0	0	0	0
		10:7:83-CO_2:O_2:N_2	0	0	0	0
Spleen	CMRL-1066 (3/10)	5:17:78-CO_2:O_2:N_2			0	
Bone marrow					0	
Thymus					0	
Circulating WBC					0	
Appendix					0	
Sacculus rotundus					0	
Peyer's patches					0	
Spleen plus appendix***					2,000	
Spleen plus bone marrow					2,000	
Spleen plus thymus					1,000	
Spleen plus Peyer's patches					180	
Spleen plus WBC					4	

* In each case, 2 to 3 x 10^7 lymphoid cells were incubated with 10^7 S-rbc.

** The ratio, in parenthesis, represents the number of successful experiments to the number attempted.

*** In the cases where mixtures of cells were cultures, 2 x 10^7 lymphoid cells of one organ were cultured with 2 x 10^7 cells of the other organ.

II IN VIVO MIGRATION PATHWAY(S) OF ANTIGEN (SRBC) STIMULATED ANTIBODY FORMING CELLS IN THE ADULT RABBIT.

Experimental procedures:

The experimental protocol is diagrammatically presented in Figure 1. Rabbits were immunized by the intravenous injection of 10^9 SRBC. They were then sacrificed at varying intervals of time and the bone marrow, spleen, thymus, lymph node (popliteal), appendix, sacculus rotundus and Peyer's patches were extirpated.

The preparation of the various cell suspensions was described in MATERIALS AND METHODS.

These cell suspensions were analyzed for their content of plaque-forming cells (PFCs) by the technique of Jerne and Nordin (244) immediately after sacrifice to determine whether these organs possessed antibody-synthesizing cells. Other aliquots of these cell suspensions were cultured for 4 days in vitro in the presence of the antigen, SRBC, and then analyzed for their content of PFCs.

The tissue culture procedure used was essentially that described by Mishell and Dutton (243) and outlined in MATERIALS AND METHODS.

One tenth ml aliquots of the lymphoid cell suspensions containing 2-4 x 10^7 mononuclear cells were cultured with 10^7 SRBC in medium CMRL-1066 containing normal rabbit serum (final concentration 20 per cent). At day four following initiation of culture, the cells were harvested and analysed for the number of hemolytic plaque-forming cells (PFCs).

Results:

The results of a typical experiment are presented in Table X. Prior to day 4 after in vivo immunization, none of the lymphoid organs possessed PFCs which could be detected by plating the cells immediately following sacrifice of the rabbit. However, following in vitro culture of the cells obtained from the rabbit sacrificed 1 to 2 days following in vivo immunization, only the Peyer's patches were capable of giving rise to PFCs. Of the organs obtained from the rabbit sacrificed 4 to 7 days following immunization, only the spleen possessed cells which could give

rise to PFCs following 4 days in culture. However, unlike the Peyer's patches cells, the spleen also contained cells which could give a PFC response if tested before culture. In other words, the spleen at this time possessed cells which were active antibody producing cells. After day 7 of immunozation, antibodies in high titer and PFCs could be detected in the circulation.

At no time were plaque-forming cells (PFCs) detected in the spleens of unimmunized rabbits irrespective whether the cell suspensions were analyzed for PFCs immediately following sacrifice of the rabbit or after 4 days in culture in the presence of the antigen. We were unable to obtain a primary in vitro response with the cells of any of the lymphoid organs of the normal rabbit.

Since only the Peyer's patches and spleen cells of the immunized rabbits gave PFCs, only results with the cells of these two organs are presented in Table XI. Here again, the Peyer's patches cells, but not the spleen cells, obtained from rabbits sacrificed 1 or 2 days after immunization uniformly gave a PFC response following culture for 4 days in the presence, but not in the absence,of

the antigen. The spleen began to give a PFC response, either immediately after sacrifice or after culture in vitro, only if the cells were obtained from the immunized rabbits 3 or more days following immunization. By this time the Peyer's patches cells either gave a minimal or no PFC response.

It must be emphasized that the PFCs detected are not memory cells since they cannot be detected in the unimmunized rabbit. Therefore, the cells detected initially in the Peyer's patches are probably immature committed AFCs, since they must mature in culture in the presence of the antigen before they are capable of giving a PFC response.

Discussion:

In view of the numerous investigations which implicate the gut-associated lymphoid tissues in the rabbit--sacculus rotundus, appendix and Peyer's patches (SAPP organs)-- in the mediation of the humoral immune response (46, 54, 55, 58, 251, 257), it was felt that these organs may act as reservoirs of virgin AFCs and/or as organs of transit and/or as sites of maturation for the stimulated AFC. The results presented tend to support the two latter suppositions. The Peyer's patches cells did not

manifest PFC activity when the cells were analyzed for PFCs immediately after sacrifice one or two days following immunization, but did give rise to PFCs following 4 days in cell culture. Since the AFC is detected in the spleen two days after it is initially detected in the Peyer's patches and after it can no longer be detected in the Peyer's patches, it is suggested that the antigen-stimulated AFC matures in the Peyer's patches for 1 to 2 days following which it migrates to the spleen, where its period of maturation terminates and it transforms into an overt PFC and antibody-synthesizing cell. A similar consideration of the cellular events culminating in antibody formation has been presented by Hanaoka and Waksman (54) with respect to the rabbit and by Cooper and Turner (58) with respect to the rat. In the former case (54), the appendix is the SAPP organ considered to be the source of the virgin AFCs while in the latter case (58) it is the Peyer's patches which are considered to assume this role. In this connection, it is noteworthy that Good et al (259) arrived at a completely different conclusion with respect to the role of the SAPP organs in the immune response.

Rabbits were immunized with SRBC by various routes (intravenously, intraperitoneally and orally) and the various lymphoid organs were analyzed for PFCs 6 days later. The greatest number of PFCs (per 10^6 lymphoid cells) were detected in the spleen and lymph nodes, a much lesser number were found in the bone marrow and almost no PFCs were detected in the SAPP organs or the thymus. These investigators (259) concluded that the stimulated AFCs do not exist in the SAPP organs and that they do not home in on these organs even if they are stimulated in another organ. However, these investigators only analyzed the organs of the immunized rabbits at 6 days post-immunization. One can only speculate as to whether they would have detected PFCs in some other lymphoid organ would they have investigated their animals at daily intervals of time post-immunization and cultured their lymphoid cells, as we did.

Henry et al (52) have reported that they obtained primary <u>in vitro</u> PFC responses to SRBC in tissue culture with cells obtained from normal adult rabbit Peyer's patches and spleen. However, whether these responses represent primary responses in the

true sense may be seriously questioned in view of the fact that the only antigen used was the SRBC, which is known to cross-react with a number of enteric bacteria and to possess the Forssman group of antigens (258, 260). The responses observed by these investigators (52) may therefore be attributed to committed AFCs, rather than virgin, uncommitted AFCs. Some of these cells may have homed in on the Peyer's patches, following their stimulation with antigen(s) which cross-react with the SRBC in some other organ. In the presence of sufficient antigen in the microenvironment of the Peyer's patches, the cells would be further stimulated and would be induced to migrate to the spleen, where they would transform and/or proliferate into overt antibody-forming cells and give rise to the memory cells. In the absence of a threshold concentration of antigen in the Peyer's patches, the stimulated AFCs would remain in this organ and would not migrate to the spleen. A similar migration pattern for the AFC has been suggested by Hanaoko and Waksman (54) and Cooper and Turner (58). Since we were unable to obtain a primary response with the cells of any of the lymphoid organs of the unimmunized rabbits, it would be fair to assume that the

rabbits used in this investigations did not possess AFCs already committed to respond to stimulation with SRBC in vitro prior to immunization.

The data obtained strongly suggest that the Peyer's patches constitute prime sites for the maturation of the SRBC-stimulated AFC.

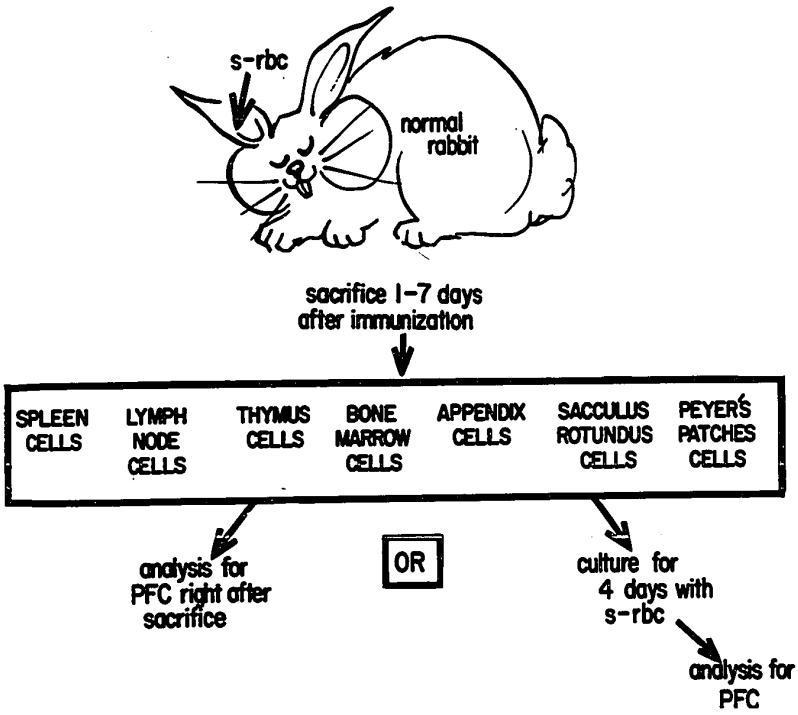

FIGURE 1. PROTOCOL FOR THE INVESTIGATION OF THE IN VIVO MIGRATION PATHWAY OF THE ANTIGEN STIMULATED ANTIBODY FORMING CELL(S).

TABLE X

THE ORGAN SOURCE OF COMMITTED PRECURSORS OF PLAQUE-FORMING CELLS (PFC) AT INTERVALS OF TIME SUBSEQUENT TO THE INTRAVENOUS ADMINISTRATION OF 10^9 SRBC.

Interval (in days) between immunization and sacrifice	The plaque-forming capacity (PFC per 10^6 lymphoid cells) of cells of organs analyzed immediately or after four days in culture.						
	Spleen	Lymph node	Thymus	Bone Marrow	Appendix	Sacculus Rotundus	Peyer's Patches
Control - not Immunized	0/0*	0/0	0/0	0/0	0/0	0/0	0/0
1	0/0	0/0	0/0	0/0	0/0	0/0	35/0
2	0/0	0/0	0/0	0/0	0/0	0/0	38/0
3	35/0	0/0	0/0	0/0	0/0	0/0	14/0
4	100/30	0/0	0/0	0/0	0/0	0/0	6/0
5	280/80	0/0	0/0	0/0	0/0	0/0	0/0
7	50/150	0/10	0/3	0/5	0/3	0/0	0/0

* The numerator and the denominator represent the PFCs observed after four days in culture and immediately after sacrifice, respectively.

TABLE XI

THE TEMPORAL RELATIONSHIP BETWEEN THE APPEARANCE OF PFCs IN PEYER'S PATCHES AND SPLEEN CELLS AT INTERVALS OF TIME FOLLOWING IMMUNIZATION WITH 10^9 SRBC.

Rabbit No.	Interval between immunization and sacrifice (days).	The plaque-forming capacity (No. of PFCs per 10^9 lymphoid cells) of cells of the Peyer's patches and spleen analyzed immediately or after 4 days in culture.	
		Peyer's patches cells	Spleen cells
1	1	35/0 *	0/0
2	1	24/0	0/0
3	1	30/0	0/0
4	1	35/0	0/0
5	1	28/0	0/0
6	2	38/0	0/0
7	2	35/0	0/0
8	2	24/0	0/0
9	2	38/0	0/0
10	2	30/0	0/0
11	3	15/0	30/0
12	3	10/0	36/0
13	3	12/0	40/0
14	3	14/0	20/0
15	4	0/0	65/30
16	4	0/0	48/22
17	4	2/0	50/28
18	4	0/0	56/42
19	4	1/0	40/15
20	5	0/0	165/95
21	5	0/0	278/80
22	5	0/0	200/75
23	5	0/0	160/65
24	7	0/0	50/150
25	7	0/0	45/180

* The numerator and the denominator represent the PFCs observed after four days in culture and immediately after sacrifice, respectively.

III THE ORGAN SOURCE OF THE CELLS MEDIATING A CELLULAR IMMUNE REACTION IN VITRO.

A. The Inhibition by Homologous Lymphoid Cells of the Plaque-Forming Cell Response by Memory Spleen Cells in Culture.

Experimental procedures:

The protocol used in these experiments is diagrammatically represented in Figure 2. Normal white rabbits were immunized by the intravenous injection of 10^9 SRBC. They were not used for tissue culture experiments until 3 to 6 months had elapsed following immunization. These rabbits are referred to as "memory rabbits".

The animals were sacrificed by the intravenous injection of nembutal (50 mg per kg body weight). The circulating leukocytes and the various lymphoid organs - thymus, bone marrow, spleen, popliteal lymph node, appendix, sacculus rotundus and Peyer's patches, were excised and processed to yield cell suspensions in the manner described in MATERIALS AND METHODS. The appendix, sacculus rotundus and Peyer's patches are collectively referred to as SAPP organs.

The *in vitro* cell culture technique was set up as described previously. Spleen cells of a memory rabbit (i.e. white rabbit) were cultured with SRBC with or without the addition of homologous (i.e. black rabbit) lymphoid cells or autologous lymphoid cells in sterile petri dishes (Figure 2). The medium used was CMRL-1066 (Microbiological Associates, Bethesda, M.D.) and the gaseous phase was 5 per cent CO_2, 18 per cent O_2 and 77 per cent N_2 since these conditions were shown to be optimal for the induction of a secondary immune response *in vitro* (see Section I of this chapter). The petri dishes were placed into a specially constructed plexiglass chamber and subjected to 6 complete horizontal excursions per minute for the duration of culture. The cultured cells were then harvested, centrifuged and analyzed for their content of hemolytic plaque-forming cells (PFC) to SRBC by the method of Jerne and Nordin (244).

To determine the need for viable effector cells, the memory spleen cells were cultured with SRBC and the various homologous or autologous lymphoid cells or with SRBC and sonicates of the homologous lymphoid cells.

Sonicates were prepared by subjecting the cells to 15,000 cycles per second for 30 seconds, using a Fisher ultrasonic probe (Fisher Scientific Co.,). Cell viability was determined by the method outlined in MATERIALS AND METHODS.

The effect of varying the relative concentrations of homologous lymphoid cells was assessed using the same protocol (Figure 2). The number of memory spleen cells cultured was kept at 2.0×10^7 and the number of homologous lymphoid cells added to the cultures was varied from 0.1×10^7 to 8.0×10^7 cells. At the end of the culture period, the cells were harvested and assayed for the content of hemolytic plaque-forming cells to SRBC as described above.

Experiments were carried out to determine the time required for the effector cells to be in the culture to inhibit the secondary immune response by the memory spleen cells. Effector cells were added to the memory spleen cells at the initiation of culture and at 24 and 48 hours thereafter. At the end of the 4 day culture period, the cells were harvested and assayed for hemolytic plaque-forming cells.

Results:

As can be seen in Table XII, the capacity of memory spleen cells to generate PFCs to SRBC in culture was not affected by the addition to the culture plates of cells of any of the other autologous lymphoid organs. In fact, the addition of bone marrow cells stimulated the memory spleen cells to proliferate and/or transform to PFCs to a greater degree than in their absence, as attested to by the large number of PFC per 10^6 cultured lymphoid cells (Table XII, column 7). On the other hand, results of a quite different nature were obtained when the memory spleen cells (white rabbit) were cultured with cells of a homologous normal rabbit (black rabbit) (Table XIII). The most startling finding was the almost complete suppression of the emergence of PFCs by the homologous SAPP cells and the circulating WBC. The spleen and lymph node cells induced approximately a 40 to 50 per cent inhibition of the PFC response whereas the bone marrow and thymus cells induced no inhibition. However, here again, the memory spleen cells cultured with homologous bone marrow cells appeared

to survive and/or proliferate to a greater degree than normal, since the number of PFC per 10^6 splenic lymphoid cells cultured was almost 3 times greater than the control value (Table XIII, column 7). Extending the cultures for 6 days rather than the customary 4 days still did not result in any inhibitory effects by the homologous thymus or bone marrow cells (not shown in table). The results were identical irrespective whether heated (56°C, 30 minutes) or unheated normal rabbit serum was incorporated into the culture medium. These results, inhibition by homologous cells of the PFC response by memory spleen cells, were consistently obtained in a series of over forty experiments. In only one case did homologous cells fail to inhibit the PFC response.

These experiments were repeated in the reciprocal manner. That is, preimmunized black rabbits were used as the sources of memory spleen cells and the organs of normal white rabbits were used as sources of effector cells. In six such experiments, identical results to those presented in Tables XII and XIII were obtained in that only the homologous (white rabbit) SAPP cells and circ-

ulating lymphocytes could inhibit the generation of PFC's from memory (black) spleen cells in culture.

As can be seen in Table XIV, the inhibitory capacity of the SAPP cells and circulating WBC is a property of the living cell since sonicates of these cells were unable to inhibit the emergence of PFC's in culture in vitro.

The inhibiting cells appear to represent a constant proportion of the cells in each of the four cell preparations which manifest inhibitory activity (appendix, sacculus rotundus, Peyer's patches and circulating WBC) since they were all equally active at cell concentrations equal to the memory spleen cells but lost activity at the same dilution (Table XV). On the other hand, incubation of the memory spleen cells with much larger numbers of homologous thymus and bone marrow cells had no deleterious effect on the capacity of the former to generate PFC's (Table XV). However, incubation of the memory spleen cells with homologous spleen and lymph node cells in a ratio of 1 to 4 (memory spleen cell to homologous lymphoid cell) resulted in total inhibition of the PFC response in culture (results not shown in Table).

The effector cells could exert their inhibitory activity only if cultured with the memory spleen cells for the entire 4 day culture period (Table XV). Addition of the effector cells at day 1 of culture (24 hours after commencement of culture) resulted in only a 30 to 40 per cent reduction in the number of PFC's while addition of effector cells at day 2 of culture produced no inhibition at all (TAble XVI).

Discussion:

The objective of this investigation was to establish an in vitro system to serve as a counterpart to the in vivo graft-versus-host (GVH) reaction. In this way, it was hoped to elucidate the organ source of the cells mediating the graft-versus-host reaction, a reaction generally considered to represent a cell-mediated immune reaction (267). The system described here permits the direct observation of allogeneic cell interaction and presumably cell death. The spleen cells of a rabbit immunized 3 to 6 months previously (memory rabbit) generates plaque-forming cells (PFC) in vitro, if incubated with sheep cells for 4 days under the appropriate culture conditions. If,

however, the memory spleen cells are prevented from either proliferating or transforming into PFC's, then no plaques will be detected when these cells are analyzed for PFCs four days after the initiation of culture. Homologous (or allogeneic) lymphoid cells obtained from the circulating blood and the appendix, sacculus rotundus and Peyer's patches (SAPP organs) are capable of interacting with the lymphoid cells of the spleen of the memory rabbit since they can prevent these splenic lymphoid cells from giving rise to PFCs following stimulation with the antigen. It must be emphasized that the lymphoid cells of only these lymphoid organs were capable of completely inhibiting the PFC response. The cells in the bone marrow and thymus had no inhibitory capacity at all while those in the lymph node and spleen exhibited low-grade activity. However, when lymph node and spleen cells were used in much greater numbers in culture (4 to 8 times the number of SAPP cells), they could effectively inhibit the induced PFC response.

An interesting finding was the stimulation, rather than inhibition, provided by allogeneic bone marrow cells for the generation of PFC's <u>in vitro</u>.

Since the rabbit bone marrow cells could not generate PFC's when cultured with SRBC in vitro (see CHAPTER FIVE, Section I) and since it has been demonstrated that they do not possess precursors of antibody-forming cells (68), it must be concluded that the bone marrow cells provide a good feeder layer for the memory spleen cells to thrive on. The stimulation provided by the bone marrow cells can only be detected if the results (Tables XII and XIII) are given as the number of PFC per original 10^6 memory spleen cells cultured and not as number of PFC per 10^6 lymphoid cells recovered.

The failure of rabbit thymus cells to demonstrate allogeneic inhibition is not surprising since it has been demonstrated by a number of investigators that thymus cells themselves do not exhibit GVH activity to any great extent. Rather, it is the recirculating thymus-derived and/or thymus dependent cells localized in the peripheral lymphoid organs which exhibit GVH activity (25, 268-271). Although evidence has been presented demonstrating the capacity of thymocytes to induce splenomegaly upon transfer to allogeneic immunoincompetent or hybrid hosts (271-274), the reactions obtained with the transfer of spleen cells (thymus derived) are

much more marked and intense (271, 275). However, it must be stressed that the relationship between the induced splenomegaly and the GVH reaction is not clear (272). The transferred cells may simply provide a stimulus toward proliferation of host mononuclear cells in a manner akin to cellular events in the Elkins' Model (238). The fact that the thymus in the adult rabbit does not appear to play a definitive role in the induction of the humoral immune response (276) suggests that the role of the thymus in the induction of both humoral and cell-mediated immunity in the immunologically mature rabbit may be more indirect than direct and it may function in a non-specific manner.

The inhibitory activity demonstrated by the SAPP and circulating cells is dose dependent in that a ratio of memory lymphoid cell to inhibitory cell of greater than 10 to 1 in the cultures permits a normal PFC response by the memory cells. It was anticipated that one of these organs might possess much greater numbers of effector cells than the others, on the assumption that these cells originate from stem cells in only one organ and are then dispersed via the circulation to other organs

where they may mature or die. However, since the SAPP cells and the circulating WBC exhibited essentially similar dose-response patterns, it would appear that the circulation and all three SAPP organs in the normal rabbit - appendix, sacculus rotondus and Peyer's patches, constitute equivalent sources of the effector cells. In the absence of evidence to the contrary, it may be assumed that the effector cells originate in the SAPP organs, spill over into the circulation and infiltrate the spleen and lymph nodes where they may survive for a time and be detected in small numbers and where they eventually die. The failure to detect any effector cells in the thymus and the bone marrow implies that these organs do not provide conditions conducive to the continued viability or proliferation of these cells. It is therefore necessary to postulate that the capacity to invoke a GVH reaction is a property of a unique cell which dies or sheds this function once it leaves the circulation. Alternatively, the GVH reactivity of the cell may be characteristic of a specific period of the lifespan of the cell and this activity is lost as the cell passes into the "old age" phase, at which time it

leaves the circulation. At this point, it may constitute the immunologically-incompetent pool of lymphocytes.

Friedman (80, 277) recently reported upon the use of the inhibition by homologous cells of the PFC response by immune mouse spleen cells as a means to detect transplantation immunity. He demonstrated that plaque formation by immune spleen cells (mouse A) could be inhibited if the cells were initially incubated with spleen cells obtained from a mouse of a different strain (mouse B), irrespective whether the latter had been previously immunized to mouse A cells. The suppression of plaque formation was found to be quantitatively related to the relative numbers of effector (mouse B) cells incubated (80, 277). Cells from hybrid mice had no effect on plaque formation by spleen cells obtained from mice of the parental strain immunized with SRBC (277). On the other hand, plaque reduction occurred when spleen cells from non-sensitized allogeneic mice were incubated with the plaque-forming spleen cells. The author concluded that the reaction reflected a homograft response <u>in vitro</u> on one spleen cell population

against another and constitutes a cell-mediated reaction.

Results of a similar nature using spleen cells of mice have most recently been reported by Hirano and Uyeki (278). Unlike the experiments of Friedman (80, 277), these investigators cultured spleen cells from mice of different strains with SRBC for a period of 4 days following which the cells were analyzed for PFC's. The degree of suppression of PFC's was related to the disparity of the H-2 allele and did not depend on complement to ensue. However, since the spleen cells of both strains of mice (allogeneic culture) incubated were capable of transforming into PFC's in vitro, it is difficult to ascertain the exact extent of allogeneic inhibition induced by cells of one spleen against the other. The results obtained with the system deployed here are much simpler to interpret since only the target memory spleen cells are capable of giving rise to PFCs in culture. Another distinction between our experiments and those of Friedman (80, 277) and Hirano and Uyeki (278) is that we investigated the "killer" capacity of cells of a large number of lymphoid organs and

the blood whereas the latter investigators were only concerned with the spleen.

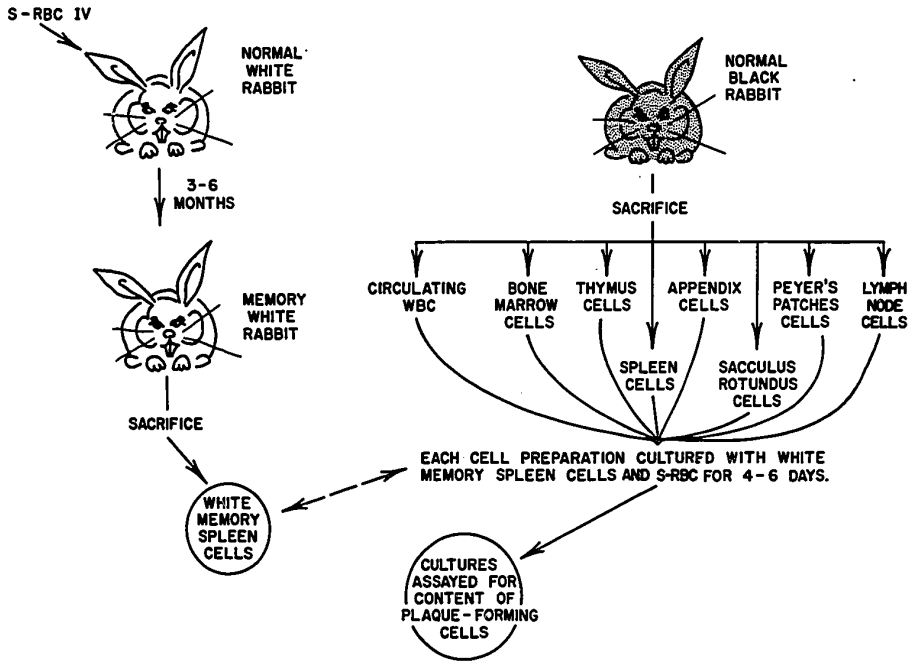

FIGURE 2. PROTOCOL FOR THE ELUCIDATION OF THE ORGAN SOURCE OF THE "VIRGIN" CELLS IN THE NORMAL IMMUNOLOGICALLY-COMPETENT RABBIT MEDIATING CELLULAR IMMUNITY IN VITRO.

TABLE XII

THE FAILURE OF AUTOLOGOUS LYMPHOID CELLS TO INHIBIT THE PLAQUE-FORMING CELL (PFC) RESPONSE BY MEMORY SPLEEN CELLS IN CULTURE.

No. of memory spleen lymphoid cells cultured*	No. of S-rbc added to culture	Organ source of autologous cells added to culture	No. of autologous cells added to culture	No. of lymphoid cells recovered	No. of PFC per 10^6 recovered lymphoid cells after 4 days of culture	No. of PFC per 10^6 cultured spleen lymphoid cells after 4 days in culture.
1.4×10^7	Nil	Nil	Nil	4.8×10^6	0	0 - 2
1.4×10^7	10^7	Nil	Nil	6.0×10^6	430	48
2.8×10^7	10^7	Nil	Nil	1.8×10^7	580	60
1.4×10^7	10^7	Bone marrow	1.4×10^7	1.7×10^7	380	155
1.4×10^7	10^7	Thymus	1.4×10^7	6.6×10^6	440	54
1.4×10^7	10^7	Lymph node	1.4×10^7	6.0×10^6	370	40
1.4×10^7	10^7	Circulating wbc	1.4×10^7	9.6×10^6	370	66
1.4×10^7	10^7	Appendix	1.4×10^7	6.0×10^6	440	39
1.4×10^7	10^7	Sacculus rotundus	1.4×10^7	8.4×10^6	360	55
1.4×10^7	10^7	Peyer's patches	1.4×10^7	6.6×10^6	440	54

* Four culture plates were set up in each instance and the cells were pooled at the termination of culture.

TABLE XIII

THE INHIBITION, BY HOMOLOGOUS LYMPHOID CELLS, OF PLAQUE-FORMING CELL (PFC) RESPONSE BY MEMORY SPLEEN CELLS IN CULTURE.

No. of memory spleen lymphoid cells cultured*	No. of S-rbc added to culture	Organ source of homologous effector cells added to culture	No. of homologous effector cells added to culture	No. of lymphoid cells recovered	No. of PFC per 10^6 recovered lymphoid cells after 4 days of culture	No. of PFC per 10^6 cultured spleen lymphoid cells after 4 days in culture
1.4×10^7	Nil	Nil	Nil	4.8×10^6	0	0 - 2
1.4×10^7	10^7	Nil	Nil	6.0×10^6	430	48
2.8×10^7	10^7	Nil	Nil	1.8×10^7	580	60
1.4×10^7	10^7	Bone marrow	1.4×10^7	1.7×10^7	390	122
1.4×10^7	10^7	Thymus	1.4×10^7	6.0×10^6	400	46
1.4×10^7	10^7	Spleen	1.4×10^7	4.8×10^6	210	20
1.4×10^7	10^7	Lymph node	1.4×10^7	7.8×10^6	240	35
1.4×10^7	10^7	Circulating wbc	1.4×10^7	1.0×10^7	28	5
1.4×10^7	10^7	Appendix	1.4×10^7	6.6×10^6	18	2
1.4×10^7	10^7	Sacculus rotundus	1.4×10^7	9.0×10^6	40	6
1.4×10^7	10^7	Peyer's patches	1.4×10^7	9.6×10^6	30	5

* Four culture plates were set up in each instance and the cells were pooled at the termination of culture.

TABLE XIV

THE REQUIREMENT OF LIVE HOMOLOGOUS EFFECTOR CELLS TO EFFECT INHIBITION OF THE PFC RESPONSE BY MEMORY SPLEEN CELLS.

No. of memory spleen lymphoid cells cultured*	No. of S-rbc added to culture	Organ source of effector lymphoid cells added to culture	No. of effector lymphoid cells (or equivalent) added to culture	No. of lymphoid cells recovered	No. of PFC per 10^6 recovered lymphoid cells after 4 days of culture
1.5×10^7	Nil	Nil	Nil	1.1×10^7	0 - 3
1.5×10^7	10^7	Nil	Nil	1.3×10^7	365
1.5×10^7	10^7	Autologous wbc	1.5×10^7	1.5×10^7	310
1.5×10^7	10^7	Homologous wbc	1.5×10^7	1.1×10^7	5
1.5×10^7	10^7	Homologous wbc sonicate	1.5×10^7	1.2×10^7	340
1.5×10^7	10^7	Autologous appendix	1.5×10^7	1.1×10^7	350
1.5×10^7	10^7	Homologous appendix	1.5×10^7	1.2×10^7	4
1.5×10^7	10^7	Homologous appendix sonicate	1.5×10^7	1.3×10^7	300
1.5×10^7	10^7	Autologous sacculus rotundus	1.5×10^7	1.1×10^7	325
1.5×10^7	10^7	Homologous sacculus rotundus	1.5×10^7	1.0×10^7	10
1.5×10^7	10^7	Homologous sacculus rotundus sonicate	1.5×10^7	1.4×10^7	350

* Four culture plates were set up in each instance and the cells were pooled at the termination of culture.

TABLE XV

INHIBITION, BY HOMOLOGOUS LYMPHOID CELLS, OF THE PFC RESPONSE IN VITRO BY MEMORY SPLEEN CELLS. THE EFFECT OF VARYING THE RELATIVE CONCENTRATION OF HOMOLOGOUS LYMPHOID CELLS.

No. of memory spleen lymphoid cells cultured*	No. of S-rbc added to culture	Organ source of homologous effector cells added to culture	No. of homologous effector cells added to culture	No. of PFC per 10^6 lymphoid cells recovered after 4 days in culture
2.0×10^7	Nil	Nil	Nil	0 - 3
2.0×10^7	10^7	Nil	Nil	320
2.0×10^7	10^7	Circulating wbc	2.0×10^7	0
2.0×10^7	10^7	Circulating wbc	0.4×10^7	165
2.0×10^7	10^7	Circulating wbc	0.2×10^7	210
2.0×10^7	10^7	Circulating wbc	0.1×10^7	300
2.0×10^7	10^7	Appendix	2.0×10^7	0
2.0×10^7	10^7	Appendix	0.4×10^7	160
2.0×10^7	10^7	Appendix	0.2×10^7	225
2.0×10^7	10^7	Appendix	0.1×10^7	308
2.0×10^7	10^7	Sacculus rotundus	2.0×10^7	0
2.0×10^7	10^7	Sacculus rotundus	0.4×10^7	175
2.0×10^7	10^7	Sacculus rotundus	0.2×10^7	240
2.0×10^7	10^7	Sacculus rotundus	0.1×10^7	290
2.0×10^7	10^7	Peyer's patches	2.0×10^7	0
2.0×10^7	10^7	Peyer's patches	0.4×10^7	180
2.0×10^7	10^7	Peyer's patches	0.2×10^7	250
2.0×10^7	10^7	Peyer's patches	0.1×10^7	300
2.0×10^7	10^7	Thymus	8.0×10^7	310
2.0×10^7	10^7	Thymus	4.0×10^7	330
2.0×10^7	10^7	Thymus	2.0×10^7	318
2.0×10^7	10^7	Bone marrow	8.0×10^7	375
2.0×10^7	10^7	Bone marrow	4.0×10^7	360
2.0×10^7	10^7	Bone marrow	2.0×10^7	342

* Four culture plates were set up in each instance and the cells were pooled at the termination of culture.

TABLE XVI

THE INHIBITION, BY HOMOLOGOUS LYMPHOID EFFECTOR CELLS, OF THE PFC RESPONSE IN VITRO BY MEMORY SPLEEN CELLS. THE EFFECT OF VARYING THE TIME OF ADDITION OF THE HOMOLOGOUS EFFECTOR CELLS.

No. of memory spleen lymphoid cells cultured*	No. of S-rbc added to culture	Organ source of homologous effects cells added to culture	No. of homologous effects cells added to culture	Effector cells added at following day after initiation of culture	No. of PFC per 10^6 recovered lymphoid cells after 4 days in culture
2.5×10^7	Nil	Nil	Nil	Nil	0 - 3
2.5×10^7	10^7	Nil	Nil	Nil	425
2.5×10^7	10^7	Circulating wbc	2.5×10^7	0	0
2.5×10^7	10^7	Circulating wbc	2.5×10^7	1	255
2.5×10^7	10^7	Circulating wbc	2.5×10^7	2	390
2.5×10^7	10^7	Appendix	2.5×10^7	0	20
2.5×10^7	10^7	Appendix	2.5×10^7	1	280
2.5×10^7	10^7	Appendix	2.5×10^7	2	410

* Four culture plates were set up in each instance and the cells were pooled at the termination of culture.

B. The Radio-Sensitivity of Normal Lymphoid Cells Mediating a Cellular Immune Reaction in Vitro.

Experimental procedures:

The protocol for the experimental procedures carried out is diagrammatically presented in Figure 3. Memory spleen cells were obtained from white rabbits immunized with 10^9 SRBC three to six months prior to sacrifice.

Effector cells were obtained from homologous black rabbits which were subjected to whole body irradiation in doses ranging from 800 to 1400R. These rabbits were sacrificed two to twelve hours after irradiation. WBC, appendix, Peyer's patches and sacculus rotundus cell suspensions were prepared as described in MATERIALS AND METHODS. These suspensions were cultured with the memory spleen cells together with SRBC for four days after which the cells were harvested and analyzed for their PFC content. As controls memory rabbits were subjected to 800 to 1200R total body irradiation and their spleens were then analyzed for the capacity to give a secondary immune response in vitro.

In other experiments, circulating white blood cells and cell suspensions of appendix, sacculus rotundus and Peyer's patches obtained from normal black rabbits were subjected to doses of irradiation varrying from 2000R to 10,000R. Each cell preparation was cultured with memory spleen cells in the presence of SRBC for four days. Culture conditions were the same as those described in MATERIALS AND METHODS. At the end of the culture period, the cells were harvested and assayed for the content of PFCs as described in MATERIALS AND METHODS.

Results and Discussion:

The capacity of cells of irradiated rabbits to inhibit the PFC response of homologous memory spleen cells in vitro is detailed in Table XVII. A dose of up to 1200R whole body irradiation did not have any effect on the SAPP cells or circulating WBC whereas the same cells of animals which had been subjected to 1400R irradiation lost the capacity to inhibit the PFC response.

The relative effect of irradiation on the capacity of memory spleen cells to give rise to PFCs or, conversely, of SAPP cells and WBC to in-

hibit the emergence of PFCs, is shown in Table XVIII. Although 1200R completely suppressed the capacity of memory spleen cells to produce PFCs in culture, SAPP cells and WBC of such irradiated animals could nevertheless inhibit the PFC response by memory spleen cells (Table XVIII).

As can be seen from Table XIX, effector cells that have been exposed to 10,000R irradiation in vitro lost their capacity to inhibit the generation of PFCs by memory spleen cells. Exposure of these cells to 4,000R irradiation in vitro markedly decreased their capacity to inhibit the PFC response by the memory spleen cells. A dose of 2000R had only a slight effect on the capacity of SAPP cells and circulating WBC to inhibit the emergence of PFCs from homologous memory spleen cells.

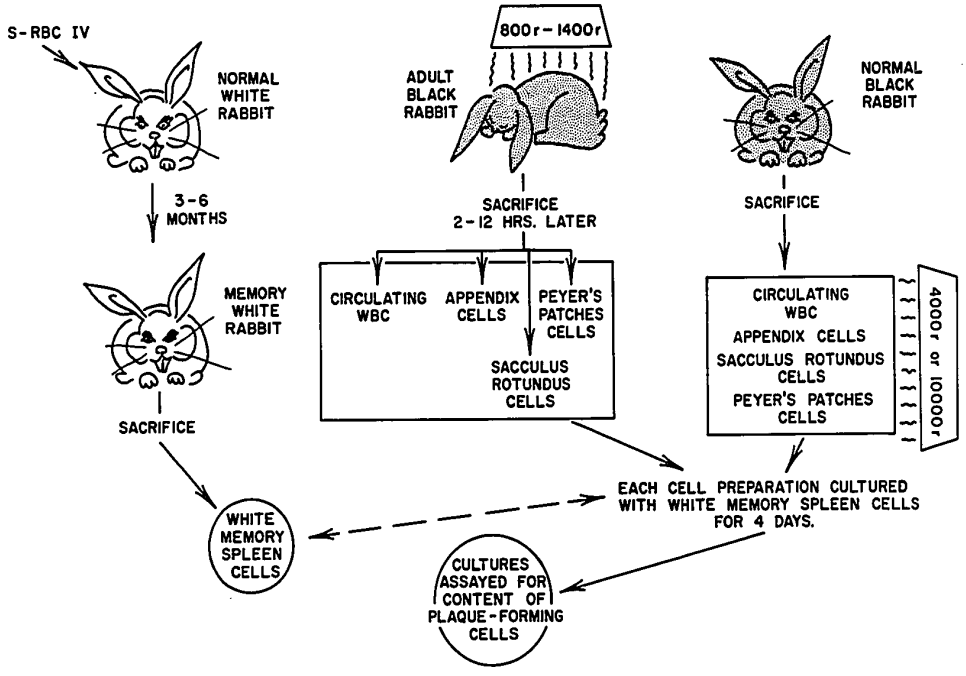

FIGURE 3. PROTOCOL FOR THE DETERMINATION OF THE RADIO-SENSITIVITY OF THE CELLS OF THE NORMAL IMMUNOLOGICALLY-COMPETENT RABBIT MEDIATING CELLULAR IMMUNITY IN VITRO.

TABLE XVII

THE INHIBITION, BY HOMOLOGOUS LYMPHOID EFFECTOR CELLS OF THE PFC RESPONSE BY MEMORY SPLEEN CELLS. THE EFFECT OF IRRADIATION OF THE HOMOLOGOUS CELL DONOR.

No. of memory spleen lymphoid cells cultured*	No. of S-rbc added to culture	Donor of homologous cells subjected to following dose of total body irradiation	Organ source of homologous effector cells added to culture (2.5×10^7)	No. of PFC per 10^6 lymphoid cells recovered after 4 days in culture
2.5×10^7	Nil	Nil	Nil	0 - 3
2.5×10^7	10^7	Nil	Nil	320
2.5×10^7	10^7	800r	Circulating wbc	0
2.5×10^7	10^7	800r	Appendix	0
2.5×10^7	10^7	800r	Sacculus rotundus	10
2.5×10^7	10^7	1000r	Circulating wbc	0
2.5×10^7	10^7	1000r	Appendix	0
2.5×10^7	10^7	1000r	Sacculus rotundus	0
2.5×10^7	10^7	1200r	Circulating wbc	0
2.5×10^7	10^7	1200r	Appendix	15
2.5×10^7	10^7	1200r	Sacculus rotundus	20
2.5×10^7	10^7	1400r	Circulating wbc	300
2.5×10^7	10^7	1400r	Appendix	290
2.5×10^7	10^7	1400r	Sacculus rotundus	320

* Four culture plates were set up in each instance and the cells were pooled at the termination of culture.

TABLE XVIII

THE EFFECT OF TOTAL BODY IRRADIATION ON THE CAPACITY OF MEMORY SPLEEN CELLS TO GIVE A PFC RESPONSE IN VITRO AND ON THE CAPACITY OF SAPP CELLS AND WBC TO INHIBIT THE PFC RESPONSE IN VITRO.

Immune status of rabbit	Dose of irradiation given	Capacity of spleen cell to give PFC in culture (No. of PFC per 10^6 splenic lymphoid cells recovered)	No. of PFC per 10^6 recovered lymphoid cells following incubation of memory spleen lymphoid cells (2×10^7) with an equal number of homologous	
			Circulating wbc	Appendix cells
Memory rabbit	Nil	200 - 400		
	800r	200 - 400		
	1200r	0		
Normal donor of effector cells	Nil		0	0
	1000r		0	0
	1200r		10	14
	1400r		280	300

TABLE XIX

THE INHIBITION BY HOMOLOGOUS LYMPHOID EFFECTOR CELLS OF THE PFC RESPONSE BY MEMORY SPLEEN CELLS. THE EFFECT OF IN VITRO IRRADIATION OF THE HOMOLOGOUS LYMPHOID CELLS.

No. of memory spleen lymphoid cells cultured*	No. of SRBC added to culture.	Homologous lymphoid cells subjected to the following dose of irradiation in vitro.	Organ source of homologous effector cells added to culture.	No. of PFC per 10^6 lymphoid cells recovered after 4 days in culture
2.8×10^7	Nil	Nil	Nil	0
2.8×10^7	10^7	Nil	Nil	360
2.8×10^7	10^7	2,000 R	Circulating WBC	30
2.8×10^7	10^7	2,000 R	Appendix	40
2.8×10^7	10^7	2,000 R	Sacculus rotundus	25
2.8×10^7	10^7	4,000 R	Circulating WBC	280
2.8×10^7	10^7	4,000 R	Appendix	300
2.8×10^7	10^7	4,000 R	Sacculus rotundus	300
2.8×10^7	10^7	10,000 R	Circulating WBC	330
2.8×10^7	10^7	10,000 R	Appendix	350
2.8×10^7	10^7	10,000 R	Sacculus rotundus	345

* Four culture plates were set up in each instance and the cells were pooled at the termination of culture.

C. Investigations to Determine the Presence of Humoral Factors in the Supernatants from PFC Inhibited Cell Cultures.

Experimental procedures:

1. Capacity of supernatants to inhibit a secondary immune response in vitro.

The protocols used in these experiments is represented diagramatically in figure 4.

A portion of the memory spleen cell preparation was suspended in Medium 199 containing 10 per cent DMSO to give a cell concentration of 10^8 per ml. The cells were then frozen in liquid nitrogen at the rate of 1 degree per minute and were maintained at -70°C until used (280). The other portion of the memory spleen cells was cultured with effector cells for 4 days in the manner described earlier. At the termination of culture, the cells were centrifuged and the supernatants were saved. These supernatants were then tested for their capacity to inhibit the PFC response by incubating them with the cultured

memory cells and complement just prior to plating of the cells.

To test whether the supernatants of PFC-inhibited cultures could inhibit the secondary immune response by memory spleen cells in vitro, the supernatants were mixed with equal volumes of fresh Medium 199 fortified with 200 units penicillin and streptomycin per ml. This latter solution was used as culture medium for memory spleen cells which were either obtained from a new memory rabbit sacrificed at this stage of the experiment or from the original memory rabbit whose spleen cells had been kept frozen during the initial four day culture period. (See figure 4). After four days of culture with 10^7 SRBC, these memory spleen cells were harvested and assayed for their content of hemolytic plaque forming cells (PFCs).

To determine the effect of anti-SRBC antibodies on the induction of a secondary immune response by memory spleen cells in vitro, the antisera was added to aliquots of medium so that the anti-SRBC titres of these media were 1/5, 1/20, 1/100 and 1/200. The memory spleen cells were cultured in this medium in the presence of 10^7 SRBC in the usual manner. After

four days in culture, the cells were harvested and assayed for plaque forming cells.

2. Capacity of supernatants to manifest a cytotoxic effect in vitro.

Supernatants of PFC inhibited cultures collected as described above were tested for their cytotoxic effect on allogeneic and syngeneic cells in vitro. For the analysis of cytotoxic activity, 0.1 ml(2×10^6) cells of the spleen cell suspension (memory spleen cells were cultured in the absence of antigenic stimulant and were collected after four days in culture) was mixed with 0.1 ml of the "cytotoxic" solution and 0.1 ml of undiluted guinea pig serum (a source of complement) in a plastic tube. The tubes were incubated for 60 minutes at 37°C at which point 0.6 ml Medium 199 and 0.3 ml of a 0.2 per cent solution of trypan blue were added (281). The cells were then analyzed microscopically to determine the number of viable cells, defined as cells which excluded the dye.

3. The absence of antibodies directed to cultured spleen cells in supernatants from PFC-inhibited cultures.

The culture supernatants were analyzed for the presence of antibodies directed to the cultured spleen lymphocytes by the fluorescent antibody (sandwich) technique, originally described by Coons et al (282). Smears of spleen memory cells used for the cultures were air-dried on microscope slides and fixed with 94 per cent ethanol for 10 minutes at room temperature. The slides were stored at 4°C until used. The slides were exposed to the potential antibody-containing supernatants for 30 minutes at room temperature, following which they were washed with buffered saline and exposed to fluorescein-conjugated horse anti-rabbit gamma-globulin for 30 minutes. The slides were then washed with buffered saline, mounted in buffered glycerol and analyzed under a Reichert fluorescence microscope (Zetopan, binolux).

Results:

The supernatants of inhibited cultures (those in which the PFC response was inhibited by homologous SAPP cells or WBC) were analyzed for cytotoxic antibodies directed to the spleen lymph-

oid cells of the original memory cell donor. These cells had been quick-frozen immediately following sacrifice and were thawed just prior to their use as target cells for cytotoxic antibodies. As can be seen in Table XX, the supernatants exhibited no cytotoxic activity even if complement were added to the incubates. Furthermore, these supernatants did not possess any cytotoxic activity directed toward the PFCs since they could not inhibit plaque formation if incubated with the PFCs obtained from cultures of the original memory rabbit for 60 minutes in the presence of complement at 37°C prior to plating (Table XXI). Nor could the supernatants inhibit the in vitro generation of PFCs if added to the medium used for culturing memory spleen cells obtained from the original memory rabbit. (The cells which were frozen in DMSO and thawed just prior to their culture with these supernatants) and SRBC or cells from an allogeneic memory rabbit and SRBC (Table XXII). At no time were anti-SRBC antibodies detected in any of the cell cultures other than the cultures consisting of the memory spleen cells stimulated by SRBC (Tables XXI and XXIII).

The addition of anti-SRBC antiserum to the medium used for culturing the memory spleen cells

and SRBC could inhibit the generation of PFCs only if the anti-SRBC titre of the medium exceed 200 (Table XXIV).

Media showing antibody titers below 100 showed little or no inhibition. No antibodies to the memory spleen lymphoid cells could be detected in any of the culture supernatants using the fluorescent antibody (sandwich) technique.

Discussion:

This series of experiments was performed with the aim of providing further evidence for the cellular nature of the reaction observed -- the inhibition of the generation of PFCs from memory spleen cells by allogeneic lymphoid cells *in vitro*. The fact that the reaction is a complement-independent one strongly favours its cell-mediated nature, since it is generally agreed that humoral antibody can express its cytotoxic properties best, if not only, in the presence of lytic complement (165, 279, 280, 283). In the studies conducted on the supernatants from PFC inhibited cultures, no antibodies directed towards the target cells could be detected using the fluorescent antibody technique.

These results suggest a cell-cell interaction but do not completely rule out the possibility of a soluble factor which may be released by the effector cells.

Results of numerous investigations have disclosed that the addition of specific antibodies to antibody-forming cells in culture will prevent active antibody formation to the specific antigen by these cells (285-287) in the same manner as the injection of antibody in vivo will inhibit specific antibody synthesis (288-291). Circulating antibodies to SRBC can often be detected in the circulation of the normal rabbit albeit in low concentration (292-293), if these antibodies are synthesized by SAPP cells their release in sufficient numbers from the cultured SAPP cells might inhibit the antibody-synthetic mechanism of the memory spleen cells present in the culture thus preventing their transformation into PFCs. However, the SAPP cells by themselves could not give rise to PFCs if cultured with SRBC (Table XIX) and, they could not synthesize anti-SRBC antibodies in detectable quantities. Furthermore, the addition of anti-SRBC antibodies to the

culture mixture could effect inhibition of the generation of PFCs to the SRBC but only if the amount of antiserum added to the medium were sufficient to confer a titre of 200 or more to the medium used. These titres were much higher than those ever measured in the supernatants obtained from memory spleen cells exhibiting a secondary immune response *in vitro*. (Tables IX and X). The presence of anti-SRBC can thus be ruled out as a factor leading to the *in vitro* inhibition of PFC by memory spleen cells.

The failure to detect antibodies directed toward the memory spleen lymphoid cells and the lack of any cytotoxic activity effect in the supernatants of PFC-inhibited cultures, even in the presence of complement, points to the cellular nature of the reaction observed. However this does not rule out the presence of other soluble factors which may play a role in the cellular interaction described.

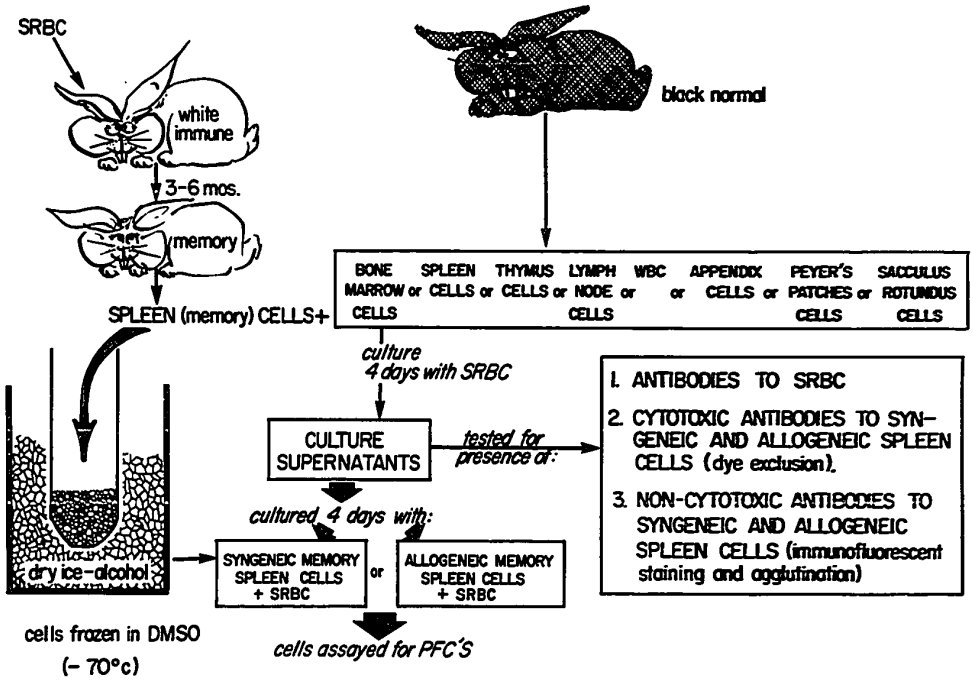

FIGURE 4. PROTOCOL FOR THE INVESTIGATION OF THE PRESENCE OF ANTIBODIES IN SUPERNATANTS OBTAINED FROM PFC-INHIBITED CULTURES.

TABLE XX

THE LACK OF CYTOTOXIC ACTIVITY OF THE SUPERNATANTS OF CULTURES OF HOMOLOGOUS EFFECTOR CELLS AND MEMORY SPLEEN CELLS.

Culture supernatant tested*	Cytotoxic index** of supernatant tested with cells of memory spleen cell donor.***	
	Complement added to test cell mixture	No complement added to test cell mixture
Memory spleen + S-rbc	14 per cent	10 per cent
S-rbc	10 per cent	10 per cent
Memory spleen + S-rbc + homologous appendix	18 per cent	16 per cent
Memory spleen + S-rbc + autologous appendix	13 per cent	12 per cent
Memory spleen + S-rbc + homologous wbc	20 per cent	18 per cent
Memory spleen + S-rbc + autologous wbc	15 per cent	10 per cent
Memory spleen + S-rbc + homologous thymus	18 per cent	18 per cent

* Supernatants were obtained at the termination of culture (4 days).
** Defined as the percentage of dead cells.
*** Spleen cells of donor rabbit were frozen in DMSO (see text) and thawed just prior to testing for cytotoxicity. The number of viable lymphoid cells is considered to represent 100 per cent.

TABLE XXI

THE LACK OF CYTOTOXIC AND ANTI-PFC ACTIVITY OF SUPERNATANTS OF PFC-INHIBITED CULTURES.

Contents of original cell cultures*	No. of PFC per 10^6 lymphoid cells of original cultures after 4 days incubation.	Hemagglutination titers of supernatants** of cultures.	Hemolytic titers of supernatants** of cultures.	The plaque forming capacity of *in vitro* generated PFCs incubated with C' and the supernatants of original cell cultures for 60 minutes prior to plating.***
Memory spleen + SRBC	120	0	8	65
Memory spleen + SRBC + Autol. wbc	90	0	0	60
Memory spleen + SRBC + Homol. wbc	0	0	0	70
Memory spleen + SRBC + Autol. App.	95	0	0	64
Memory spleen + SRBC + Homol. App.	0	0	0	68

* Cultures consisted of 2×10^7 splenic lymphoid cells, 10^7 SRBC and, where stated, 2×10^7 autologous or homologous circulating wbc or appendix cells. The gaseous phase used was 5% CO_2, 18% O_2 and 77% N_2.

** The supernatants were obtained by centrifugation of the cultures after incubation for 4 days at 37°C.

*** The memory spleen cells were obtained from the same rabbit whose cells were used in the original cultures (Column 1). These cells had been incubated with SRBC for 4 days, following which they were harvested, incubated with the suspected cytotoxic supernatants and complement for 60 minutes and then analysed for their content of PFC's (see text).

TABLE XXII

THE LACK OF CAPACITY OF SUPERNATANTS OF PFC-INHIBITED CULTURES TO INHIBIT THE GENERATION OF PFCs FROM MEMORY SPLEEN CELLS IN VITRO.

Supernatants obtained from the following primary cultures*	No. of PFCs per 10^6 lymphoid cells following culture of supernatants with memory spleen cells (secondary culture) obtained from the following rabbits:	
Memory spleen cells (white rabbit A) cultured with:	The same donor as used for primary cultures** (Rabbit A)	A homologous white memory rabbit (Rabbit C)
Nil	192	325
SRBC	205	305
Autologous WBC + SRBC	160	270
Autologous thymus + SRBC	180	275
Autologous appendix + SRBC	160	290
Homologous WBC (normal black rabbit B) + SRBC	170	265
Homologous thymus (normal black rabbit B) + SRBC	155	290
Homologous appendix (normal black rabbit B) + SRBC	180	275

* Supernatants were obtained at the termination of culture (4 days) of memory spleen cells of rabbit A and cells of autologous or homologous black rabbit.

** Spleen cells of rabbit A were frozen in DMSO (see text) at the time of sacrifice and thawed just prior to use in culture with the suspected cytotoxic and non-cytotoxic supernatants.

TABLE XXIII

ANTIBODY SYNTHESIS BY RABBIT LYMPHOID CELLS CULTURED IN VITRO.

Immune status of rabbit	Cells of organ cultured	Antigen (S-rbc) added (10^7 cells)	Antibody (anti-S-rbc) titers of supernatants after 4 days in culture*
Memory	Spleen	No	0
Memory	Spleen	Yes	32 - 64
Normal	Spleen	No	0
Normal	Spleen	Yes	0
Normal	Appendix	No	0
Normal	Appendix	Yes	0
Normal	Sac. Rotundus	No	0
Normal	Sac. Rotundus	Yes	0
Normal	Peyer's Patches	No	0
Normal	Peyer's Patches	Yes	0

* The titer is defined as the maximum dilution capable of effecting hemolysis of SRBC. Titers less than 2 are considered to be negative.

TABLE XXIV

THE EFFECT OF ANTI-SRBC ANTISERUM ON THE GENERATION OF PFCs BY MEMORY SPLEEN CELLS IN VITRO.

No. of memory spleen cells cultured	No. of SRBC added to culture	Organ source of homologous effector lymphoid cells added to culture	No. of homologous lymphoid cells added to culture	Hemagglutination titer of the culture medium following the addition of the anti-SRBC antiserum	No. of lymphoid cells recovered	No. of PFCs recovered per 10^6 lymphoid cells after 4 days of culture
2.3×10^7	nil	—	—	—	1.8×10^7	0
2.3×10^7	1×10^7	—	—	—	1.6×10^7	360
2.3×10^7	1×10^7	—	—	0 (NRS)	1.6×10^7	350
2.3×10^7	1×10^7	—	—	1:5	1.7×10^7	310
2.3×10^7	1×10^7	—	—	1:20	1.8×10^7	240
2.3×10^7	1×10^7	—	—	1:100	1.5×10^7	170
2.3×10^7	1×10^7	—	—	1:200	1.7×10^7	40
2.3×10^7	1×10^7	Homol. thymus	2.3×10^7	1:5	1.8×10^7	290
2.3×10^7	1×10^7	Homol. thymus	2.3×10^7	1:20	1.4×10^7	210
2.3×10^7	1×10^7	Autol. appendix	2.3×10^7	1:5	1.9×10^7	280
2.3×10^7	1×10^7	Autol. appendix	2.3×10^7	1:20	2.0×10^7	200

IV IN VIVO MIGRATION PATHWAY(S) FOLLOWING SPECIFIC ANTIGENEIC STIMULATION, OF THE CELLS MEDIATING THE CELLULAR IMMUNE REACTION IN VITRO.

Experimental procedures:

The experimental design of this series of experiments is illustrated in Figure 5. White rabbits were immunized by the intravenous administration of 10^9 SRBC. Three to six months later they were subjected to partial appendectomy and the cells obtained from the extirpated appendix were washed several times and suspended in medium 199. Aliquots of appendix cells (about $5x10^8$ cells) were administered intravenously to black rabbits which were then sacrificed at specified times following immunization and their lymphoid organs were extirpated. Cell suspensions of bone marrow, thymus, circulating white blood cells, lymph nodes (popliteal), spleen, appendix, Peyer's patches and sacculus rotundus were prepared from the immunized black rabbits as described in MATERIALS AND METHODS. Each of these cell suspensions was cultured with an equal number of spleen cells

from the original white memory rabbit whose appendix had been used for immunization. Following four days of culture under the conditions described earlier the cells were harvested and analysed for their content of PFCs.

Results and Discussion:

As can be seen from Table XXV immunization of the donor of the effector cells with appendix cells of the prospective memory rabbit resulted in a change with respect to the organ sources of the cells capable of effecting the inhibition of the PFC response by memory spleen cells. Cells from the sacculus rotundus, appendix, Peyer's patches and circulating white blood cells obtained three days following immunization no longer inhibited the PFC response of memory spleen cells to SRBC *in vitro*. In contrast, the thymus cells were capable of markedly inhibiting the PFC response. Bone marrow, spleen and lymph node cells showed no change in their pattern of inhibition on day three following immunization from the pattern seen when these cells were obtained from unimmunized normal (black) rabbits and cultured with memory spleen cells in a similar

fashion. On day ten following immunization thymus cells still had the capacity to almost completely inhibit the generation of PFCs in vitro. SAPP cells and WBC, on the other hand, began to regain their inhibitory capacity. Spleen cells could induce a 75 per cent inhibition whereas lymph node cells induced about 50 per cent inhibition. By day 18 after immunization with memory appendix cells the organs possessing effector cell activity were the same as those in normal rabbits (See Table XXV). SAPP cells induced almost complete suppression of the emergence of PFCs. Spleen cells exhibited approximately a 70 per cent inhibition whereas thymus, bone marrow and lymph node cells induced no inhibition of the PFC response in vitro.

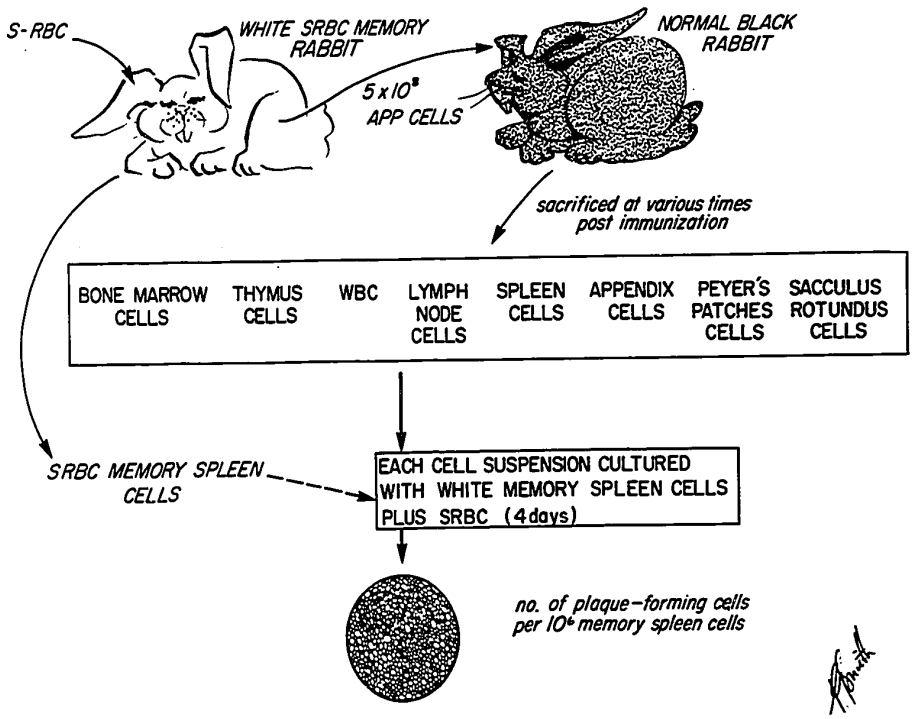

FIGURE 5. PROTOCOL FOR THE STUDY OF THE MIGRATION OF THE CELLS MEDIATING THE IN VITRO GRAFT-VERSUS-HOST REACTION IN THE RABBIT.

TABLE XXV

THE INHIBITION BY HOMOLOGOUS LYMPHOID EFFECTOR CELLS OF THE PFC RESPONSE IN VITRO BY MEMORY SPLEEN CELLS. THE EFFECT OF IMMUNIZATION OF THE DONOR OF THE EFFECTOR CELLS WITH APPENDIX CELLS OF THE MEMORY RABBIT.

No. of memory (white rabbit) spleen cells cultured. (total lymphoid cells)	No. of SRBC added to culture	Organ source of homologous effector cells added to culture. Cells obtained from black rabbit immunized with appendix cells of memory white rabbit*	No. of homologous (black rabbit) lymphoid cells added to the culture	No. of PFC per 10^6 recovered lymphoid cells after 4 days of culture. The effector cells were obtained from the black rabbit at the following days after immunization with appendix cells of white memory rabbit.			
				DAY 3	DAY 10	DAY 10	DAY 18
2.1×10^7	nil	nil	nil	0	0	0	0
2.1×10^7	10^7	nil	nil	300	300	300	300
2.1×10^7	10^7	bone marrow	2.1×10^7	220	ND	ND	135
2.1×10^7	10^7	thymus	2.1×10^7	0	15	25	310
2.1×10^7	10^7	lymph node	2.1×10^7	175	160	180	290
2.1×10^7	10^7	spleen	2.1×10^7	210	95	70	80
2.1×10^7	10^7	wbc	2.1×10^7	300	85	80	30
2.1×10^7	10^7	sacculus rotundus	2.1×10^7	280	100	120	10
2.1×10^7	10^7	appendix	2.1×10^7	265	130	150	5
2.1×10^7	10^7	Peyer's patches	2.1×10^7	220	75	80	0

* Appendix cells for the sensitization of the normal black rabbits were obtained by partial appendectomy of the prospective white memory rabbit whose spleen cells were subsequently used as target cells.

CHAPTER SIX

GENERAL DISCUSSION

Although the optimal conditions required for inducing a secondary immune response were achieved, their use in attempts to induce a primary immune response with lymphoid cells from unimmunized rabbits were mostly non-productive. It is noteworthy that the only successes attained were with a mixed culture of normal spleen and either normal appendix or bone marrow cells and to a lesser extent with a mixture of spleen and thymus cells. However, this response proved to be very inconsistent as only 3 out of 10 experiments attempted gave the results referred to in Table IX. It would appear that under appropriate conditions attained with the cells of a minority of rabbits a marked humoral immune response could be obtained only with mixed cell cultures and not with individual organ cultures.

Notwithstanding the results described here, other investigators have apparently been more successful at inducing a primary response in vitro with rabbit lymphoid cell cultures. Henry et al (52) obtained a primary PFC response to SRBC with cultures of normal

rabbit Peyer's patches cells and spleen cells and Theis and Thorbecke (246) observed a primary plaque-forming cell response to SRBC with normal rabbit spleen cells *in vitro*. However, in both instances, the responses observed with cells of normal, previously unimmunized rabbits were consistently much lower than those observed with spleen cells of previously immunized rabbits. Whether these responses represent primary responses in the true sense of the word may be seriously questioned in view of the fact that the *in vitro* responses were obtained with the cells of a single normal lymphoid organ whereas the primary response *in vivo*, in both the mouse and the rabbit, is mediated by the interaction of at least two organ specific, functionally - distinct lymphocytes, the virgin antigen-reactive cell (ARC) and the virgin antibody-forming cell (AFC) (15, 30, 247, 248). In the mouse, the virgin ARC is localized in the thymus (30, 252-255) and the virgin AFC in the bone marrow (30, 252). In the normal rabbit, the virgin ARC is localized in the bone marrow (50, 64, 248, 256) and the source of the virgin AFC is not yet definitely known although one source may be the appendix (55). However,

in both of these animal species, cells of two distinct lymphoid organs must interact in order for humoral antibody formation to be induced. In the unimmunized rabbit, the spleen does not appear to possess the necessary complement of virgin immunocompetent cells (50, 248). Since the only antigen used to evaluate the primary immune response in vitro has been the sheep red blood cell, which is known to cross-react with the enteric bacteria (258, 260) it may be that the virgin immunocompetent lymphoid cells in the rabbit are, in fact, being continually stimulated with subthreshold or even threshold concentrations of antigens cross-reacting with the SRBC. It may therefore be speculated that the immunocompetent cells detected by virtue of their capacity to give a primary immune response in vitro (52, 246) are probably committed cells and therefore should not be considered to be virgin AFCs. This conclusion is based on previous findings from this laboratory which disclosed cells in the sacculus rotundus of the normal, unimmunized rabbit capable of responding with antibody formation following stimulation with SRBC (60). These cells were, in fact, committed AFCs (60). Since outbred rabbits

vary in their functional immunologic responsive pathways (295), and since the stimuli provided by cross-reacting micro-organisms may vary in degree, it may be that committed AFCs responsive to SRBC may be localized in any or all of the organs in the normal rabbit in which virgin immunocompetent cells have been detected, namely the spleen (246, 310), sacculus rotundus (60), appendix (55) and Peyer's patches (52).

In view of the numerous investigations which implicate the gut associat
in the rabbit, - sacculus rotund
Peyer's patches (SAPP, in the m
humoral immune response) (46, 5
it was felt that these organs m
of virgin AFCs and/or as organ
stimulated AFC, which may actu
where. The results presented
tend to support one or both of
Since PFC could not be detect
patches if the cells were anal
diately after sacrifice one o
immunization, but did give ri
4 days in cell culture, it ma

Peyer's patches serve as a primary store of virgin AFCs. Certainly, sacrificing the rabbit one day following immunization with threshold antigen allows sufficient time for the antigen-stimulated ARC to vacate the bone marrow and impart its "message" to the virgin AFC (64, 248, 256). Since the AFC is detected in the spleen two days after it is initially detected in the Peyer's patches and after it can no longer be detected in the r's patches, it is suggested that the stimulat- matures for 1 to 2 days in the Peyer's (or other SAPP organs in other strains of following which it migrates to the spleen, matures into an overt PFC and antibody- zing cell (Figure 6). A similar consider- the cellular events culminating in anti- mation has been presented by Hanaoka and (54) with respect to the rabbit and by nd Turner (58) with respect to the rat. ormer case (54), the appendix is the SAPP nsidered to be the source of the virgin ile in the latter case (58), it is the patches which assume this role. It is le, however, that following subthreshold

immunization with SRBC that the sequence of events may be aborted at different stages of the immune response with the result that -

(a) either the ARC is not sufficiently stimulated following immunization so that the "message" does not leave the bone marrow ARC (64) or

(b) that the antigenic stimulus is sufficient to permit stimulation of the AFC in the Peyer's patches but there is not sufficient antigen to induce the cell to vacate this organ and to migrate to the spleen (Figure 7).

Instead, the stimulated AFCs remain in the Peyer's patches where they transform to overt antibody-forming cells and into memory cells. Such a situation may, in fact, occur in the normal rabbit as a result of continuous stimulation locally with enteric bacterial antigens which cross-react with the SRBC (258, 260). This interpretation may explain why normal rabbits possess circulating antibodies to SRBC but do not possess detectable numbers of PFCs in the spleen.

In order to establish whether the observations regarding the immunocompetence of the

various SAPP organs represent a general or universal phenomenon, it would be necessary to repeat the experiments using a number of non-cross reacting antigens. It may be significant that the cells in the sacculus rotundus of the normal rabbit capable of responding to the SRBC were unable to respond to any of the other antigens used, thus demonstrating the antigenic specificity of the response of the sacculus cells (60). Furthermore, the kinetics of the *in vitro* primary responses observed with either normal rabbit spleen cells (246) or normal rabbit Peyer's patches cells (52) appeared to be similar to those observed with memory spleen cells in that the optimal response appeared to occur by the fourth day in culture. Since, *in vivo*, the kinetics of the primary and secondary immune responses are considerably different (see discussion in reference 60), it behooves the investigator to explain the seemingly anomalous behaviour seen in the *in vitro* responses where these differences appear not to exist. Furthermore, it might be interesting to repeat these same *in vitro* experiments with cells obtained from neonatal or very young rabbits. On the assumption that the response

observed by Theis and Thorbecke (246) and Henry et al (52) are largely facilitated by the cross-reactive nature of SRBC and enteric bacteria, it might be anticipated that the lymphoid tissue of the neonatal rabbit, not having been exposed to these micro-organisms, would not exhibit immunocompetence in vitro. This, in fact, has been demonstrated by Sterzl et al (298) using neonatal rabbits. They demonstrated that the spleen of a rabbit under 20 days of age does not possess any plaque-forming cells directed to SRBC. However, at 30 days of age, plaque-forming cells could be detected in the spleen (298). Since even newborn rabbits can be stimulated to produce plaque-forming cells in the spleen by 5 days of age following immunization with SRBC (294), it is obvious that the animal is fully immunocompetent and could therefore be likewise stimulated by cross-reacting enteric micro-organisms.

The apparent ease with which a primary humoral response can be initiated in vitro with normal mouse spleen cells (243, 311-314) beckons investigators to carry out similar studies using other animal species in order to ascertain the universality of these results. However, the enthusiasm generated by the repeated successes

with normal mouse spleen cells is tempered somewhat by the very rigid conditions imposed onto the system in order to facilitate a positive in vitro response. Several of these conditions are as follows:

1. A primary response can only be obtained with cells of certain inbred strains of mice, i.e. CBA (300, 312). Attempts to induce a primary response in vitro with cells of other strains of mice have not been successful (300, 312).
2. Even in the strains of mice in which reconstitution, following immunosuppressive irradiation, can be achieved by the transfer of spleen cells (15, 249, 303) or a mixture of thymus and bone marrow cells (15, 67b, 249), the in vitro counterpart of this model, namely the initiation of a response in vitro using mixed cultures of thymus and bone marrow cells, has not been achieved.
3. The antigens which have been used successfully to induce a primary response

in vitro are limited in number. The most dramatic successes have been noted with the sheep red cell (SRBC) as antigen (243, 299, 300, 302, 303, 311-314) although success has also been reported using the dinitrophenyl determinant (304). The fact that the SRBC contains the Forssman group of antigens (258, 260), that the normal mouse spleen invariably invariably possess large numbers of background plaque-forming and rosette-forming cells to SRBC (260, 305-307, 312), and that antibodies to SRBC can be regularly detected in the sera of normal mice (308, 309) has not tempered the general enthusiasm of the investigators concerned. However, it has recently been suggested that what was being detected in vitro was not a primary response but a combination of a primary and secondary response (260, 309, 312).

4. The great rapidity with which the "primary" response can be induced with mouse cells in vitro mitigates

to its being a true primary response. Although the optimal PFC response usually is not detected *in vivo* until about 4-5 days have elapsed following antigen administration (80), the *in vitro* PFC response reaches high levels within 3-4 days of culture (243, 299, 311, 312).

We were unable to obtain consistent *in vitro* PFC responses with any of the organ cultures prepared from the various lymphoid organs of the normal rabbit, using a variety of media and gaseous conditions. The small number of successful experiments was overshadowed by the very large number of unsuccessful ones and may represent "immune" normal rabbits as a result of active immunization of these rabbits with cross-reacting enteric micro-organisms. The explosive response observed with mixed cell cultures would be more significant would they be reproducibly and consistently obtained. However, since they were observed in only a small minority of the experiments attempted, little significance can be attached to them.

Another objective of this investigation was to establish an *in vitro* system to serve as a counterpart to the *in vivo* graft-versus-host (GVH) reaction. In this way, it was hoped to elucidate

the organ source of the cells mediating the graft-versus-host reaction, a reaction generally considered to represent a cell-mediated immune reaction (267). The system described here permits the direct observation of allogeneic cell interaction and presumably cell death. The spleen cells of a rabbit immunized 3 to 6 months previously (memory rabbit) generates plaque-forming cells (PFC) in vitro if incubated with sheep cells for 4 days under the appropriate culture conditions. If, however, the memory spleen cells are prevented from either proliferating or transforming into PFCs, then no plaques will be detected when these cells are analyzed for PFCs four days after initiation of culture. Homologous (or allogeneic) lymphoid cells obtained from the circulating blood and the appendix, sacculus rotundus and Peyer's patches (SAPP organs) are capable of interacting with the lymphoid cells of the spleen of the memory rabbit since they can prevent these splenic lymphoid cells from giving rise to PFCs following stimulation with the antigen. It must be emphasized that the lymphoid cells of only these lymphoid organs are capable

of completely inhibiting the PFC response. The cells in the bone marrow and thymus have no inhibitory capacity at all while those in the lymph node and spleen exhibit low-grade activity. However, when lymph node and spleen cells were used in much greater numbers in culture (4 to 8 times the number of SAPP cells), they could effectively inhibit the induced PFC response.

An interesting finding was the stimulation, rather than inhibition, provided by allogeneic bone marrow cells for the generation of PFCs in vitro. Since the rabbit bone marrow cells could not generate PFCs when cultured with SRBC in vitro and since it has been demonstrated that it does not possess precursors of antibody-forming cells (68) it must be concluded that bone marrow cells provide a good feeder layer for the memory spleen cells to thrive on. The stimulation provided by the bone marrow cells can only be detected if the results (Tables I and II) are given as the number of PFC per original 10^6 memory spleen cells cultured and not as number of PFC per 10^6 lymphoid cells recovered.

The failure of rabbit thymus cells to

demonstrate allogeneic inhibition is not surprising since it has been demonstrated by a number of investigators that thymus cells themselves do not exhibit GVH activity to any great extent. Rather, it is the recirculating thymus-derived and/or thymus dependent cells localized in the peripheral lymphoid organs which exhibit GVH activity (25, 268-271). Although evidence has been presented demonstrating the capacity of thymocytes to induce splenomegaly upon transfer to allogeneic immunoincompetent or hybrid hosts (271-274), the reactions obtained with spleen cells (thymus derived) are much more marked and intense (271, 275). However, it must be stressed that the relationship between the induced splenomegaly and the GVH reaction is not clear (272). The transferred cells may simply provide a stimulus toward proliferation of host mononuclear cells in a manner akin to cellular events in the Elkins' Model (238). The fact that the thymus in the adult rabbit does not appear to play a definitive role in the induction of the humoral immune response (276) suggests that the immunologic role of the thymus in the immunologically mature rabbit may be more indirect than direct and it may function in a non-specific manner.

The inhibitory activity demonstrated by the SAPP and circulating cells is dose dependent in that a ratio of memory lymphoid cell to inhibitory cell of greater than 10 to 1 in the cultures permits a normal PFC response by the memory cells. It was anticipated that one of these organs might possess much greater numbers of effector cells than the others, on the assumption that these cells originate from stem cells in only one organ and are then dispersed via the circulation to other organs where they may mature or die. However, since the SAPP cells and the circulating WBC exhibited essentially similar dose-response patterns, it would appear that the circulation and all three SAPP organs in the normal rabbit-appendix, sacculus rotundus and Peyer's patches, constitute equivalent sources of the effector cells. In the absence of evidence ot the contrary, it may be assumed that the effector cells originate in the SAPP organs, spill over into the circulation and infiltrate the spleen and lymph nodes where they may survive for a time and be detected in small numbers and where they eventually die (Figure 8). The failure to detect any effector cells in the thymus and bone

marrow implies that these organs do not provide conditions conducive to the continued viability or proliferation of these cells (Figure 8). It is therefore necessary to postulate that the capacity to invoke a GVH reaction is a property of a unique cell which dies or sheds this function once it leaves the circulation. Alternatively, the GVH reactivity of the cell may be characteristic of a specific period of the lifespan of the cell and this activity is lost as the cell passes into the "old age" phase, at which time it leaves the circulation. At this point, it may constitute the immunologically-incompetent pool of lymphocytes.

Friedman (80, 277) recently reported upon the use of the inhibition by homologous cells of the PFC response by immune mouse spleen cells as a means to detect transplantation immunity. He demonstrated that plaque formation by immune spleen cells (mouse A) could be inhibited if the cells were initially incubated with spleen cells obtained from a mouse of a different strain (mouse B), irrespective whether the latter had been previously immunized to mouse A cells. The suppression of plaque formation was found to be quantitatively related to the relative numbers of effector (mouse B) cells incubated (80, 277). Cells

from hybrid mice had no effect on plaque formation by spleen cells obtained from mice of the parental strain immunized with SRBC (277). On the other hand, plaque reduction occurred when spleen cells from non-sensitized allogeneic mice were incubated with the plaque-forming spleen cells. The author concluded that the reaction reflected a homograft response in vitro of one spleen cell population against another and constitutes a cell-mediated reaction.

Results of a similar nature using spleen cells of mice have most recently been reported by Hirano and Uyeki (278). Unlike the experiments of Friedman (80, 277), these investigators cultured spleen cells from mice of different strains with SRBC for a period of 4 days following which the cells were analyzed for PFCs. The degree of suppression of PFCs was related to the disparity of the H-2 allele and did not depend on complement to ensue. However, since the spleen cells of both strains of mice (allogeneic culture) incubated were capable of transforming into PFCs in vitro, it is difficult to ascertain the exact extent of allogeneic inhibition induced by cells of one spleen

against the other. The results obtained with the system deployed here are much simpler to interpret since only the target memory spleen cells are capable of giving rise to PFCs in culture. Another distinction between our experiments and those of Friedman (80, 277) and Hirano and Uyeki (278) is that we investigated the "killer" capacity of cells of a large number of lymphoid organs and the blood whereas the latter investigators were only concerned with the spleen.

What is the evidence in favour of the graft-versus-host (GVH) nature of the reaction observed here? Is it necessarily a cell-mediated reaction and can antibodies secreted by the effector cell be ruled out either as the essential mediator or participant of the reaction? Several characteristics of the inhibition of the PFC response by effector cells, as observed here, are compared and contrasted with the counterpart characteristics of the humoral immune response in Table XXVI. A consideration of the properties listed would tend to dissociate the two immune reactions at least insofar as the primary mechanisms are concerned. The cell types are different as is their sensitivity

to irradiation; complement is required for antibody-mediated cytolysis but it is not required for the reaction observed here; the periods of *in vitro* incubation required for optimal manifestations of the two immune reactions are also different. The fact that the reaction observed here is a complement-independent one strongly favors the cell-mediated nature of the reaction, since it is generally agreed that humoral antibody can express its cytotoxic properties best, if not only, in the presence of lytic complement (165, 279, 280, 283). In our laboratory, antilymphocyte serum is effective as a cytotoxic agent in rabbit cells only if complement is added to the system (284).

A very different interpretation of the findings reported here is possible. Results of numerous investigations have disclosed that the addition of specific antibodies to cells in culture will prevent active antibody formation by these cells (285-287) in the same manner as the injection of antibody *in vivo* will inhibit specific antibody synthesis (288-290). Furthermore, it has been demonstrated that 19S antibody will stimulate, whereas 7S antibody will inhibit, active antibody synthesis (288) and the generation of PFCs (288).

Since rabbit cells do not possess the Forssman Antigen (57) which the SRBC and many enteric bacteria possess (57), it has been suggested that the rabbit is constantly being subjected to subthreshold antigenic stimuli, the latter provided by the enteric bacteria and other micro-organisms (57). This assumption might explain why circulating antibodies to SRBC can often be detected in the rabbit albeit in low concentration (292, 293). Since the SAPP organs provide the first line of defence with respect to enteric micro-organisms, it may be argued that antibody forming cells would normally be generated in these organs. Thus, incubation of these cells in vitro might result in the release of sufficient numbers of anti-SRBC antibodies to inhibit the antibody-synthetic mechanism of the memory spleen cells present in the culture preventing their transformation into PFCs. However, this interpretation cannot be seriously considered as the correct one for the following reasons: -

(1) Although the normal SAPP cells or WBC could inhibit the emergence of PFCs from homologous memory spleen cells in culture, these

cells by themselves could not give rise to PFCs if cultured with SRBC (See Table IX). Thus, they could not synthesize anti-SRBC antibodies.

(2) Memory spleen cells could generate PFCs in culture even if incubated with large numbers of autologous SAPP cells or WBC. These latter cells should be as effective as homologous cells to produce antibodies to SRBC, if in fact they had been endowed with this capacity.

(3) Supernatants of "inhibited" incubates were <u>not</u> capable of inhibiting the generation of PFCs by other memory spleen cells in culture. Certainly, if the theory advanced above is valid, then these supernatants should have been rich in anti-SRBC antibodies and therefore very inhibitory to the generation of PFCs. However, such was not the case.

(4) Although the primary response can usually be inhibited by passively-

transferred antibody, the secondary response generally cannot be so inhibited. The response evoked here is a secondary immune response *in vitro*.

(5) Generally, Forssman antibodies are of the 19S class following primary immunization and of the 7S class following prolonged immunization (316, 317). Since the effector cells were usually obtained from young (2-3 months old) rabbits, it might be expected that these rabbits would still be synthesizing 19S anti-Forssman antibodies and that their cells in culture would secrete 19S antibodies which would stimulate, not inhibit, further antibody synthesis by the homologous memory spleen cells. Conversely, since the memory rabbits were usually much older (6-8 months old), it might be expected that they would be synthesizing 7S antibodies and that their cells in culture would release 7S antibodies which should inhibit, not stimulate, further antibody

synthesis by the memory spleen cells. In fact, the opposite occurred in the sense that SAPP cells and WBC of young rabbits inhibited the generation of PFCs by homologous memory cells, whereas these cells obtained from the older immunized rabbits had no inhibitory activity.

(6) Optimal inhibition of PFC generation was observed when the ratio of effector cell to memory spleen cell was 1:1. When the ratio was increased to 1:5, the inhibition was very slight. This stochiometric relationship between effector and memory cells suggests a cell-cell interaction, rather than interaction between the memory spleen cell and a soluble factor released by the effector cell.

On the basis of these considerations, it is hardly likely that the inhibitory activity mediated by the SAPP cells and WBC is effected by conventional type antibody molecules.

An intriguing question is whether the reaction observed here, the inhibition of the in vitro generation of PFC from memory spleen cells, is the result of interaction with a single immunocompetent effector cell or a number of functionally distinct effector cells. Since the activity can be manifested by cells from a single organ-either appendix, sacculus rotundus or Peyer's patches, it is hardly likely that two different cells are involved. Otherwise, it is necessary to postulate that the two or more cells mediating the response are localized in the same proportion in each of these organs. This latter possibility is not very likely and it is more probable that the GVH or cell-mediated immune reaction is mediated by a single effector cell (318).

The finding of the effector cell in the circulation in apparently its most active state would support the contention that the reaction described in vitro is a cell-mediated one since other cell-mediated immune reactions, such as the mixed leukocyte culture (MLC) reaction, graft rejection and passively-transferred autoimmune disease are also mediated by circulating lymphoid

cells. The mixed leukocyte culture reaction (MLC) is observed when leukocytes of one individual are cultured with those of another under specified culture conditions (319). After 4 to 5 days in culture, numerous blast cells appear, the number of which can be correlated to the degree of genetic non-identity of the donors of the white cells (319-325). Nisbet et al (326) and Simonsen (231, 232, 327) have demonstrated that circulating lymphoid cells can induce graft-versus-host reaction in the chick embryo. Brunet and Boyer (330), Simons and Fowler (331), Szenbert et al (332) and Coppleson and Michie (333) have observed that circulating lymphoid cells can induce the graft-versus-host reaction in the chick chorioallantoic membrane. Saleh et al (334) and Gordon et al (335) have also demonstrated, using a modification of the Elkin model (237), that circulating leukocytes can induce the GVH reaction if injected under the kidney capsule in cyclophosphamide-treated rats. The blastogenic response of circulating lymphoid cells to phytohemagglutinin (PHA) is also considered to represent an index of cellular, rather than humoral, immunity (199, 297, 336, 338, 339). Notwithstanding the above demonstrations of the

cell-mediated immune capacity of the virgin circulating lymphocyte, it is of more than academic interest to note that the virgin immunocompetent cells mediating the humoral immune response are not normally found in the circulation but are localized to specific lymphoid organs, the thymus (antigen-reactive cells) (248, 252-255) and bone marrow (antibody-forming cells (248, 252-255) in the mouse and bone marrow (antigen-reactive cells) (50, 68, 248) in the rabbit. The organ source of the virgin antibody-forming cell in the rabbit has not as yet been elucidated. However, one may speculate as to its source. In the rat and mouse, evidence has been presented demonstrating the bone marrow origin of the AFC (248, 252-255) and the cells mediating the delayed skin reaction (248, 340, 341) and the graft-versus-host reaction (248, 342). Since the SAPP organs appear to be the sources of the virgin cells mediating the CMI reaction in the rabbit, might not these same organs constitute the sources of the virgin AFCs? A number of investigations (9, 250, 343) have demonstrated a definite role for the SAPP organs in the humoral immune response in the rabbit. Extirpation of these organs in the neonatal state or in the adult

state followed by irradiation resulted in a marked inhibition of humoral antibody formation when the rabbit was immunized at a later date. Our findings indicate that cells of only a single organ-appendix, sacculus rotundus or Peyer's patches, are capable of inducing the GVH reaction in a wholly in vitro system. These effector cells are also identified in equivalent numbers in the circulation. Thus, the SAPP organs may function in the rabbit in a manner analogous to the bone marrow in the rat and mouse, that is, to provide a source of both the virgin afcs mediating the humoral immune response and the effector cells mediating the cellular immune response.

Whether this virgin uncommitted effector cell is a short-lived or long-lived lymphoid cell is not known at the present time. McGregor et al (344) have recently demonstrated that the immunologically committed (immune) effector does not recirculate via the thoracic duct lymph and is short lived whereas the virgin presumable unstimulated effector cell recirculates repeatedly from blood lymph and potentially has a long life span (344).

The organ source of the virgin effector cells mediating the cellular immune reaction has not yet been unequivocally elucidated. Furthermore, the studies which have been conducted to clarify this problem have been limited to only mice and rats. Willard and Smith (345) described a technique used to determine the organ source of effector cells which was based on rejection of the erythropoietic component of homografted bone marrow by transplanted isologous cells in an irradiated recipient mouse. The degree of erythropoiesis was determined by the extent of uptake of Fe^{59} by the spleen 7 days after the homotransplant and allograft. The circulating leukocytes were by far the most effective cells in their ability to reject grafted bone marrow. Lymph node and spleen cells exhibited about 50 per cent of the leukocyte activity, while peritoneal fluid cells, marrow cells and thymus cells showed almost no activity. The authors speculated that GVH activity of the marrow cells could have been masked by the proliferation of erythropoietic cells of the homologous and isologous marrow. Lonai and Feldman (346) sensitized rat lymphocytes on C3H mouse

fibroblast monolayers and then tested the lytic effect of these cells against C3H cells. As observed by Willard and Smith (345), Lonai and Feldman (346) also reported that thymus cells manifested very low lytic activity while the spleen and especially the lymph node cells manifested substantial activity. However, they did not test the cells of any of the other lymphoid organs. Celada and Carter (347) also demonstrated that spleen cells can mediate a GVH reaction but no attempt was made to compare the spleen cells with those of the other lymphoid organs for GVH activity. They also observed that the GVH activity was much more resistant to irradiation than was the antibody-forming mechanism (347) and suggested that the capacity to participate in or mediate these two types of immune responses may be attributed to two different somatic cell lines or differentiation states. Our data (Table XVII) support this thesis since 1200r whole body irradiation could totally inhibit the humoral immune response although lymphoid cells of these animals exhibit normal GVH activity in in vitro culture. Coppleson and Michie (333) reported that incubation of the chorioallantoic membrane of a developing chick embryo with living adult chick leukocytes developed white focal lesions

within a few days. These lesions are considered to be an expression of the GVH reaction. However, here again, cells of only one source, the circulation, were tested for GVH activity.

A number of investigators have reported the induction of the GVH reaction using a combination of bone marrow and thymus cells where neither cell suspension by itself could induce the GVH reaction (348-350). It has been speculated that the role of the thymus cell is to recognize and interact with the allogeneic antigen while that of the bone marrow cell is non-specific or non-immunologic, confined to a proliferative response (348, 349). Notwithstanding these reports, Stuttman and Good (275) were unable to demonstrate a thymus-bone marrow synergism in the GVH reactions. Cantor and Asofsky (70) have observed a synergism between spleen cell or lymph node cells combined with thymus cells in the induction of the GVH reaction. However, it is important to note that SAPP organs or circulating cells were not investigated for their GVH activity in any of the studies enumerated above.

The only investigation which conferred a role in cell-mediated immunity to the SAPP organs

is that of Jacobson et al (351). They observed that mice which had been irradiated with one Peyer's patch shielded were as capable of rejecting homologous bone marrow grafts as were sham-operated mice. They also observed that applying supralethal irradiation to the previously shielded Peyer's patch within 10 minutes after the whole body irradiation nevertheless resulted in rejection of the homografted marrow, thus demonstrating the rapid emigration of cells from the Peyer's patch into the circulation, presumably to repopulate the peripheral lymphoid organs.

On the other hand, it is interesting to note that bone marrow-derived cells constitute the majority of the cells infiltrating a skin site in which a delayed hypersensitivity skin reaction has been induced (341, 352, 353). These results suggest that the effector cells reside preferentially in the bone marrow. Since the reconstitution of the capacity to give a cell-mediated response has been shown by a number of investigators to involve the participation of both thymus-derived and bone marrow-derived cells (348-350), it may be that the function of the thymus cell is that of recognition of, and interaction with, the antigen (9, 349, 350). A

product released from the cell following this interaction (information?) would then act on the virgin competent uncommitted bone marrow effector cell to transform it into an actively sensitized committed effector cell, capable of infiltrating sites in the skin of the sensitized animal challenged with the antigen.

Can the effector cells be detected by other than GVH reactions in vitro? It has been speculated that the degree of the in vitro blastogenic response of the lymphocytes to phytohemagglutinin (PHA) and allogeneic leukocytes may serve as a barometer of the numbers of cells capable of participating in cellular immune reactions (199, 297, 336, 338, 339). However, the relationship between the blastogenic response to mitogens in vitro and the capacity to give a GVH reaction is not at all clear-cut insofar as the rabbit is concerned. It has been demonstrated that cells of all the lymphoid organs are capable of reacting to stimulation with PHA, with the greatest response given by the cells of the sacculus rotundus (297). The appendix and sacculus rotundus cells gave good responses when stimulated with xenogeneic and allogeneic

leukocytes (297). However, the greatest response was obtained with lymph node cells, although it may be significant that thymus and bone marrow cells gave only negligible responses (297). Somewhat unexpected, however, is the rather close relationship observed between the blastogenic response of the lymphoid cells to GARIG (Goat anti-rabbit immunoglobulin serum) (297) and their capacity to mediate the GVH reaction. The greatest blastogenic responses were given by sacculus rotundus, appendix and circulating lymphocytes. Much lesser responses were obtained with spleen and lymph node cells and negligible responses were obtained with thymus and bone marrow cells (297). Peyer's patches cells were not tested. These data, in concert with the data presented here, suggest that the cells mediating the GVH reaction or any other reaction of cellular immunity possess immunoglobulin recognition sites of their surfaces. In fact, Greaves et al (354) have unequivocally demonstrated that human lymphocytes treated with anti-light chain antiserum (rabbit) were sharply depressed in their capacity to give a blastogenic response when stimulated with allogeneic leukocytes (the mixed lymphocyte reaction) or with PPD. They con-

cluded that immunoglobulin light chains probably form parts of the antigen receptor sites for the initiation of cell-mediated immune reactions. The studies by Mason and Warner (355) confirm those referred to above (354) in that they demonstrated that pre-treatment of normal adult mouse spleen cells or peritoneal exudate cells of a sensitized mouse with antiserum to light chains dramatically suppressed the ability of these cells to induce the GVH reaction and the transfer of delayed hypersensitivity, respectively. Anti-heavy chain serum did not suppress the immunologic capacity of these cells, suggesting that antigen recognition as it relates to cellular immunity involves an immunoglobulin light chain bound to the surface of the cell (355). Davie and Paul (356) have also presented evidence of the existence of immunoglobulin receptor sites on cells mediating the cellular immune response. Ramseier and Lindeman (357) have presented direct evidence in favour of immunoglobulin recognition sites on effector cells. They injected adult F_1 hybrid animals with lymphoid cells from one of the parental strains. The antisera obtained were capable of specifically inhibiting recognition by cells of the parent of transplantation antigens of

the other parent present on the F_1 hybrid cell. Recognition of and interaction with other unrelated cells was not affected by pretreatment of the cells with the antiserum. The authors concluded that F_1 hybrids can form antibodies against recognition structures on syngeneic cells which they specifically lacked (357).

It has also been demonstrated that the virgin immunocompetent cells which participate in the induction of the primary humoral immune response (the ARC and/or the AFC) also possess immunoglobulin-like antigen recognition sites on their surfaces (284, 358-365) and that these sites are probably composed of light chains as well (360, 362). One may therefore conclude that the virgin immunocompetent cells mediating humoral and cellular immunity both possess immunoglobulin recognition sites on their surfaces through which they interact with the antigen. These sites are probably present on lymphoid cells arising from two different cell lines, one thymus derived and the other bursal-derived. It has been observed that the cells which respond to stimulation with phytohemagglutinin (PHA) (339, 366-369) and those capable of mediating the

GVH reaction (271, 370-372) are thymus derived whereas those cells which participate in humoral antibody formation are bursal derived (373). Whether or not an antibody-mediated or cell-mediated immune response will ensue following immunization will therefore depend, not so much on the interaction of an immunocompetent cell with the antigen by means of a recognition site but on whether a second cell type, the AFC, is recruited by the immune cell pathway activated (318). In cellular immunity, it is postulated that no information is transferred from the antigen reactive cell (ARC); rather, it undergoes transformation and/or proliferation into sensitized or committed effector cells (318), which are capable of mediating the delayed hypersensitivity skin reaction *in vivo* and of releasing the migration inhibitory factor (MIF) if stimulated by antigen *in vitro* (283). In the case of humoral immunity, it is postulated that information is transferred from the ARC, following its interaction with the antigen, to the virgin, pluripotential uncommitted AFC, which then transforms and proliferates giving rise to antibody-forming, unipotent, committed AFCs (267, 284, 318).

Since the virgin AFC (9, 250, 343) and the virgin effector cells participating in humoral and cellular immunity, respectively, both appear to originate in one or more of the SAPP organs would result in the loss of ability to elicit humoral and cellular immune responses. Extirpation of the SAPP organs in the neonate or the young rabbit combined with irradiation does, in fact, result in inhibition of the capacity to give a humoral immune response (9, 250, 343). However, extirpation of the SAPP organs in the adult rabbit without ensuing irradiation does not affect the capacity to synthesize humoral antibody in the primary immune response (374), suggesting that the virgin AFCs had vacated the SAPP organs in the immediate neonatal period and infiltrate, reside and proliferate in the peripheral lymphoid organs as SAPP-derived cells (374). Furthermore, Cooper et al (9) observed that the delayed skin reaction and homograft rejection could still be obtained in rabbits which had been sensitized with tuberculin in complete Freund's adjuvant or been given a skin homograft subsequent to extirpation of the SAPP organs. Since nothing is known about the number of sensitized cells required to mediate the

delayed hypersensitivity reaction, it is possible that the SAPP-derived effector cells in the spleen and lymph nodes of the SAPPed rabbit may suffice.

In summary, it has been demonstrated that the gastro-intestinal-associated lymphoid organs - the sacculus rotundus, appendix, and Peyer's patches (SAPP), possess cells capable of reacting with allogeneic cells in culture. Allogeneic memory spleen cells capable of giving rise to plaque-forming cells in vitro were used as the target cells for these effector cells. The circulating lymphocytes exhibited the same degree of effector activity, the spleen and lymph node much less and the bone marrow and thymus could not at all inhibit the generation of PFC from allogeneic memory spleen cells in culture. In fact, the bone marrow cells, whether autologous or homologous, facilitated a much greater PFC response than normally observed in cultures of only spleen memory cells. The reaction observed has been shown to be cell-mediated and not humoral-mediated. Whether or not this cell-mediated reaction in vitro may serve as an indicator of the capacity of cells to mediate a graft-versus-host reaction remains

to be determined.

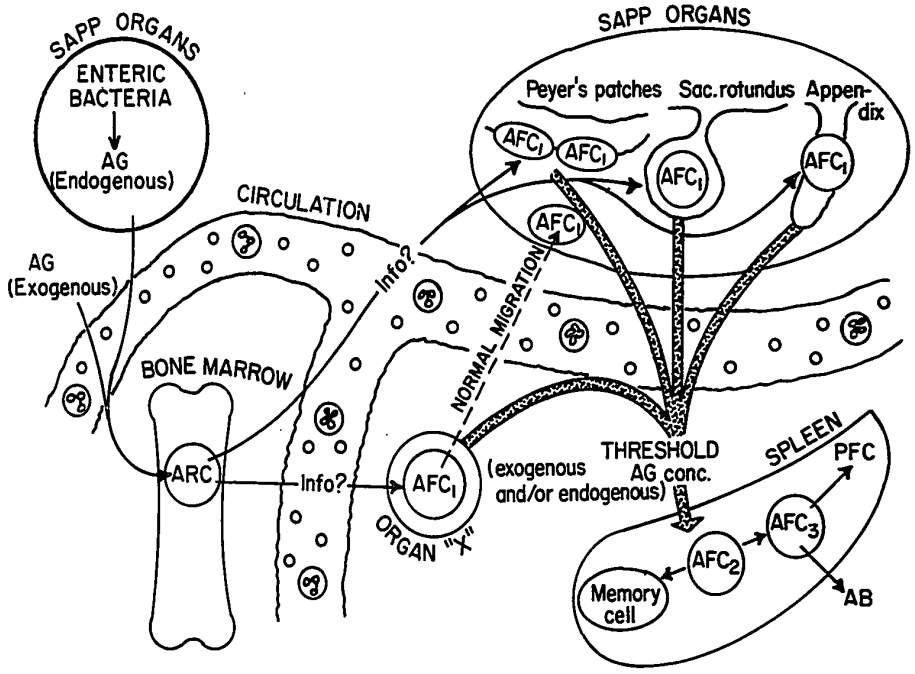

FIGURE 6. MIGRATION PATHWAYS OF THE IMMUNOCOMPETENT CELLS IN THE NORMAL RABBIT FOLLOWING STIMULATION WITH THRESHOLD ANTIGEN (SRBC).

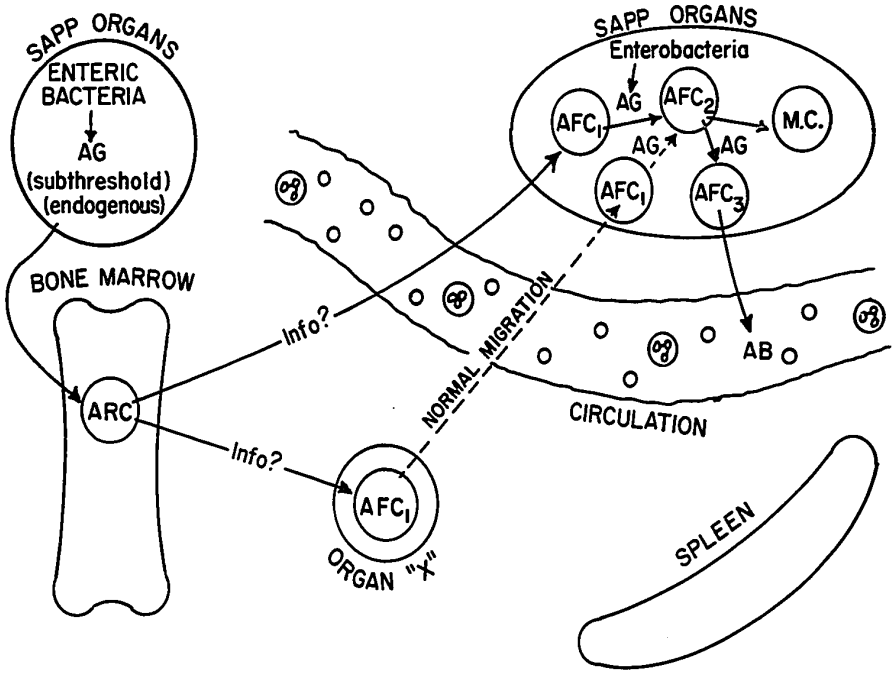

FIGURE 7. MIGRATION PATHWAYS AND SITES OF MATURATION OF IMMUNOCOMPETENT CELLS IN ABSENCE OF CIRCULATING THRESHOLD CONCENTRATION OF ANTIGEN (SRBC) IN THE RABBIT. THRESHOLD CONCENTRATION OF AG IN SAPP ORGANS ONLY.

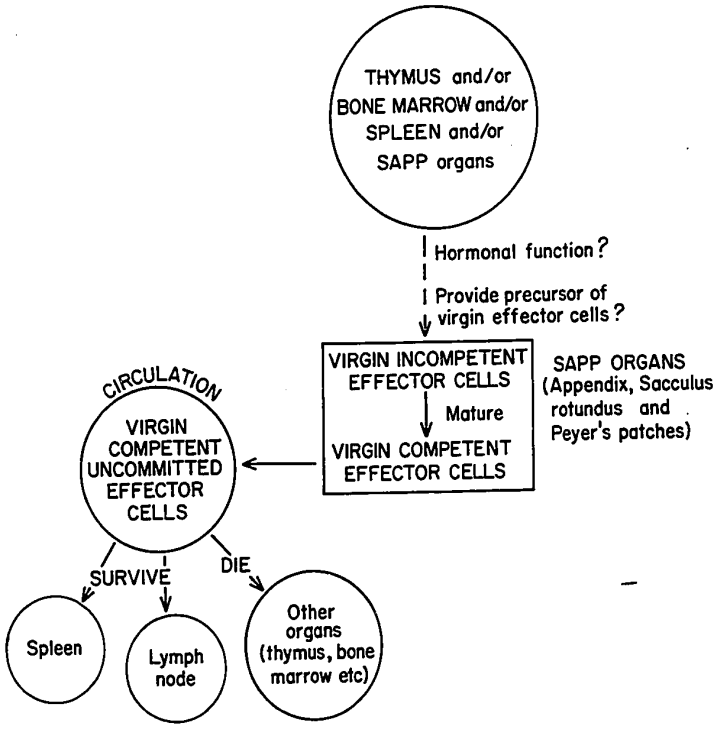

FIGURE 8. THE ORGAN SOURCE OF THE VIRGIN IMMUNO-COMPETENT EFFECTOR CELLS MEDIATING THE CELLULAR IMMUNE REACTION.

A SCHEMATIC REPRESENTATION.

TABLE XXVI

EVIDENCE IN FAVOUR OF THE NON-HUMORAL NATURE OF THE INHIBITION IN VITRO OF THE HOMOLOGOUS PLAQUE-FORMING CELL (PFC) RESPONSE BY APPENDIX, SACCULUS ROTUNDUS AND PEYER"S PATCHES LYMPHOCYTES (SAPP CELLS) AND CIRCULATING LEUKOCYTES (WBC).

Properties characteristic of Humoral Immune Mechanisms	Properties characteristic of the Inhibition of The Homologous PFC Response in Vitro by SAPP Cells and Circulating WBC
1. Complement is required for the lysis of cells by antibody.	Complement is not required for this reaction.
2. The primary humoral immune response is inhibited in rabbits subjected to 800r whole body irradiation prior to antigen administration.	This reaction occurs equally well with SAPP cells and WBC obtained from either normal or 800r-irradiated rabbits.
3. The bone marrow ARC cell is required for the induction of the primary response in vivo.	Bone marrow cells are not required nor are they effective.
4. Spleen cells from previously immunized rabbits give rise to plaque-forming cells following antigenic stimulation in vitro.	Spleen cells are not required nor are they effective.
5. SAPP cells and WBC do not participate in humoral response in vitro.	SAPP cells and WBC are the only lymphoid cells which are effective.
6. Time required in culture to elicit optimal (a) secondary PFC response - 6-8 days (b) secondary immune response (synthesis and release of antibody) 10-18 days.	Time required in culture to inhibit homologous PFC response - 4 days or less.
7. Spleen cells of memory rabbit subjected to 1200r whole-body irradiation cannot give a PFC response in culture.	Appendix and circulating cells of rabbit subjected to 1200r whole-body irradiation retain capacity to inhibit homologous PFC response in vitro.
8. Antibodies can be detected and identified.	No antibodies can be detected in supernatants of cultures of memory spleen and homologous SAPP cells or WBC.

CHAPTER SEVEN

SUMMARY AND CONCLUSIONS

Experiments were carried out to determine the optimal conditions for the induction of a secondary immune response *in vitro*. Rabbits immunized with sheep red blood cells (SRBC) three to six months prior to sacrifice (memory rabbits) were used as a source of lymphoid cells. Cell suspensions prepared from the spleen, bone marrow, thymus, popliteal lymph nodes, circulating WBC, sacculus rotundus, appendix and Peyer's patches were cultured *in vitro* with SRBC using a variety of commercially available media as culture solutions.

Optimal conditions for the successful induction of a secondary immune response to SRBC *in vitro* were determined. Only the spleen cells obtained from the memory rabbits could generate hemolytic plaque-forming cells (PFCs) following *in vitro* stimulation, with an optimal response observed at 6 - 8 days following initiation of culture. Two media, Joklick and CMRL-1066, consistantly facilitated maximum immune responsiveness *in vitro*, using a gaseous phase of 5 percent CO_2, 18 percent O_2 and 77 percent N_2. A very low or

no response was obtained unless the cultures were maintained on a rocker platform and rocked at 6 - 7 complete cycles per minute for the duration of culture. The addition of hydrocortisone or L-asparagine considerably inhibited the PFC response of the memory spleen cells. The PFC response was found to be specific in the sense that no response in vitro could be initiated if the memory spleen cells were cultured with a non-cross reacting antigen, horse red blood cells. Sheep red blood cell sonicates and stroma failed to induce a response in vitro.

Attempts were made to induce a primary response in vitro with the different lymphoid cells of normal rabbits. Only six out of sixteen experiments were successfull in this regard. In three such experiments, spleen cells elicited a low grade PFC response. In three other experiments mixed cultures of spleen and appendix cells or spleen and bone marrow cells gave very strong PFC responses after four days in culture. No other combinations of lymphoid cells in culture gave any positive response.

Rabbits were immunized with SRBC and sacrificed at varying intervals of time. Cell suspensions of their lymphoid organs were prepared and analyzed for their content of PFCs immediately follow-

ing sacrifice while other aliquots of the same cell suspensions were cultured for four days in vitro in the presence of SRBC and then analyzed for their content of PFCs. None of the lymphoid organs possessed any PFCs prior to day 4 after in vivo immunization when tested immediately following sacrifice. However, when cultured in vitro, only the Peyer's patches cells obtained from rabbits sacrificed 1 to 2 days following in vivo immunization were capable of giving rise to PFCs. Of the organs obtained from rabbits sacrificed 4 to 7 days following immunization, only the spleen possessed cells which gave rise to PFCs when tested immediately after sacrifice and after 4 days in culture. It is concluded that the cells detected initially in the Peyer's patches are probably immature committed AFCs, since they must mature in culture in the presence of antigen before they are capable of giving rise to a PFC response, whereas the spleen harbours active antibody producing cells by 3 to 4 days following in vivo immunization. The results presented tend to support the view that the Peyer's patches may act as organs of transit and/or as sites of maturation for the stimulated AFC. It is postulated that the antigen stimulated AFCs mature in the Peyer's patches for 1 to 2 days following which they migrate to

the spleen where their period of maturation terminates and they transform into overt PFCs and antibody synthesizing cells.

Spleen cells of a memory rabbit (ex. white rabbit) were cultured with SRBC with or without the addition of homologous (ie black rabbit) lymphoid cells. It was demonstrated that the gastro-intestinal associated lymphoid organs - the sacculus rotundus, appendix and Peyer's patches, - possess effector cells capable of reacting with the allogeneic memory cells in culture on the basis of their capacity to inhibit the generation of PFCs from the memory spleen cells. The circulating lymphocytes exhibited the same degree of effector activity, the spleen and lymph node much less whereas the bone marrow and thymus could not at all inhibit the generation of PFC from allogeneic memory spleen cells in culture. The reaction observed has been shown to be cell-mediated and not humoral-mediated. The effector cells were found to be radio-resistant up to a dose of 1200R total body irradiation of the effector cell donor. Exposure of effector cells to 4,000R irradiation in vitro markedly decreased their capacity to inhibit the generation of PFCs from the memory spleen cells. The reaction could only be med-

iated by viable cells. The effector cells constitute a constant proportion of the cells in each of the four cell preparations which manifest inhibitory activity since the cells of each of these organs displayed similar reactivity when tested in equal numbers. Supernatants from PFC-inhibited cultures were tested to determine the presence of humoral factors which might be responsible for this inhibition. No antibodies directed toward the memory spleen lymphoid cells could be detected in these supernatants. In addition, these supernatants exhibited no cytotoxic activity toward the memory spleen cells even in the presence of complement. The presence of anti-SRBC has been ruled out as a factor leading to the in vitro inhibition of PFCs by memory spleen cells. These results strongly suggest that the reaction observed is a cell mediated one.

Preliminary experiments were carried out to determine the in vivo migration pathway(s) of the effector cells. Rabbits were immunized with appendix cells of the prospective memory rabbit and were sacrificed at intervals of time thereafter. The cells of the different lymphoid organs were then cultured with spleen cells of the memory rabbit and sheep red

blood cells. It was observed that the sacculus rotundus, appendix and Peyer's patches cells were devoid of PFC inhibitory activity or displayed minimal activity if obtained from rabbits 4 days following immunization with the allogeneic appendix cells. On the other hand the thymic cells displayed marked activity. The situation with respect to the organ localization of effector cells appeared to return to normal after day 17 post-immunization.

CHAPTER EIGHT

CLAIMS TO ORIGINALITY

1- A secondary immune response could be initiated *in vitro* with spleen cells of previously immunized (memory) rabbits. None of the other lymphoid organs of the memory rabbit possessed this capacity.

2- It was demonstrated that the media and gaseous phase which facilitated the optimal induction of the secondary immune response *in vitro* are different from those which are generally used in similar experiments with mouse cells.

3- In the instances were a primary immune response was obtained *in vitro* with cells of normal rabbits the media which facilitated the induction of such a response were different from those used to induce a secondary immune response *in vitro*.

4- It was demonstrated that the Peyer's patches constitute either the organ of origin and/or the organ of transit and/or the organ of maturation of the antibody forming cell (AFC) directed to SRBC as antigen.

Following immunization with SRBC immature AFCs or their precursors could be detected in the Peyer's patches before they could be detected in any of the other lymphoid organs.

5- It was demonstrated that circulating lymphoid cells as well as cells obtained from gastrointestinal lymphoid organs - sacculus rotundus, appendix and Peyer's patches - possess the capacity to inhibit the secondary immune response of memory spleen cells in culture. Cells possessing this type of inhibitory activity are referred to as effector cells. Cells of thymus and bone marrow did not appear to contain any effector cells whereas the spleen and lymph nodes contained small numbers of these cells.

6- It was demonstrated that the inhibition by SAPP cells and circulating lymphocytes, of the generation of PFCs by memory spleen cells in vitro is a cell mediated and not a humoral-mediated reaction.

7- It was demonstrated that the radiosensitivity of the effector cell differed from that of the memory cell.

8- It was demonstrated that the effector cell vacates the sacculus rotundus, appendix and Peyer's patches and homes into the thymus by four days following *in vivo* stimulation with allogeneic lymphoid cells. During the next 12 days the cells leave the thymus and return to the original organs of origin. This migration pathway of the effector cell is therefore different from that of the antibody forming cell.

1. Glick, B., Chang, T.S. and Jaap, R.G.,
Poultry Sci. 35: 224, 1956.

2. Glick, B.,
Poultry Sci., 37: 240, 1958.

3. Cooper, M.D., Peterson, R.D.A., South, M.A. and
Good, R.A.,
J. Exp. Med. 123: 75, 1966.

4. Miller, J.F.A.P., Doak, S.M.A. and Cross, A.M.,
Proc. Soc. Exp. Biol. Med. 112: 785, 1963.

5. Good, R.A., Dalmasso, A.P., Martinez, C.,
Archer, O.K., Pierce, J.C. and Papermaster, B.W.,
J. Exp. Med. 116: 773, 1962.

6. Gain, W.A., Cooper, M.D. and Good, R.A.,
Nature, 217: 87, 1968.

7. Dent. P.P. and Good, R.A.,
Nature, 207: 491, 1965.

8. Auerbach, R.,
in The Thymus, Ciba Foundation Symposium.
C.E.W. Wolstenholme and R. Porter (Eds) p. 39, 1966.

9. Cooper, M.D., Perey, D.Y., Gabrielsen, A.E.,
Sutherland, D.E.R., McKnealley, M.F. and Good, R.A.,
Int. Arch. of Allergy 33: 65, 1968.

10. Van Furth, R., Schuit, H.R.E. and Hymans, W.,
Immunol. 11: 19, 1966.

11. Parrot, D.M., De Sousa, A.B. and East, J.,
J. Exp. Med. 123: 191, 1966.

12. Trentin, J.N., Wolf, N., Cheng, V., Fahlberg, W.,
Weiss, D., and Bonhag, R.,
J. Immunol. 98: 1326, 1967.

13. Tyan, M.L. and Cole, L.J.,
Nature 208: 1223, 1965.

14. Nossal, G.J.V., Cunningham, A., Mitchell, G.F. and
Miller, J.F.A.P.,
J. Exp. Med. 128: 839, 1968.

15. Claman, H.N., Chaperon, R.H. and Triplett, R.F.,
Proc. Soc. Exp. Biol. N.Y. 122: 1167, 1966.

16. Wu, A.M., Till, J.E., Siminovitch, L. and McCulloch, E.A., J. Exp. Med. 127: 455, 1968.

17. Wu, A.M., Till, J.E., Siminovitch, L. and McCulloch, E.A., Proc. Natl. Acad. Sc., 59: 1209, 1968.

18. Richter, M. and Algom, G.D., In Press.

19. Parrot, D.M., and De Sousa, M. Clin. Exp. Immunol. 8: 663: 1971.

20. Miller, J.F.A.P., Lancet 11: 748, 1961.

21. Miller, J.F.A.P., Proc. Roy. Soc. B., 156: 415, 1962.

22. Humphrey, J.H., Parrot, D.M.V. and East, J., Immunology 7: 419, 1964.

23. Miller, J.F.A.P., Nature (London) 208: 1337, 1965.

24. Weissman, I.L., J. Exp. Med. 126: 291, 1967.

25. Miller, J.F.A.P. and Osoba, D., Physiol. Rev. 47: 437, 1967.

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

27. Miller, J.F.A.P., Mitchell, G.F. and Weiss, N.S., Nature 214: 992, 1967.

28. Metcalf, D., Nature 208: 1336, 1965.

29. Jeejeebhoy, H.F., Immunology 9: 417, 1965.

30. Mitchell, G.F. and Miller, J.F.A.P., J. Exp. Med. 128: 821, 1968.

31. Mitchell, G.E. and Miller, J.F.A.P., Proc. Natl. Acad. Sci. 59: 296, 1968.

32. Doenhoff, M.J., Davies, A.J.S., Leuchars, E. and Wallis, V., Proc. Royal Soc. B. 176: 69, 1970.

33. Raff, M.C. and Wortis, H.H., Immunology 18: 931, 1970.

34. Moller, G., Transp. Proc. 3: 15, 1971.

35. Schlesinger, M. and Hurvitz, O., Transplant 7: 132, 1969.

36. Dumonde, D.C., Wolstencroft, R.A., Panayi, G.S., Mathew, M., Morley, J. and Howson, W.T., Nature (London) 224: 38, 1969.

37. Peterson, R.D.A., Cooper, M.D. and Good, R.A., Am. J. Med. 38: 579, 1965.

38. Good, R.A., J. Lab. Clin. Med. 46: 167, 1955.

39. Good, R.A., Kelly, W.D., Rotstein, J. and Vargo, R.L., Prog. of Allergy. Karger, Basel, New-York. 6: 187, 1962.

40. Di George, A.M., In Immunologic Differences in Man. The National Foundations. Edit. Bergsman and Good 1968.

41. Nezelof, C., Jammet, M.L., Lertholary, P., Labrune, B. and Lamy, M., Arch. Franc. Pediat. 21: 897, 1964.

42. Jankovic, B.D., Waksman, B.H. and Arnason, B.G., J. Exp. Med. 116: 159, 1962.

43. Archer, O.K., Sutherland, D.E.R. and Good, R.A., Nature (London) 200: 337, 1963.

44. Archer, O.K., Sutherland, D.E.R. and Good, R.A., Lab. Invest. 13: 259, 1964.

45. Sutherland, D.E.R., Archer, O.K. and Good, R.A., Proc. Soc. Exp. Biol. N.Y. 115: 673, 1964.

46. Konda, S. and Harris, T.N., J. Immunology 97: 805, 1966.

47. Cornes, J.S., Gut. 6: 225, 1965.

48. Cooper, M.D., Perey, D.Y., McKneally, M.F., Gabrielsen, A.E., Sutherland, D.E.R. and Good, R.A., Lancet I: 1388, 1966.

49. Jacobson, L. O., Robson, M.J. and Marks, E.K., Proc. Soc. Exp. Biol. N.Y. 75: 145, 1950.

50. Richter, M., Rose, B. and Abdou, N.I., Int. Arch. of Allergy 38: 269, 1970.

51. Armstrong, W.D., Diener, E. and Shellam, G.R., J. Exp. Med. 129: 393, 1969.

52. Henry, C., Faulk, W.P., Kuhn, L., Yoffey, J.M., Fudenberg, H.H., J. Exp. Med. 131: 1200, 1970.

53. Hanaoka, M., Nomolo K. and Waksman, B.H., J. Immunology 104: 616, 1970.

54. Hanaoka, M. and Waksman, B.H. Cell Immun. 1: 316, 1970.

55. Ozer, H. and Waksman, B.H., J. Immunology 105: 791, 1970.

56. Warner, N.D. and Szenberg, A., Am. Rev. of Microbiol. 18: 253, 1964.

57. Boyd, W.C., In Fundamentals of Immunology - Interscience Publishers N.Y., 1966.

58. Cooper, G.N. and Turner, K., Aus. J. Exp. Biol. Med. Sci. 45: 363, 1967.

59. Cooper, G.N. and Turner, K., J. Reticuloendoth. Soc. 6: 419, 1969.

60. Abdou, N.I. and Richter, M., J. Immunol. 104: 1087, 1970.

61. Perey, D.Y.E. and Good, R.A., Lab. Invest. 18: 15, 1968.

62. Evans, E.P., Ogden, D.A., Ford, C.E. and Micklem, H.S., Nature (London) 216: 36, 1967.

63. Cornes, J.S., Gut 6: 230, 1965.

64. Singhal, S.K. and Richter, M.,
J. Exp. Med. 128: 1099, 1968.

65. Davies, A.J.S., Leuchars, E., Wallis, V., Marchant, R. and Elliott, E.V.,
Transplantation 5: 222, 1967.

66. Davies, A.J.S., Leuchars, E., Wallis, V. and Koller, P.C.,
Transplantation 4: 438, 1966.

67. Miller, J.F.A.P. and Mitchell, G.F.,
J. Exp. Med. 128: 801, 1968.

68. Richter, M. and Abdou, N.I.,
J. Exp. Med. 129: 1261, 1969.

69. Abdou, N.I., Rose, B. and Richter, M.,
J. Exp. Med. 130: 867, 1969.

70. Cantor, H. and Asofsky, R.,
J. Exp. Med. 131: 235, 1970.

71. Evans, R. and Alexander, P.,
Nature (London) 228: 620, 1970.

72. De Weck, A.L. and Schneider, C.H.,
Bayer Symp. 1: 32, 1970.

73. Govaerts, A.,
J. Immun. 85: 516, 1960.

74. Roseneau, W. and Moon, H.D.,
J. Nat. Cancer Instit. 27: 471, 1961.

75. Brunner, K.T., Mauel, J. and Schindler, R.,
Immunol. 11: 499, 1966.

76. Holm, G. and Perlmann, R.,
Immun. 12: 525, 1967.

77. Lundgren, G., Zuloski, C.H.F. and Moller, E.,
Clinical Exp. Immun. 3: 817, 1968.

78. Brondz, B.D.,
Folia Biol. 10: 164, 1964.

79. Perlmann, P. and Holm, G.,
Mechanisms of Inflammation Induced by Immun. Reactions.
Miescher and Grabar Eds, Sehwabe, Basel, 1968.

80. Friedman, H.,
Science 145: 607, 1964.

81. Granger, G.A. and Weiser, R.S.,
Science 145: 1427, 1964.

82. Brondz, B.D.,
Transplantation 3: 356, 1965.

83. Merril, J.P., Hanan, C. and Hawes, M.D.,
Ann. N.Y. Acad. of Sci. 87: 266, 1960.

84. Old, L.J., Boyse, E.A., Bennett, B. and Lilly, F.,
Cell Bound Antibody (Amos and Koprowski Eds)
Wistar Institute Puss. Philadelphia 1963.

85. Bennett, B.,
J. Immunol. 95: 656, 1965.

86. Koprowski, H. and Fernandes, M.V.,
J. Exp. Med. 116: 467, 1962.

87. Berg, O. and Kallen, B.,
Acta Path. Microbiol. Scand. 58: 72, 1963.

88. Moller, G. and Moller, E.,
Nature 208: 260, 1965.

89. Green, H., Fleischer, R.A., Barrow, P. and
Goldberg, B.,
J. Exp. Med. 109: 511, 1959.

90. Klien, G. and Perlman, P.,
Nature 199: 451, 1963.

91. Kirata, A.A.,
J. Immunol. 91: 625, 1963.

92. Holm, G. and Perlmann,P.,
Nature 207: 818, 1965.

93. Holm, G. and Perlmann, P.,
Immunol. 12: 525, 1967.

94. Wigzel, H.,
Transplant 3: 423, 1965.

95. Sanderson, A.R.,
Nature, 204: 250, 1964.

96. MacLennan, I.C.M. and Loewi, G.,
Clin. Exp. Immunol. 3: 385, 1968.

97. Berke, G., Ax, W., Ginsburg, H. and Feldman, M.,
Immunol. 16: 643, 1969.

98. Brunner, K.T., Mauel, J., Cerottini, J.C. and Chapuis, B., Immunol. 14: 181, 1968.

99. Perlmann, P., Perlmann, H. and Holm, G., Science 160: 306, 1968.

100. Granger, G.A. and Williams T.W., Nature 218: 1253, 1968.

101. Granger, G.A. and Kolb, W.P., J. Immunol. 101: 111, 1968.

102. Taylor, H.E. and Culling, C.F.A., Lab. Invest. 12: 884, 1963.

103. Moller, E., J. Exp. Med. 122: 11, 1965.

104. Ulrich, K. and Kieler, J., Proc. Soc. Exp. Biol. Med. 130: 1297, 1969.

105. Takasugi, M. and Klein, E., Transplantation 9, 219, 1970.

106. Wilson, D.B., J. Cellular Comp. Physiol. 62: 273, 1963.

107. Perlmann, P., Perlmann, H., Muller-Eberhard, H.J. and Manni, J.A., Science 163: 937, 1969.

108. Hellstrom, K.E. and Moller, G., Prog. in Allergy 9: 158, 1965.

109. Hellstrom, I. and Hellstrom, K.E., Science 156: 981, 1967.

110. Hellstrom, I. Pierce, G.E. and Yang, J.P.S., Nature 220: 1352, 1968.

111. Hellstrom, I., Pierce, G.E. and Bill, A.H., Proc. Nat. Acad. Sci. 60: 1231, 1968.

112. Roseneau, W. and Morton, D.L., J. Nat. Cancer Ins. 36: 825, 1966.

113. Brunner, K.T., Mauel, J. and Schindler, R., Nature 213: 1246, 1967.

114. Brunner, K.T., Jauel, J., Cerottini, J.C. and Chapuis, B., Immunologic 14: 181, 1968.

115. Wilson, D.B.,
J. Exp. Med. 122: 143, 1965.

116. Brunner, K.T., Mauel, J., Rudolf, H. and Chapuis, B.,
Immunol. 18: 501, 1970.

117. David, J.R.,
Proc. Nat. Acad. Sci. U.S., 56: 72, 1966.

118. Bloom, B.R. and Bennett, B.,
Science 153: 80, 1966.

119. Cline, M.J. and Swett, V.C.,
J. Exp. Med. 128: 1309, 1968.

120. Roseneau, W.,
Cell Bound Antibodies, Amos and Koprowski Eds. p.75
Wistar Instit. Press Philadelphia.

121. Brondz, B.D. and Bartova, L.M.,
Folia Biol. 12: 431, 1966.

122. Gerughty, R.M., Roseneau, W. and Moon, H.D.,
J. Immunol. 97: 700, 1966.

123. Wilson, D.B. and Wecker, E.E.,
J. Immunol. 97: 512, 1966.

124. Bondevick, H. and Mannick, J.A.,
Proc. Soc. Exp. Biol. Med. 129: 264, 1968.

125. Wilson, D.B.,
Transplantation 5: 986, 1967.

126. Perlmann, P., Perlmann, H., Wasserman, J. and
Packalen, T.H.,
Int. Arch. Allergy and Applied Immunol. 1969.

127. Abdou, N.I. and Richter, M.,
Adv. Immunol. 12: 201, 1970.

128. Mauel, J., Rudolf, H., Chapuis, B. and Brunner, K.T.,
J. Immunol. 18: 517, 1970.

129. Roseneau, W. and Moon, H.D.,
J. Immunol. 96: 80, 1966.

130. Brunner, K.T., Mauel, J. and Schindler, R.,
Immunol. 11: 499, 1966.

131. Askonas, B.A. and Humphrey, J.H.,
Biodhem. J. 68: 252, 1958.

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

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

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

135. Dutton, R.W. and Park House,
in Molecular and Cellular Basis of Antibody Formation
(J. Sterzl et al Ed.) p567, 1965.
Academic Press, N.Y.

136. Lewis, M.R. and Lewis, W.H.,
Anat. Rec. 5: 277, 1911.

137. Lewis, M.R. and Lewis, W.H.,
Anat. Rec. 6: 207, 1912.

138. Vogelaar, J.P.M. and Erlichman E.,
Am. J. of Cancer 18: 28: 1933.

139. Baker, L.E. and Ebeling, A.H.,
J. Exp. Med. 69: 365, 1939.

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

141. Eagle, H.,
J. Biol. Chem. 214: 839, 1955.

142. Eagle, H.,
J. Exp. Med. 102: 37, 1955.

143. Eagle, H.,
J. Exp. Med. 102: 595, 1955.

144. Evans, V.J., Bryant, J.C., Fioramonti, M.C.,
McQuilkin, W.T., Stanford, K.K. and Earle, W.R.,
Cancer Res. 16: 77, 1956.

145. Haff, R.F. and Swim, H.E.,
Proc. Soc. Exp. Biol. N.Y. 94: 779, 1957.

146. Swim, H.E. and Parker, R.F.,
Arch. Biochem. and Biophys. 78: 46, 1958.

147. Parker, R.C.,
Methods of Tissue Culture "3 1/2" Edition.
Hoeber Inc. N.Y.

148. Neuman, R.E. and Tytell, A.A.,
Exp. Cell Res. 15: 637, 1958.

149. Healy, G.M., Fisher, O.C. and Parker, R.C.,
Cand. J. Biochem. and Physiol. 32: 327, 1954.

150. Healy, G.M., Fisher, O.C. and Parker, R.C.,
Proc. Soc. Exp. Biol. and Med. 89: 71, 1955.

151. Harris, M.,
J. Exp. Zool. 125: 85, 1954.

152. Swim, H.E. and Parker, R.F.,
J. Biophys. Biochem. Cytol. 4: 525, 1958.

153. Eagle, H.,
Arch. of Biochem. 61: 356, 1956.

154. Swim, H.E.,
Am. Rev. of Microbiol. 13: 141, 1959.

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

156. Parkhouse, R.M.E. and Dutton, R.W.,
Federation Proc. 23: 389, 1964.

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

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

159. Trowell, O.A.,
Cells and Tissues in Culture (E.M. Willmer Ed.)
Vol. 2, p96. Academic Press N.Y.

160. Wasserman, J. and Packalen, T.H.,
Immunology 9: 1, 1965.

161. MacLennan, I.C.M. and Loewi, G.,
Nature 219: 1069, 1968.

162. MacLennan, I.C.M. Loewi, G. and Howard, A.,
Immunology 17: 897, 1969.

163. Taylor, H.E. and Culling, C.F.A.,
Nature 220: 506, 1968.

164. Lundgren, G. Collste, L. and Moller, G.,
Nature 220: 289, 1968.

165. Perlmann, P. and Holm, G.,
Adv. Immunol. 11: 117, 1969.

166. Holm, G. and Perlmann, P.,
Antibiot. chemotherap. 15: 295, 1969.

167. Lo Buglio, A.F., Cotran, and Jandl, J.H.,
Science 158: 1582, 1967.

168. Holm, G. and Perlmann, P.,
Transplant Proc. 1: 420, 1969.

169. Oppenheim, J.J.,
Fed. Proc. 27: 21, 1968.

170. Ruddle, N.H. and Waksman, B.H.,
Science 157: 1060.

171. Ruddle, N.H. and Waksman, B.H.,
J. Exp. Med. 128: 1237, 1968.

172. Ruddle, N.H. and Waksman, B.H.,
J. Exp. Med. 128: 1255, 1968.

173. Ruddle, N.H. and Waksman, B.H.,
J. Exp. Med. 128: 267, 1968.

174. Roseneau, W.,
Fed. Proc. 27: 34, 1968.

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

176. Malchow, H., Zeiss, I., Ax, W., Szenberg, A. and Fisher, H.,
Int. Arch. Allergy and Applied Immunol. 35: 495, 1969.

177. Holm, G. and Perlmann, P.,
J. Exp. Med. 125: 721, 1967.

178. Holm, G., Perlmann, P. and Johansson, B.,
Clin. Expl. Immunol. 2: 351, 1967.

179. Williams, T. and Granger, G.A.,
J. Immunol. 102: 911, 1969.

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

181. Moller, G., Beckman, V. and Lundgren, G.,
Nature 212: 1203, 1966.

182. Holm, G.,
Exp. Cell Res. 48: 334, 1967.

183. Muller-Eberhard, H.J., Dalmasso, A.P. and
Calcott, M.A.,
J. Exp. Med. 123: 33, 1966.

184. Cooper, N.R. and Muller-Eberhard, J.J.,
Immunochemistry 5: 155, 1968.

185. Gowans, J.L. and McGregor, D.D.,
Preg. Allergy 9: 1, 1965.

186. Marchesi, V.T. and Gowans, J.L.,
Proc. Roy. Soc. (Biol.) 159: 283, 1964.

187. Weiner, J., Spiro, D. and Russell, P.S.,
Am. J. Path. 44: 319, 1964.

188. Cell-bound Antibodies (Amos B. and Koprowski, H. Eds.)
Wistar Instit. Press Philadelphia 1963.

189. Abdou, N.I. and Richter, M.,
J. Exp. Med. 129: 757, 1969.

190. Mediators of Cellular immunity (Lawrence, H. and
Landy, M. Eds.) Academic Press N.Y., 1970.

191. Najarian, J.S., May, C. and Cochrum, K.C.,
Am. N.Y. Acad. Sci. 129: 76, 1966.

192. Strober, S. and Gowans, J.C.,
J. Exp. Med. 122: 347, 1965.

193. Barker, C.F., Billingham, R.E. and Shaffer, C.F.,
Transpl. Proc. 1: 597, 1969.

194. Chanana, A.D., Cronkhite, E.P. and Joel, D.D.,
Transpl. 7: 459, 1969.

195. Russell, P.S. and Monaco, A.P.,
Biology of Tissue Transplantation
Little, Brown and Co., Boston, 1965.

196. Text book of Immunopathology.
(Miescher, P.A. and Muller-Eberhard, Grune
and Straton Eds.) N.Y., 1969.

197. Clark, D.S., Foker, J.E., Good, R.A. and
Varlo, R.L.,
Lancet 1: 8, 1968.

198. Richter, M. and Singhal, K.,
J. Exp. Med. 128: 1099, 1968.

199. Naspitz, C.K. and Richter, M.,
Prog. Allergy 12: 1, 1968.

200. Richter, M. and Naspitz, C.,
Int. Arch. Allergy 33: 411, 1968.

201. Turk, J.L.,
Brit. Med. Bull. 23: 3, 1967.

202. Koch, R.,
Dent. Med. Wochschr, 16: 1029, 1890.

203. Landsteiner, K. and Chase, M.W.,
Proc. Soc. Exp. Biol. Med. 49: 688, 1942.

204. Boughton, B. and Spector, W.G.,
J. Path. Bact. 85: 371, 1963.

205. Turk, J.L., Heather, C.J. and Dirngdon, J.V.,
Int. Arch. Allergy 29: 278, 1966.

206. Chase, M.W.,
Proc. Soc. Exp. Biol. Med. 59: 134, 1945.

207. Zinsser, H. and Mueller, J.H.,
J. Exp. Med. 41: 159, 1925.

208. Freund, J.
J. Immunol. 11: 383, 1926.

209. Metaxas, M. and Metaxas-Buhler, M.,
J. Immunol. 75: 333, 1955.

210. Jeter, W.S., Tremaine, M.M. and Seebohm, P.M.,
Proc. Soc. Exp. Biol. Med. 86: 251, 1954.

211. Cummings, M.M., Patnode, R.A. and Hudgins, P.C.,
Am Rev. of Tuberc. 73: 246, 1956.

212. Feldman, J.D. and Najarian, J.S.,
J. Immunol. 91: 306, 1963.

213. McCluskey, R.T., Benacerraf, B. and McCluskey, J.W.,
J. Immunol. 90: 466, 1963.

214. Lubaroff, D.M. and Waksman, B.H.,
Science 157: 322, 1967.

215. Uhr, J.W., Salvin, S.B. and Pappenheimer, A.M. Jr.,
J. Exp. Med. 105: 11, 1957.

216. Gell, P.C.H.,
Int. Arch. Allergy and Appl. Immunol. 18: 39, 1961.

217. Holtzer, J.D. and Winkler, K.C.,
Immunol. 12: 701, 1962.

218. Karush, F. and Eisen, H.N.,
Science 136: 1032, 1962.

219. Benacerraf, B. and Lexine, B.B.,
J. Exp. Med. 115: 1023, 1962.

220. Gell, P.G.H. and Silverstein, A.M.,
J. Exp. Med. 115: 1037, 1962.

221. Scholssman, S.F. and Levine, H.,
J. Immunol. 98: 211, 1967.

222. Inderbitzin, T., Keel, A. and Blumental, G.,
Int. Arch. Allergy and Appl. Immunol. 29: 417, 1966.

223. Spector, W.G. and Willoughby, D.A.,
Mechanism of Inflammation Induced by Immune Reactions.
(Miescher and Crabar Eds.) Schwabe, Basel 1968.

224. Svejcar, J., Pekarek, J. and Johanovsky, J.,
Immunol. 15: 1, 1968.

225. Asherson, G.L. and Loewi, G.,
Immunol. 11: 277, 1966.

226. Asherson, G.L.,
Immunol. 13: 441, 1967.

227. Dempster, W.J.,
Brit. J. Surg. 40: 447, 1953.

228. Simonsen, M.,
Acta Pathol. Microbiol. Scand. 32: 36, 1953.

229. Billingham, R.E. and Brent, L.,
Transpl. Bull. 4: 67, 1957.

230. Billingham, R.E.,
The Harvey Lectures Series 62: 21, 1966-67.

231. Simonsen, M.,
Prop. Allergy 6: 349, 1962.

232. Simonsen, M.,
Cold Spring Harbor Symposia 32: 517, 1967.

233. Elkins, W.L. and Palm, J.,
Am. N.Y. Acad. Sci. 192: 573, 1966.

234. Billingham, R.E., Defendi, V., Silvers, W.K. and Steinmuller, D.,
J. Nat. Cancer Inst. 28: 365, 1962.

235. Martinez, C., Dalmasso, A.P., Blaise, M. and Good, R.A.
Proc. Soc. Exp. Biol. and Med. 111: 404, 1962.

236. McBride, R.A.,
Cancer Res. 26: 1135, 1966.

237. Elkins, W.L.,
J. Exp. Med. 120: 329, 1964.

238. Elkins, W.L.,
J. Exp. Med. 123: 103, 1966.

239. Elkins, W.L. and Guttmann, R.D.,
Science 159: 1250, 1968.

240. Brent, L. and Medawar, P.B.,
Brit. Med. J. II: 269, 1963.

241. Brent, L. and Medawar, P.B.,
Proc. Roy. Soc. Biol. 165: 281, 1966.

242. Brent, L. and Medawar, P.B.,
Proc. Roy. Soc. Biol. 165: 413, 1966.

243. Mishell, R.I. and Dutton, R.,
J. Exp. Med. 126: 423, 1967.

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

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

246. Theis, G.A. and Thorbecke, G.J.,
J. Exp. Med. 131, 979, 1970.

247. Richter, M. and Abdou, N.I.,
J. Exp. Med. 129: 1261, 1969.

248. Abdou, N.I. and Richter, M.,
Adv. Immunol. 12: 201, 1970.

249. Strober, S. and Law, L.W.,
Proc. Nat. Acad. Sci. 62: 1023, 1969.

250. Perey, D.Y.E., Frommel, D., Hong, R. and Good, R.,
Lab. Invest. 22: 212, 1970.

251. Sussdorf, D.H. and Draper, L.R.,
J. Infec. Dis. 99: 129, 1956.

252. Miller, J.F.A.P. and Mitchell, G.F.,
Transpl. Rev. 1: 3, 1969.

253. Davies, A.J.S.,
Transpl. Rev. 1: 43, 1969.

254. Claman, H.N. and Chaperon, E.A.,
Transpl. Rev. 1: 92,1969.

255. Taylor, R.B.,
Transpl. Rev. 1: 114, 1969.

256. Abdou, N.I. and Richter, M.,
J. Exp. Med. 129: 757, 1969.

257. Perey, D.Y.E., Frommel, D. Hong, R. and Good, R.,
Lab. Invest. 22: 212, 1970.

258. Aitken, I.D.,
J. Inf. Dis. 114: 174, 1964.

259. Good, R.A., Cain, W.A., Perey, D.Y.E., Dent, P.B.,
Meuwissen, H.G., Rodey, G.E. and Cooper, M.D.,
Adv. in Exp. Biol. 5: 33, 1969.

260. Cheng, V. and Trentin, J.J.,
Proc. Soc. Exp. Biol. Med. 126: 467, 1963.

261. Gabrielsen, A.E. and Good, R.A.,
Adv. in Immunol. 6, 1967.

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

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

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

265. Strander, H.,
Immunol. 10: 44, 1966.

266. Cohen, I.R., Stavy, L. and Feldman, M.,
J. Exp. Med. 1055, 1971.

267. Turk, J.L.,
In delayed hypersensitivity (John Wiley and Sons Edit.) N.Y., 1967.

268. Metcalfe, D.,
In the Thymus (Springer-Verlag Publishers)
N.Y.,1966.

269. Hess, M.,
In Experimental Thymectomy Possibilities and Limitations (Spring-Verlag Publishers)
N.Y., 1968.

270. Good, R.A., Gabrielson, A.E., Peterson, R.D.A., Finstad, J. and Cooper, M.D.,
Ciba Foundation Symposium J 2A Churchill Ltd.,
London, 1966.

271. Sosin, H., Hilgard, H. and Martinez, C.,
J. Immunol. 96: 189, 1966.

272. Knight, S. and Thorbecke, G.J.,
Cell Immunol. 1: 545, 1971.

273. Blomgren, H. and Anderson, B.,
Cell Immunol. 1: 545, 1971.

274. Colley, D.G., Shih Wu Ay and Waksman, B.H.,
J. Exp. Med. 132: 1107, 1970.

275. Stuttman, O. and Good, R.A.,
Proc. Soc. Exp. Biol. Med. 130: 848, 1969.

276. Abdou, N.I. and Richter, M.,
M. Path and Microbiol. 37: 73, 1971.

277. Friedman, H.,
Transpl. Proc. 1: 574, 1969.

278. Hirano, S. and Uyeki, E.M.,
J. Immunol. 106: 619, 1971.

279. Raff, M.C.,
Nature, 224: 378, 1969.

280. Symes, M.O. and Riddell, A.G.,
Lancet 1: 1052, 1968.

281. Motta, R.,
Rev. Franc. Etudes Clin. Biol. 13: 713, 1968.

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

283. Winn, H.J.,
Ciba Foundation Symposium 122, 1961.

284. Richter, M., Kopersztych, S. and de la Noue, H.C.,
In preparation.

285. Gusdon, J.P. Jr.,
J. Reticulo Soc. 7: 441, 1970.

286. Pierce, C.W.,
J. Exp. Med. 130: 365, 1967.

287. Mishell, R.I. and Dutton, R.W.,
J. Exp. Med. 126: 423, 1967.

288. Henry, C. and Jerue, N.K.,
J. Exp. Med. 128: 133, 1968.

289. Wigzell, H.,
J. Exp. Med. 124: 953, 1966.

290. Cerottini, J.C., McConaley, P.J. and Dixon, F.,
J. Immunol. 3: 268, 1969.

291. Pearlman, D.S.,
J. Exp. Med. 126: 127, 1968.

292. Draper, L.R. and Owens, J.R.,
J. Immunol. 99: 142, 1967.

293. Talmage, D.W., Freter, G.G. and Taliaferro, W.H.,
J. Inf. Dis. 98: 241, 1956.

294. Riha, I.,
Folia Microbiol. 8: 1, 1963.

295. Haasz, R. and Richter, M.,
In preparation.

296. Abdou, N.I. and Richter, M.,
Immunol. 18: 833, 1970.

297. Daguillard, F. and Richter, M.,
J. Exp. Med. 130: 1187, 1969.

298. Sterzl, J., Veseley, J., Jilek, M. and Mandel, L.,
In Molecular and Cellular Basis of Antibody Formation
(Sterzl J. Ed.) Czechoslovak Acad. of Sciences
Prag. 1965.

299. Robinson, W.A., Mabrook, J. and Diener, E.,
J. Exp. Med. 126: 347, 1967.

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

301. Cross, A.M., Leuchare, E. and Miller, J.F.A.P.,
J. Exp. Med. 119: 837, 1969.

302. Nossal, G.J.V., Bussard, A.E., Lewis, H. and
Mazie, J.C.,
J. Exp. Med. 131: 894, 1970.

303. Bussard, A.E., Nossal, G.J.V., Mazie, J.C. and
Lewis, H.,
J. Exp. Med. 131: 917, 1970.

304. Segal, S. Globerson, A., Feldman, M., Haimovitch, J.
and Sela, M.,
J. Exp. Med. 131: 93, 1970.

305. Biozzi, G., Stiffel, C. and Mouton, D.,
Cancer and Chemotherapy (Mihich E. Ed.),
Acad. Press, 1967.

306. Biozzi, G., Stiffel, C., Mouton, D., Bouthillier, C.
and Decrenserond, C.,
Immunol. 14: 7, 1968.

307. Laskov, R.,
Nature 219: 973, 1968.

308. Stern, K. and Davidson, I.,
J. Immunol. 72: 209, 1954.

309. Stimpelling, J.H.,
J. Immunol. 85: 530, 1960.

310. Greaves, M.F. and Flad, H.D.,
Nature 219: 975, 1968.

311. Marbrook, J.,
Clin. Exp. Immunol. 3: 367, 1968.

312. Haskill, J.S., Byrt, P. and Marbrook, J.,
J. Exp. Med. 131: 57, 1970.

313. Mozier, D.E.,
J. Exp. Med. 129: 351, 1969.

314. Pierce, C.W.,
J. Exp. Med. 130: 345, 1969.

315. Cooper, M.D., Peterson, R.D.A. and Good, R.A.,
Nature 205: 143, 1965.

316. Carpenter, P.L.,
In Immunol. and Serology (Saunders W.B.),
Philadelphia Pa 1965.

317. Taliaferro, W.H.,
In reticuloendothelial structure and function.
(Heller, J.H., Ed.) Ronald Press Co., N.Y. 1960.

318. Richter, M.,
Proc. Nat. Acad. Sci. 66: 1127, 1970.

319. Bain, B., Vas, M. and Lowenstein,L.,
Blood 23: 108, 1964.

320. Bain, B. and Lowenstein, L.,
Science (Washington) 145: 315, 1964.

321. Wilson, D.B.,
J. Exp. Med. 126: 625, 1967.

322. Wilson, D.B., Silvers, W.K. and Nowell, P.C.,
J. Exp. Med. 126: 655, 1967.

323. Sullivan, C.E., Stevens, L.E., Main, R.K., Jones,
M.J. and Reemstma, K.,
Transpl. 11: 190, 1971.

324. Bach, F. and Hirschorn, H.,
Science 143: 813, 1964.

325. Rubin, A.L., Stenzel, K.H., Hirschorn, K. and Bach, F.,
Science 143: 815, 1964.

326. Nisbet, N.W., Simonsen, M. and Zaleski, M., J. Exp. Med. 129: 459, 1969.

327. Simonsen, M., Acta. Path. et Microbiol. Scand. 40: 480, 1957.

328. Simonsen, M., Sym. Quant. Biol. 32: 517, 1967.

329. Simonsen, M., Progress Allergy 6: 349, 1962.

330. Burnet, F.M. and Boyer, G.S., J. Path. Bact. 81: 141, 1961.

331. Simons, M.J. and Fowler, R., Nature 209: 588, 1966.

332. Szenberg, A., Warner, N.L., Burnet, F.M. and Lind, P.E., Brit. J. Exp. Path. 43: 129, 1962.

333. Coppleson, L.W. and Michie, D., Proc. Royal Soc. B. 163: 555, 1965.

334. Saleh, W.S., MacLean, L.D., Gordon, J. and Lamoureux G., Transpl. 8: 524, 1969.

335. Gordon, J. Saleh, W.S. and MacLean, L.D., Excerpta Medica Int. Congress Series 197: 185, 1970.

336. Rodey, G.E. and Good, R.A., Int. Arch. Allergy 36: 399, 1969.

337. Daguillard, F. and Richter, M., J. Exp. Med. 130: 1187, 1969.

338. Daguillard, F. and Richter, M., J. Exp. Med. 131: 119, 1970.

339. Greaves, M.F., Roitt, I.M. and Rose, M.E., Nature (London) 220: 293, 1968.

340. Lubaroff, D.M. and Waksman, B.H., J. Exp. Med. 128: 1425, 1968.

341. Lubaroff, D.M. and Waksman, B.H., J. Exp. Med. 128: 1437, 1968.

342. McGregor, D.D.,
J. Exp. Med. 127: 953, 1968.

343. Good, R.A. and Papermaster, B.W.,
Adv. Immunol. 4: 1, 1964.

344. McGregor, D.D., Koster, F.T. and Mackaness, G.B.,
J. Exp. Med. 133: 389, 1971.

345. Willard, H.G. and Smith, L.H.,
Transpl. 4: 56, 1966.

346. Lonai, P. and Feldman, M.,
Transpl. 10: 372, 1970.

347. Celada, F. and Carter, R.R.,
J. Immunol. 89: 161, 1962.

348. Hilgard, H.R.,
J. Exp. Med. 132: 317, 1970.

349. Barchilon, J. and Gershon, R.K.,
Nature 227: 71, 1970.

350. Argyris, B.F.,
Transpl. 8: 538, 1969.

351. Jacobson, L.O., Marks, E.K., Simmons, E.L. and Gaston, E.O.,
Proc. Soc. Exp. Biol. Med. 108: 487, 1961.

352. Kosunen, T.U.,
Immunol. 19: 117, 1970.

353. Liden, S.,
Acta Path. Microbiol. Scand. 70: 58, 1967.

354. Greaves, M.F., Torrigioni, G. and Roitt, I.M.,
Nature 222: 885, 1969.

355. Mason, S. and Warner, N.L.,
J. Immunol. 104: 762, 1970.

356. Davie, J.M. and Paul, W.E.,
Cell. Immunol. 1: 404, 1970.

357. Ramseier, H. and Lindemann, J.,
Path. et Microbiol. 34: 379, 1969.

358. Metzger, H.,
Ann. Rev. Biochem. 39: 889, 1970.

359. Singhal, S.K. and Wigzell, H.,
Progr. Allergy - in press.

360. Warner, N.L., Byrt, P. and Ada, G.L.,
Nature 226: 942, 1970.

361. Coombs, R.R.A., Feinstein, A. and Wilson, A.B.,
Lancet 11: 1157, 1969.

362. Lesley, J. and Dutton, R.W.,
Science 169: 487, .970.

363. Jones, G., Morcuson, E.C. and Roitt, I.M.,
Nature 227: 1051, 1970.

364. Coombs, R.R.A., Guyner, B.W., Janeway, C.A. (Jr.),
Wilson, A.B., Gell, P.G.H. and Kelus, A.S.,
Immunol. 18: 417, 1970.

365. Sell, S. and Gell, P.G.H.,
J. Exp. Med. 122: 423, 1965.

366. Di George, A.M.,
National Foundation, N.Y. 4: 116, 1968.

367. Kretchmer, R., Lay, B., Brown, D. and Rosen, F.S.,
N. Eng. J. Med. 279: 1295, 1968.

368. Meuwissen, H.J., Van Altan, Bach, F.H. and Good, R.A.,
National Foundation, N.Y. 4: 253, 1968.

369. Dukor, P. and Dietrich, M.F.,
Int. Arch. Allergy 32: 521, 1967.

370. Stutman, O., Yunis, E.J., Teague, P.O. and Good, R.A.,
Transpl. 6: 514, 1968.

371. Cohen, M.W., Thorbecke, G.J., Hochwald, G.M. and
Jacobson, E.B.,
Proc. Soc. Exp. Biol. Med. 114: 242, 1963.

372. Yunis, E.J., Hilgard, H., Sjodin, K., Martinex, C.
and Good, R.A.,
Nature (London) 201: 784, 1964.

373. Meuwissen, H.J., Van Altan, P.J. and Good, R.A.,
Clin. Exp. Immunol. 5: 155, 1969.

374. Richter, M., Haasz, R., de la Noue, H.C., Koperstych, S.
and Perey, D.Y.E. - In preparation.