Ph. D. Experimental Medicine

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THE FUNCTIONAL HETEROGENEITY OF THE LYMPHOCYTE

ABSTRACT

Rabbit and human lymphocytes were studied for their capacity to respond with blastogenesis and mitosis to various stimuli in vitro. The circulating lymphocytes of two agammaglobulinemic subjects responded to PHA but not to anti-immunoglobulin serum. The cells of all the rabbit lymphoid organs tested (thymus, bone marrow, sacculus rotundus, appendix, lymph node and blood) responded to stimulation with PHA and mitomycin-C inactivated leucocytes. Only the thymocytes failed to respond to stimulation with goat-anti-rabbit immunoglobulin serum (GARIG). They also adsorbed much less GARIG-I^{125}. Pretreatment of normal rabbit lymphocytes with GARIG and complement resulted in a loss of responsiveness to GARIG but not to PHA. Immune rabbit cells pretreated with GARIG could not respond to stimulation with antigen in vitro nor could they synthesize antibodies following their transfer into irradiated recipients. There was an additive effect in vitro between PHA and antigen or PHA and GARIG.

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OF THE LYMPHOCYTE

by

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CHAPTER I

GENERAL INTRODUCTION AND OBJECTIVES OF THE STUDY

The past decade has been witness to the establishment of the lymphocyte as the critical mediator of both humoral and cellular immunity and to a radical shift from the concept of the homogeneous nature of the lymphocytes to one of marked heterogeneity. Until recently, the lymphocytes were thought to constitute a relatively homogeneous population of cells in the animal body, albeit segregated into the different parenchymal lymphoid organs with only the size of the mature cell serving as a distinguishing criterion - the thymocyte being smaller than the lymphoid cells of the other lymphoid tissues. It has been demonstrated that, far from being even relatively homogeneous, the lymphocytes constitute an extremely heterogeneous population of cells, using the following non-immunologic considerations as criteria:-

a. The life span of the cell (short-lived versus long-lived lymphocytes),

b. The differential migratory pathways of the lymphoid cells of the various lymphoid organs,

c. The physical fractionation of the cells on a density gradient.

Most important from an immunologic point of view has been the recognition of the central or peripheral origin of the lymphocyte and the

dependency of this cell for its maturation on the thymus or the gut-associated lymphoid organs (the homologues of the Bursa of Fabricius).

It is now established that in the bird, the lymphocyte in the course of its differentiation may proceed along two separate pathways, one under the influence of the thymus and the other under the influence of the gut-associated Bursa of Fabricius: the former, the thymus-dependent lymphocyte, is responsible for the mediation of cellular immunity, i.e., delayed skin manifestations, graft-versus-host reaction and homograft rejection; the latter, the gut-dependent lymphocyte, gives rise to the antibody-producing cell.

Whatever line of differentiation it eventually takes, the potential immunologically competent lymphocyte, referred to as the immunocyte, must, when it becomes immuno-competent, acquire a specific recognition device through which it will eventually react with a predestined antigen. This receptor site is thought to assume the shape and composition of an antibody molecule similar to that which the cell will eventually synthesize. The demonstration by Sell and Gell that anti-immunoglobulin antisera could transform <u>normal</u> rabbit lymphocytes in vitro lends support to the assumption that antibody-like receptors must exist on the lymphocyte surface.

The mechanism whereby an antigen can stimulate the lymphoid cells of a <u>presensitized</u> individual or animal in vitro to undergo

blastogenesis and mitosis is also considered to involve the interaction of the antigen with an antibody receptor at the cell surface.

Several other substances are known to stimulate lymphocytes in vitro, namely phytohemagglutinin, pokeweed, streploysin S, staphylococal filtrate, antilymphocyte serum and allogeneic leucocytes. These so-called general stimulants are thought to stimulate all the lymphocytes and little is known about the receptor sites with which they may interact. However, the concept that functionally different lymphocytes might be distinguished on the basis of their responses to the different mitogenic substances is an attractive one. It is indeed the purpose of this study to demonstrate that the lymphocytes of man and rabbits can be differentiated on the basis of their response to specific antigens, PHA, anti-immunoglobulin serum and allogeneic and xenogeneic leucocytes. The results presented in this investigation leave no doubt as to the functional heterogeneity of the lymphocyte.

CHAPTER II

HISTORICAL REVIEW

1 FUNCTIONAL DIVISION OF THE IMMUNE SYSTEM

Probably one of the most important advances in the field of immunobiology has been the recognition of the role of the central lymphoid organs (i.e., thymus and Bursa of Fabricius) on the development of the immune system and the division of the immune system into two separate humoral and cellular branches.

1.1 THE "ANIMAL" EXPERIMENTAL MODEL

Following the observation by Glick (1) in 1956 that antibody production is decreased in neonatally bursectomized chickens, a series of elegant investigations established the role of the bursa as a "central lymphoid organ" in the expression of the immune response (2-4). At the same time, the studies of Miller and Good (5-8) established the role of the thymus as a central lymphoid organ, capable of influencing both humoral and cellular immunity in mice and rabbits (5), but responsible solely for the development of cellular immunity in the chicken (4).

The central lymphoid organs exert their function either by populating the peripheral lymphoid organs directly with immunocompetent cells, by favoring the differentiation of immigrant lymphoid stem cells which subsequently reside in the peripheral lymphoid organs (spleen and lymph node), or by exerting a humoral influence on cells situated elsewhere. The exact participation of each one of these mechanisms will be reviewed in greater detail.

Results of studies based on the migration of cells in parabiotic animals (9), repopulation of thymus grafts (10-12), repopulation of the thymus in irradiated recipients (13), and the location of chromosomally-marked cells in the intact animal (14) have established that lymphoid cells enter the thymus from without. On the other hand, although there is still a lack of evidence for large scale cell migration from the thymus to the peripheral lymphoid organs (15), several experiments indicate that thymus cells enter the spleen and lymph node. Ford and his colleagues, working with mice united in parabiosis, have shown that the lymph nodes of these mice contained fewer lymphocytes bearing the marked chromosome of their partner if the latter had been thymectomized before the establishment of parabiosis (9). Studies utilizing chromosomally-marked thymus grafts have indicated that a proportion of cells in the spleen (10, 16), lymph node (11) and peripheral blood (17) of

mice are of thymus origin. Using a less sophisticated method (migration of thymic lymphoid cells labelled in situ with tritiated thymidine), other investigators (18-21) have also demonstrated that a small number of thymocytes migrate to lymph node and spleen.

It has also been demonstrated that there is a traffic of cells through the bursa. Chromosome studies of bursal cells from chicken embryos of different sex united in parabiosis indicate that blood borne cells enter the bursa in high proportion (22, 23). A transport of cells from the bursa to the periphery has also been reported (24).

The origin and nature of the cells which migrate to the central lymphoid organs or mature under their influence are still uncertain. Certain experimental findings, however, indicate that some of these cells are of bone marrow origin. It has been demonstrated that bone marrow cells enter the thymus in significantly larger numbers than do other lymphoid cells (9, 13). Also, experiments using irradiated mice suggest that immunologically-competent cells can develop from precursors in the bone marrow under thymus influence (16, 25-35).

1.2 THE "HUMAN" EXPERIMENTS OF NATURE

More than the experiments of the laboratory, almost two decades of alert clinical research have contributed to the recognition of the dicothomy of the immune response (cellular versus humoral).

Since the description of the first case of agammaglobulinemia in 1952 by Bruton (36), the study of a variety of clinical syndromes, now referred to as immunologic deficiencies, has added to our knowledge of the complexity and heterogeneity of factors and cells involved in immune mechanisms. For the sake of clarity we will not discuss the chronology of the discovery of the different clinical entities but rather present them in a systematic way since it now seems established that they can be divided into three distinct categories: cellular immune defects, humoral immune defects and combined (humoral and cellular) defects.

Nezelof (37) described in 1964 a case of congenital immunologic deficiency characterized by thymic hypoplasia in the presence of plasma cells and normal serum immunoglobulins; this case is similar in many respects to the one described one year later by DiGeorge (38, 39) except that the congenital absence of the thymus in the DiGeorge syndrome is accompanied by the absence of the

parathyroid glands. This double absence is caused by a common embryological defect, for both thymus and parathyroid glands derive from the third and fourth pharyngeal cleft pouches of the embryo. Seven cases of the DiGeorge syndrome have been reported in the literature to date (40-42). In two cases, foetal thymus transplantations seem to have restored immunological functions to the patients (42, 43). The immunologic status of these children closely resembles that of the thymectomized chicken, in that humoral immunity is intact with immunoglobulins present in the serum in normal amount and adequate antibody formation is evident. On the other hand, these children cannot manifest or acquire delayed hypersensitivity to common antigens nor can they reject skin allografts. Histological studies of the lymph nodes of these children (41) showed that the deep cortical area, the thymus dependent area of Parrot et al (44), was hypocellular; however, the follicules were well formed and the medulla contained a normal number of plasma cells.

The immunologic defect described by Bruton (36) as well as the other forms of congenital and "primary" acquired agammaglobulinemia, all of which may be classified under the heading "antibody deficiency syndrome" (45), look very much like the defect which follows bursectomy of the chicken (46). Those affected by this syndrome exhibit an impairment of humoral immunity but their cellular immune reactions by and large appear to be normal.

Which lymphoid organ possesses bursal function in man is still unclear. The work of Good and his colleagues (46) indicates that the gut-associated lymphoid organs (Sacculus Rotundus, Peyer's patches and appendix) may have this function in the rabbit.

The "Swiss type agammaglobulinemia" described in 1957 by Cottier (47) displays an impairment of both cellular and humoral immunity. The children suffering from this disease have a severe lymphopenia and a very low level of circulating gamma globulin. The absence of a common precursor cell (stem cell) for the lymphocytes mediating humoral and cellular immunity has been suggested as an explanation for the pathogenesis of this disease (48, 49). Hitzig (50) has recently reviewed seventy cases which were all characterized by very low levels of all three immunoglobulins (γG, γM and γA), severe lymphopenia, absence of delayed hypersensitivity and impairment of graft rejection. An attempt to restore immunologic function was successful in one of these patients by utilizing blood buffy coat and bone marrow cells of an immunocompetent sibling (51).

A comparison of the immunologic functions in the various immune-deficiency states is presented in Table I. Figure 1 presents, in diagrammatic form, the functional differentiation of the immunocytes in man and animal.

It indeed seems that a precursor stem cell, possibly of bone marrow origin, may differentiate under the influence of the central lymphoid organs to generate two separate lines of immunocyte, respectively responsible for cellular or humoral immunity.

TABLE 1

IMMUNOLOGIC FUNCTIONS IN IMMUNOLOGIC DEFICIENCY SYNDROMES

SYNDROME	HUMORAL FUNCTIONS		CELLULAR FUNCTIONS	
	Immunoglobulins	Antibody Response	Delayed Hypersensitivity	Graft Rejection
HUMORAL DEFECTS Congenital agammaglobulinemia Acquired (primary) agammaglobulinemia	very low	none	normal	normal
CELLULAR DEFECTS Alymphocytosis (Nezelof syndrome) Thymic aplasia (DiGeorge syndrome)	normal	normal	absent	delayed or absent
COMBINED CELLULAR AND HUMORAL DEFECTS Alymphocytic agammaglobulinemia (Swiss type agamma) Congenital thymic dysplasia	very low	none	absent	absent

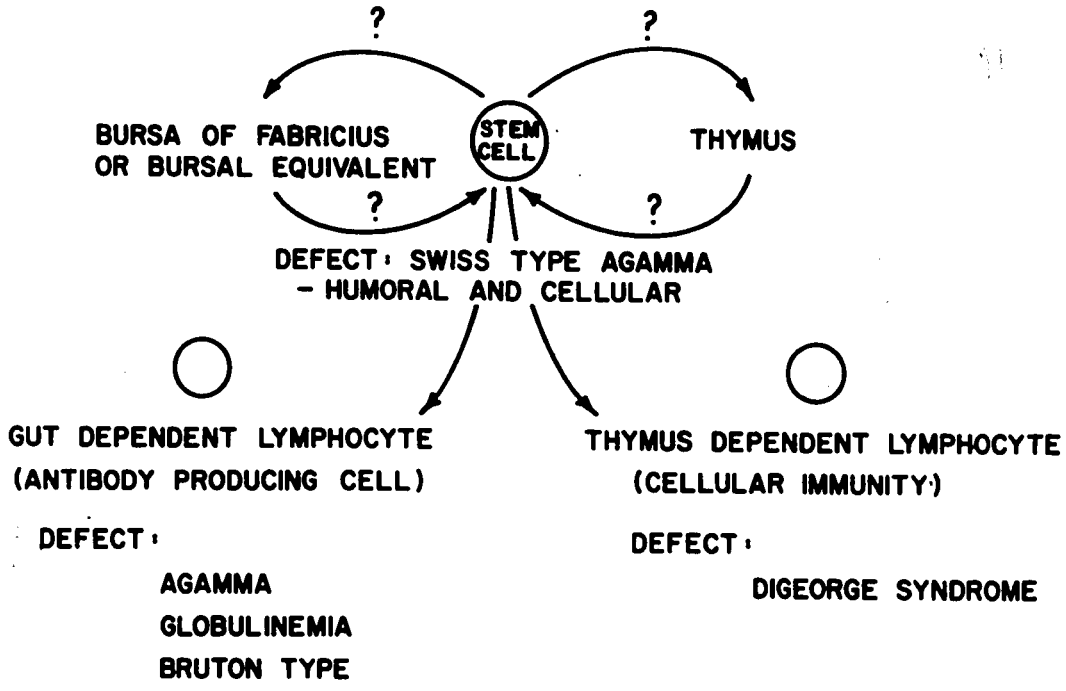


FIGURE 1

LYMPHOCYTE ABNORMALITIES AND IMMUNOCYTE MATURATION: THE RELATION BETWEEN THE CENTRAL LYMPHOID INFLUENCE ON THE MATURATION OF THE STEM CELL AND THE IMMUNE DEFICIENCY RESULTING FROM FUNCTIONAL DEFECT(S) IN THE MATURATION PROCESS. A DIAGRAMATIC REPRESENTATION.

2 THE IMMUNOCOMPETENCE OF LYMPHOID ORGANS

This section of the historical review will be concerned with the manifestation and acquisition of immunocompetence by different lymphoid cell populations.

Whatever form the immune response may take (cellular or humoral) the main characteristics of an immunocompetent organism is its ability to discriminate between the self and the non-self, and its enhanced ability to recognize a previously encountered antigen (52).

Generally, immunocompetence is thought to occur during the maturation and differentiation of the immunocyte through the acquisition of a specific device allowing for the recognition of the antigen (53, 54).

We will briefly review here:

a. the capacity of central and peripheral lymphoid organs or cells to manifest immunocompetence,
b. the influence of the central lymphoid organs on the acquisition of immunocompetence by peripheral lymphoid organs and,
c. the evidence in favor of the existence of a specific antigen-recognition site on the cell surface and its nature and characteristics.

2.1 MANIFESTATIONS OF IMMUNOCOMPETENCE

The immunocompetence of organs or cell populations has generally been demonstrated in the following ways:

a. production of antibody in vitro by cell suspensions or fragments of the organ,

b. transfer of the capability to produce antibodies or reject graft to an immuno-incompetent recipient and,

c. production of graft-versus-host reaction in a non-responsive host.

A graft-versus-host reaction can be evaluated either by the production of lesions on the chorioallantoic membrane of the chicken (Simonsen model), by a local lesion in the kidney at the cell injection site (Elkin's tumor) or by the production of runt disease and evaluation of the spleen size (spleen index) of the runting animal.

If the concept expressed in Figure 1 holds true one would expect the organ source of the stem cells (presumably the bone marrow) or the central lymphoid organs to contain a lesser number of the fully immunocompetent cells than the peripheral lymphoid organs.

2.1.1 PERIPHERAL LYMPHOID ORGANS

We will consider here, separately and in combination, the immunocompetence of lymphocytes obtained from the spleen, lymph node, blood, thoracic duct and peritoneal exudate. Such a consideration

would appear to be legitimate since it has been shown that small lymphocytes recirculate between the lymph and the blood through the spleen and lymph node (55).

A primary antibody response has been induced in vitro upon culture of lymph node, spleen or peritoneal exudate cells with the antigen (56-69). Antibody production has also been shown in vitro upon incubation of lymph node or spleen of animals previously immunized in vivo (70-100). Upon in vitro rechallenge with the specific antigen, an enhanced production of antibody (secondary response) is often observed (101-117).

Cells from lymph nodes, spleen, peritoneal exudate, peripheral blood or thoracic duct, when transferred to irradiated recipients, confer to the host the capacity to produce antibodies (118-135). Lymph node, spleen, thoracic duct and peripheral blood cells can also transfer the capacity to reject an allograft (136, 137, 299, 300) or can mediate a graft-versus-host reaction in a non-responsive host (138-145).

2.1.2 CENTRAL LYMPHOID ORGANS

Although it has been possible to detect immunoglobulins in tissue cultures of the thymus gland (146-148), production of specific antibodies has not been shown in vitro upon culture of

thymus fragments or thymus cell suspension obtained from normal or immunized animals (146-150). Normal thymocytes injected with antigen into irradiated rabbits or mice do not confer the capacity to form antibody (28, 35, 119, 146). A weak response or no response has been reported following transfer of immune thymocytes to irradiated animals (146, 151). Recently, Chaperon et al confirmed these results and reported the appearance in the mouse thymus late after the injection of antigen (131) of a small number of cells capable of producing 7 S antibodies and of transferring the capacity to synthesize humoral antibody. They interpreted their results as being caused by a small number of recirculating immunocompetent cells.

The ability of thymocytes to induce graft-versus-host reaction has also been assessed. Thymus cells were found to be less effective in this function than spleen and lymph node cells (152), but in general, when a sufficient number of cells is transferred thymocytes can repeatedly cause chorioallantoic lesions or runting (153-160).

Very few studies have concerned themselves with the immunocompetence of bursal cells: in general these cells have been found to be unable to produce antibodies (161) to cause graft-versus-host reactions (159, 160).

2.1.3 THE BONE MARROW

Normal bone marrow has been shown to contain plasma cells which, upon incubation in vitro can synthesize immunoglobulins (147). Singhal and Richter were able to induce the appearance of antibody producing cells, detectable by immunofluorescence (162) in normal rabbit marrow cultured in vitro. Marrow removed during the course of the primary immunization of mice could not form antibody in vitro (163). However, after several injections of antigen to an animal, the bone marrow cultured in vitro synthesized specific antibodies (80, 164).

As mentioned earlier, bone marrow requires thymus collaboration in order to confer to lethally irradiated mice the capacity to form antibodies (16, 26, 28, 31-35). On the other hand, irradiated rabbits injected with only bone marrow cells possess plaque-forming cells in their spleens seven days following cell transfer and immunization (165). These plaque-forming cells have been found to be of host origin (166). This latter finding suggested to the authors that rabbit marrow contains antigen reactive cells but no antibody forming cells.

Soon after the injection of antigen and during the early course of the primary immune response, mouse marrow cannot transfer to irradiated recipients the capacity to form antibodies (131, 167).

However, late in the primary response this capacity appears in the bone marrow (131, 167). The late appearance of this capability and the type of cells found to be engaged in antibody production (plasma cells alone in the bone marrow as opposed to small, medium-size and large lymphocytes as well as plasma cells in spleen and lymph nodes) prompted several authors to speculate that the cells producing antibodies were from without but recirculated through the bone marrow (131, 147, 163).

Mouse marrow without thymus collaboration cannot correct the deficient capacity of irradiated mice to reject a graft (25, 29). However, although they are very weak mediators of the graft-versus-host reaction in non-responsive rodents (168), bone marrow cells have repeatedly been able to produce this phenomenon in primates, including man (168-170).

Whatever form the direct manifestations of immunocompetence by bone marrow cells may take the likelihood of contamination with cells from without must be kept in mind when evaluating the results, particularly in view of the demonstration of an interchange of lymphocytes between marrow and blood (171).

In conclusion, it seems plausible to state that immune competence is more fully manifested by cells in the peripheral lymphoid organs (lymph node, spleen, thoracic duct and blood) than in the bone marrow, thymus or bursa.

2.2 ACQUISITION OF IMMUNE COMPETENCE: INFLUENCE OF CENTRAL LYMPHOID ORGANS

The experimental models used to demonstrate the influence of the central lymphoid organs on the acquisition of immunocompetence by an organism are essentially similar. Most encompass one or more of the following procedures:

a. removal of the organ early in the life of an animal (neonatal or prenatal period),

b. removal of the organ in the adult animal with or without total body irradiation, and

c. grafting of the central organ into an immunodeficient animal.

These investigations have helped to demonstrate that, in the chicken, mouse, rat and rabbit, the central lymphoid organs (thymus and bursa or bursal equivalent) definitely influence the immunocompetence of the animals.

2.2.1 THE THYMUS AS A CENTRAL LYMPHOID ORGAN; ROLE AND INFLUENCE

2.2.1.1 EFFECT OF THYMECTOMY

Thymectomy of the chicken in the neonatal period produces marked impairment of cellular immune functions: the rejection of skin homograft is delayed (4, 172, 173) and the capacity of these

animals to manifest delayed type skin reactions or initiate graft-versus-host reactions is depressed (4). In general, thymectomized chickens show a normal level of circulating immunoglobulins and produce adequate amounts of antibodies in response to most antigens (4, 173).

The effect of thymectomy in the mammal, however, is not limited only to the cellular functions. Neonatally thymectomized mice tolerate allografts (5-7), are more susceptible to runting and death following the injection of histo-incompatible spleen cells (5) and cannot induce graft-versus-host reaction (5, 27). They also exhibit diminished antibody response to several antigens (5, 7, 163). However, the diminished antibody response is not directed toward all antigens for it has been reported that the responses of these mice to ferritin, hemocyanin, T4 phage and pneumococcal polysaccharide are normal (174, 175).

The response of neonatally-thymectomized mice to sheep erythrocytes (SRBC) deserves special mention. The antibody response to SRBC is decreased and/or delayed following neonatal thymectomy (27, 176, 177). However, a 100-fold increase in antigen dose elicits in these mice an antibody response equal to that of sham thymectomized controls (177).

The circulating lymphocytes of neonatally thymectomized mice are also less capable, when injected with antigen into lethally irradiated recipients, of transferring hemolytic plaque forming capacity (27).

Neonatally thymectomized rats (178) show markedly impaired delayed hypersensitivity and fail to develop significant titers of antibody.

Thymectomy in early life decreases also the capacity of the rabbit to form antibodies (5, 179, 180) but does not seem to cause a delay in the rejection of skin homograft (5).

Adult thymectomy has little effect on the immune response of mice to antigens (181, 182). However, 6 to 10 months after thymectomy, both the ability of mouse spleen cells to induce graft-versus-host reactions and to form hemolytic plaques in response to SRBC stimulation is markedly decreased (181). Adult thymectomy combined with whole body irradiation affects immune capacities more quickly. As for the neonatally thymectomized mouse, a 1000-fold increase in antigen concentration is necessary to reach the number of plaque-forming cells produced in the non-irradiated non-thymectomized mouse (183).

Large doses of irradiation necessitates the injection of liver or marrow cells to permit survival of the host (301). Irradiated, thymectomized mice protected with bone marrow show a marked inability

to reject grafts (25, 29, 184) or to produce antibodies (26, 35, 184). Furthermore, their spleens contain less antigen reactive cells, i.e., cells which upon transfer with antigen to irradiated recipients allow production of antibodies (26).

In young rabbits, thymectomy followed by irradiation caused a distinct diminution of antibody response to conalbumin, but a normal response can be elicited with Salmonella H antigen (185).

Thymectomy of adult rats seems to have little effect on their immunologic functions even several months later; however, lethal irradiation of thymectomized rats depresses both delayed skin manifestations and antibody formation (186).

2.2.1.2 THYMUS GRAFT: INFLUENCE AND ROLE

Generally, thymic grafts have been able to restore wholely or partly impaired immunologic functions caused by thymectomy. The following types of grafts and grafting procedures have been used:

a. Isologous or homologous thymus grafts placed subcutaneously or beneath the kidney capsule can restore both cellular and humoral functions of neonatally thymectomized mice (8, 187);

b. A thymus graft in a millipore diffusion chamber placed intraperitoneally can restore the hemolysin response to sheep erythrocytes of neonatally thymectomized mice (188) and the ability of these

mice to reject foreign grafts within the normal rejection period (189). Homologous thymic tissue in millipore chambers can also restore the ability of neonatally thymectomized rats to manifest delayed type hypersensitivity (190) and the capacity of neonatally thymectomized rabbits to produce antibodies to human γ globulin (191);

c. Xenogeneic (calf) or allogeneic thymic extracts can also increase the antibody-forming capacity of neonatally thymectomized mice (192) or the capacity of their spleen cells to cause graft-versus-host reactions (193);

d. Thymus cells in suspension have been injected into thymectomized animals. However, this form of immunologic restoration is generally less effective than if thymus is given as a whole organ graft (8, 194);

e. Thymus cells injected together with antigen (SRBC) into neonatally thymectomized (28) mice or to thymectomized-irradiated adult mice protected with bone marrow (35) restores the plaque-forming capacity of their spleens.

The potentially different mode of action of each one of these forms of restoration immediately brings up the question of the nature of the thymus influence which has briefly been discussed earlier (Chapter 1.1).

The fact that thymus graft in a millipore chamber or thymus extracts are effective indicates that the thymus can exert its action

via a humoral factor, possibly a hormone. The demonstration that cells from without (9-14) can enter the thymus and that thymic cells seed in the periphery (9-11, 16-19) does not rule out the possibility that cells immigrating into the thymus benefit in situ from this hormonal action. The accepted idea that the thymus graft helps in the maturation and differentiation of a stem cell, probably of bone marrow origin, finds support in the following evidence:

a. thymectomized irradiated mice protected with marrow cells never recover immune capacity; however sham-thymectomized irradiated controls protected with the same number of marrow cells recover their immune capacity (25, 26, 29, 31);

b. fully differentiated immune cells of thoracic duct, spleen and lymph node, but not marrow, can restore the immunologic functions of neonatally thymectomized mice or adult thymectomized irradiated mice (25, 27, 33).

The activity of thymus cell suspensions injected along with antigen (SRBC) into neonatally thymectomized mice (28) or into irradiated-thymectomized adult mice reconstituted with bone marrow (35) brings up another aspect of the thymus-marrow interaction. The work of Davies and others using chromosomally marked thymus grafts have established that thymus cells (16, 30) multiply in response to antigen but do not engage in antibody production (28, 32). This latter function is restricted to the bone marrow cells (28, 32). The nature

of the participation of thymus cells in this system is not clear although some experiments of Miller (showing that antigen has to react with thymus cells first in order to be effective), indicate that these cells operate as antigen-reactive cells. It is known that the lymphocytes in a thymus graft in a marrow-protected irradiated host is soon replaced by cells from the innoculated marrow (195). It is possible that the bone marrow cells which traffic through the thymus (thymus derived cells) or mature in the peripheral organs under the influence of the thymus (thymus dependent lymphocytes) may both have the same function in the mouse: the capacity to react with antigen and later influence effector cells, also of bone marrow origin.

There is no indication up to now that the bone marrow effector cells which confer the capacity to form antibody (plaque formation) to mouse spleen cells mature under the influence of another central lymphoid organ.

In summary, it may be said that, in the mammal (particularly the mouse), thymic functions seem to encompass both humoral and cellular branches of the immune system. This is in contrast with the situation in birds where the thymus seems to influence only cell-mediated immunity. In mice, thymus derived and/or dependent lymphocytes do not form antibody but influence antibody production in a so far undefined manner.

2.2.2 THE BURSA OF FABRICIUS: ROLE AND INFLUENCE

2.2.2.1 EFFECT OF SURGICAL OR HORMONAL BURSECTOMY

The role of the bursa has been elucidated using chickens which have been surgically bursectomized at an early age (1) or which have been reared from eggs that had been injected with testosterone derivatives (196); this latter treatment of the eggs results in a failure of the bursa to develop.

Glick and co-workers (1, 2) in 1956 reported that hens that had been surgically bursectomized at an early age were very susceptible to infection by Salmonella typhimurium and did not produce any detectable antibody. If coupled with total body irradiation, bursectomy at hatching is more effective in reducing the immunoglobulin level and antibody formation to several antigens (4). Mueller et al (3) did not detect any precipitins to bovine serum albumin (BSA) at six weeks of age in hormonally bursectomized chickens. Pierce et al made a similar observation and also noted that the gamma globulin in these chickens did not attain normal levels (197). It has also been possible to bursectomize chickens as embryos and demonstrate subsequent defects in both circulating immunoglobulins (198) and antibody synthesis (199). Recently, Cooper et al (199) have compared the effect of surgical bursectomy at hatching or during the late stages of embryonation: bursectomy was more effective at

day 17 of embryonic life than at day 19 or at the time of hatching (199). They interpreted their results as indicating that the bursa seeds cells committed to become plasma cells to the peripheral tissues.

Bursectomized chickens reject skin homografts within a normal period of time (4, 172, 173, 200). Similarly, they exhibit normal delayed reactions to tuberculin (201), and can initiate graft-versus-host reactions (4, 200). In contrast with neonatal or prenatal bursectomy, late bursectomy (at 10 weeks or later) does not affect antibody production (200, 202).

2.2.2.2 BURSAL GRAFT: INFLUENCE AND ROLE

Attempts to restore bursal functions by grafting have not always been gratifying. Autologous bursal cells or grafts were found to have no effect on immunologic restoration of bursectomized chickens (200, 302) although one study indicates that homologous bursal grafts can partly restore the capacity of bursectomized chickens to produce antibodies against sheep red blood cells (203).

Extracts from the bursa have restored only slightly (204) or not at all (205) the capacity of bursectomized chickens to form antibody.

Earlier studies (206, 207) had indicated that bursa fragments in millipore diffusion chambers can restore the antibody

forming capacity of bursectomized birds. However, more recent work has cast doubt on these results and suggests that the enhanced antibody production could have been caused by bacterial products released from contaminated diffusion chambers (303).

The failure to demonstrate a clear humoral effect of the bursa and also the demonstration that bursal cells seed to the periphery (24) have brought several authors to conclude that the bursa exerts its function by a direct seeding of cells (199, 205).

2.2.3 THE BURSAL EQUIVALENT IN THE MAMMAL

Limited experimental data suggest that the gut-associated lymphoid organs (appendix, Sacculus Rotundus and Peyer's patches) may play a role in the rabbit analogous to that of the chicken bursa. Sutherland et al (180) reported that the production of antibody to bovine gamma globulin was lowered in neonatally thymectomized and/or appendectomized rabbits. If neonatal appendectomy is accompanied by the removal of the Sacculus Rotundus and the Peyer's patches, the antibody response to several antigens as well as the level of circulating immunoglobulins are depressed (208). Young rabbits irradiated following the removal of their appendix (185) or their appendix plus Sacculus and Peyer's patches (210) also produce less antibodies following immunization.

If the neonatal removal of these organs is followed by lethal body irradiation, necessitating reconstitution with rabbit fetal liver cells to keep these animals alive, the antibody response to brucella abortus is completely suppressed (211).

Removal of the gut-associated lymphoid organs of the rabbit with or without irradiation does not affect the graft rejection or delayed skin hypersensitivity reactions (208, 210).

From the above experiments, it may be concluded that the intestinal lympho-epithelial tissues of the rabbit apparently play a central lymphoid function and influence to a certain extent the differentiation of the immunocyte capable of producing antibodies.

In man, there have been repeated observations of intestinal dysfunctions in the antibody deficiency states. Numerous reports have indicated a high incidence of steatorrhea in hypogammaglobulinemic patients (45). Some authors have also described the association of hypogammaglobulinemia with lymphoid hyperplasia of the small intestine (212-217). This certainly is not enough to lead to the conclusion that the intestinal lympho-epithelial organs of man influence plasma cell differentiation and antibody production.

However, it is established that in man, as in the chicken, the cellular and humoral immune functions are clearly distinct. This distinction is not so clear in mice, rats and rabbits.

2.3 IMMUNE COMPETENCE AND ANTIGEN RECOGNITION; CHARACTERISTICS OF THE RECEPTOR FOR ANTIGEN AT THE CELL SURFACE

That immunocompetent cells carry "specific devices" allowing for the identification of the antigen against which they will eventually react is a necessary corollary of Burnet's theory of cellular precommittment (52).

These receptor sites must be situated on the outer membrane of the cells since it has been possible, using glass bead columns coated with a given antigen, to isolate the immunocytes capable of reacting specifically with that antigen from a normal or immune cell population (218-220). These receptors are assumed to be antibody molecules (53, 54), since the only biological structure known to react specifically with antigen is an antibody; it is therefore reasonable to assume that the specific antigen-recognition site at the cell surface has partly or wholly the conformation and characteristics of the molecule that the cell will be induced to produce. This section will deal with studies concerned with the existence of an antibody receptor on the lymphocytes. We will, however, mention findings which conflict with this concept.

2.3.1 LYMPHOCYTE AND ANTIBODY RECEPTOR

The evidence for the existence of an antibody receptor on

the lymphocyte surface is of great importance in view of the critical role of this cell in both cellular and humoral immunity. Small lymphocytes can initiate the homograft rejection (221), cause graft-versus-host reaction (135) and mediate both primary and secondary humoral immune responses (135, 222, 223).

Antibody has been found to be associated with lymphocytes, using several methods. Lymphocytes have been shown to exhibit specific fluorescence when examined by fluorescence microscopy using fluorescein-conjugated anti-immunoglobulin antiserum, thus indicating the presence of cell-bound immunoglobulins (147, 224, 225). Specific adherence of bacteria (226), red cells (227, 228) or antigen coated red cells (229) to immune lymphocytes has also been demonstrated using the immunocytoadherence technique. Rosette formation by immune lymph node cells in the presence of the specific antigen (red cells or antigen-coated red cells) could be inhibited by prior treatment of the cells with anti-immunoglobulin sera or with free antigen (228, 229).

However, these studies do not prove that the antibodies present on the lymphocyte are antigen recognition receptors. They may well be molecules of antibody produced and not yet released by the antibody-producing cells. This consideration is worth noting in view of the demonstration by agar plaque (230, 231) and micro-drop studies (232) that individual small lymphocytes may produce

antibodies. The possibility also exists that antibodies formed by other cells and released into the circulation can, due to their high affinity for leucocytes, be firmly bound by different cells. So-called cytophilic antibodies were first described by Boyden (233) in rabbit immune sera. They bind to the surface of the cells of non-sensitized animals and confer to these cells the capacity to retain antigen. Cytophilic antibodies have also been described in the sera of several other animal species (234-236). In the guinea pig, they are found in the 7S fraction of the serum and show great predilection for the macrophages to which they attach by their Fc fragment (234, 235). Recently, Fidalgo and Najjar described leucophilic antibodies in dog sera; these could be isolated in one fraction of a chromatographic separation and bind mostly to polymorphonuclear cells (236).

The cytophilic antibody help in the capture and phagocytosis of the antigen (234-236) and by so doing can enhance the processing of the antigen. This can explain partly the role of the macrophage in the immune response (238-241). However, cytophilic antibodies only passively sensitize cells and there are no indications that they are actually produced by these cells.

Direct evidence in favor of the antibody nature of the receptor at the lymphocyte cell surface is provided by the demonstration by Mitchison (242) that pretreatment of immune lymphocytes with anti-immunoglobulin antisera could inhibit the synthesis of specific

antibodies by cells exposed to the specific antigen before their transfer to irradiated hosts. Merler and Janeway (243) were able to isolate from immune lymphocytes, by mercaptoethanol treatment, material possessing properties of immunoglobulins and recognition sites for the antigens to which the donors had been immunized. Other evidence in favor of an antibody receptor on the lymphocyte surface is the demonstration by Sell and Gell that anti-immunoglobulin sera could transform lymphocytes (244-246). We will elaborate on this finding in Chapter 4.1.3.

Results of several studies using competitive inhibition either by antibodies or by antigen also indicate that the receptor has the characteristics and properties of an antibody:

a. A primary immune response can be suppressed by immune sera injected to the animal (247-250). More avid sera (late antisera) have in general been found to be more active. The antibody is suppressive probably by covering the antigenic determinant, for antibody to one antigenic determinant is not suppressive for another antigenic determinant on the same molecule (251).
b. Sensitized lymphocytes can be stimulated in vitro by hapten-protein conjugates which were used to immunize the donor animals; an excess of free hapten can inhibit this in vitro stimulation (242, 252, 253).
c. Free antigen prevents attachment of normal precommitted cells or immune cells to glad bead antigen coated columns (218-220).

d. The ability of an antigen to stimulate immune guinea pig lymphoid cells to undergo blastogenesis in vitro is directly related to its ability to bind to lymphoid cells; the affinity of the receptor site for the antigen is closely related to the affinity of the antibody produced (254, 255). These studies imply that the receptor resembles antibody in its binding properties.

2.3.2 RECOGNITION WITHOUT ANTIBODY RECEPTOR

The arguments against the existence of a specific antibody receptor on the cell surface are generally those which challenge Burnet's clonal selection theory. Simonsen and others have presented data which seem incompatible with single cellular committment, in that cells capable of responding against a particular histo-incompatibility antigen with a graft-versus-host reaction appear to be present in a random sample of as few as 50 donor lymphocytes (143, 256, 257). That such a relatively high percentage (2%) of the lymphocytes of an animal could react with a single representative of the antigenic spectrum was deemed to disprove the Burnet clonal selection theory.

The study of progenitor cells capable of forming antibodies has revealed however that these cells represent a very small proportion of the spleen cells. Several investigators have estimated

the number of progenitor cells capable of responding to sheep red cells in a non-primed animal at 1 per 10^6 (128, 258, 259). A comparable number of cells were found to produce allohemolysin following injection of allogeneic white cells to mice and hamsters (260). The high percentage of cells (2%) capable of responding to a histo-incompatibility antigen seems to be a characteristic of the cellular, rather than the humoral, response toward that antigen. The great number of cells found to be capable of responding in vitro to a strong histo-incompatibility antigen as demonstrated in the mixed leucocyte culture technique (261, 262) tends to favor this idea. This will be discussed in greater detail in Chapter 4.1.4.

The second argument which challenged the concept of an antibody receptor at the cell surface as the sole mediator of antigen recognition derived from studies on the nature and characteristic of the transfer factor in man.

Lawrence in 1955 (263) demonstrated that extracts of hypersensitive leucocytes could transfer specific delayed type hypersensitivity (transfer factor). Fractionation of the active dialyzable principle suggested a molecular weight of less than 10,000 (264). The low molecular weight of the transfer factor indicates that the active component is not an antibody molecule. The possibility that transfer factor is an informational molecule, i.e., messenger RNA, is unlikely

because of its resistance to RNAse (265). Moreover, the expected molecular weight of a messenger RNA which could code for only the light chain of an immunoglobulin was calculated to be in the order of 250,000 (266, 267). Transfer factor has been found to produce its effect within 6 hours after transfer at skin sites previously injected with the allergen (265). We can hardly envision how, within such a short period of time, the de novo production of an antibody molecule could be induced capable of conferring to non-sensitized cells reactivity for an antigen.

In conclusion, it can be stated that most of the arguments challenging the existence of a specific receptor on the outer membrane of the cell as a prerequisite for antigen recognition are based on experiments testing the cellular, rather than the humoral, parameters of the immune response. In the light of our own findings, we will elaborate more on this point in the discussion.

3 IMMUNE COMPETENCE OF THE SEPARATED LYMPHOID CELLS

Several attempts have been made to correlate the presence of a certain population of lymphoid cells with a given function. These studies have contributed to demonstrate the heterogeneity of lymphocytes.

3.1 SIZE AND FUNCTION

The small lymphocyte constitutes about 95% of all lymphocytes, the larger ones - medium, intermediate or large as the case may be - constituting the remaining 5%. Several of the methods which we will mention here have in fact been used to separate small lymphocytes from large and medium-sized ones. However, as properties other than size may have influenced this segregation, we will consider separately the results obtained by different methods. Here, we will deal with non-manipulated lymphocytes, identified as large, medium or small by the microscope. There have been attempts to correlate the size of the lymphocyte with certain electron microscopic features thought to be suggestive of a given function. An endoplasmic reticulum, which seems to indicate the capability to secrete proteins, has been seen in large, intermediate and small lymphocytes (230, 231, 268). On analysis of 1000 lymphocytes obtained from the thoracic duct fluid of 5 human subjects, Zuker-Franklin observed that all the cells

classified as intermediate cells (2%), the few plasma cells (0.3%) and 12% of the remaining small lymphocytes showed a considerable amount of endoplasmic reticulum (268). As all of the intermediate sized lymphocytes contained endoplasmic reticulum and also since the number of intermediate cells in the thoracic duct lymph correlated well with the number of cells staining for γ globulin by immunofluorescence, she suggested that intermediate cells synthesize γ globulin (268). Immunofluorescent studies have also demonstrated that large and medium size lymphocytes, in addition to plasma cells, could stain for gamma globulin or specifically for IgG, IgA and IgM (147, 224, 225, 269). Small lymphocytes were positive only for IgM (147, 225). The nature and characteristics of cells disclosing positive immunocyto-adherence (rosette) (226-229) or producing antibodies in microdrop or agar plaque studies (230-232) has also been investigated. Lymphocytes of all sizes were found to form rosettes (227-229), although some authors noticed a predominance of small lymphocytes (228, 229). Cells identified as antibody producers by microdrops or agar plaque studies varied in size (232) and/or degree of development of the endoplasmic reticulum (230, 231).

3.2 SURVIVAL IN TISSUE CULTURE AND FUNCTION

In general, large lymphocytes die more quickly in cell culture than small lymphocytes. Using this method, Gowans (221) and Billingham (141) found that incubated cell suspensions containing only very few large lymphocytes could cause runting and death of non-responsive rats. Such small lymphocyte populations were found to be active in conferring to irradiated hosts the capacity to reject a graft or mount a humoral immune response (221-223).

3.3 IMMUNOCOMPETENCE OF LYMPHOCYTES SEPARATED ON GLASS BEAD AND GLASS WOOL COLUMNS

Almost pure populations of small lymphocytes obtained by filtration through glass wool columns can cause runt disease and death of newborn mice (270). Plotz and Talal (271) fractionated rat splenic lymphoid cells on glass bead columns. The cell population sticking to the beads and subsequently eluted with ethylenediamine-tetraacetic acid was enriched in antibody forming cells as well as granulocytes and large mononuclear cells. The fraction passing through the column consisted mostly of small mononuclear cells displaying poor plaque forming capacity. Nossal et al (272) fractionated normal and immune thoracic duct cells of mice on glass bead columns. Small lymphocytes recovered by this method were

unable or less capable of transferring to adoptive hosts the capacity to form antibodies to polymerized Salmonella adelaide flagellin. However, these small lymphocytes could mediate a primary immune response to sheep erythrocytes or initiate a graft-versus-host reaction (272).

3.4 DENSITY GRADIENT FRACTIONATION AND FUNCTION

Morrison and Toepfer (273) isolated from rat marrow, by combined glass wool filtration and dextran gradient fractionation a cell population containing a high percentage of lymphocytes and exhibiting a 5-fold increase in the capacity to protect lethally irradiated animals. With density gradient techniques it was also possible, using bone marrow cells or regenerating spleen cells of mice, to obtain up to thirty-fold increases in the proportion of colony forming units (274, 275), i.e., hematopoietic stem cells (276).

By centrifugation on a discontinuous albumin gradient, it was possible to fractionate splenocytes into different fractions: one of the fractions showed a 10-fold increase in the concentration of colony forming units and more than a 10-fold decrease of graft-versus-host activity (168).

Some studies have tried to correlate the density distribution of fowl blood lymphocytes (277) or rat spleen cells (272, 278)

with specific immune functions. Small lymphocytes of fowl blood purified by cotton wool and glass bead columns were subsequently separated on a linear gradient of albumin: the graft-versus-host activity revealed marked density heterogeneity with well-defined peaks and was distinct from the total population of small lymphocytes (277). In rat spleen the density profile of antigen sensitive cells disclosed at least 4 - 6 peaks (272, 278). The relative importance of each peak was found to vary from one experiment to another. The suggestion was made that the cells exist in many distinct metabolic states or alternatively that different lines of cells are active (277).

Raidt et al (279) separated normal and immune spleen cells by differential flotation in a discontinuous albumin gradient. In the normal animal the majority of antibody producing cells and precursor cells were initially found in denser regions of the gradients. After in vivo stimulation, antibody producing cells and precursor cells were found in less dense regions of the gradient. Similarly, in rats, after antigenic stimulation, the most dense components disappeared from their usual position; two major components were observed in the less dense region in their place (278).

Mage et al (280) studied the plaque-forming distribution of immune spleen cells of mice after sedimentation at unit gravity in a sucrose gradient. Cells forming hemolytic antibody plaques

sedimented more rapidly than the bulk of the non-plaque forming cells, resulting in 10 - 18-fold enrichment of their starting concentration.

The density distribution of cells forming 19 S hemolytic antibody in blood, thoracic duct lymph, spleen and lymph node cells of rats were studied following stimulation with sheep erythrocytes. The density profile of thoracic duct lymph and blood cells was far less complex than that of spleen and lymph node cells. This finding suggested that only a given class of cells enter the circulation (281).

Finally, counter current distribution studies of mouse marrow, using an aqueous two phase polymer system, has provided a partial separation between antibody producing cells and colony forming cells (304).

3.5 LIFE SPAN AND FUNCTION

The life span of the lymphocytes has been estimated by studies utilizing the ability of cells to incorporate labelled DNA precursors repeatedly or continuously administered to rats.

Small lymphocytes of rats can be divided into at least two population of cells: short lived cells having a life span of less than two weeks, and long lived cells having a life span of

several months (282, 283). The proportion of these two populations of lymphocytes has been found to vary in different lymphoid compartments (282).

There are also indications that some small lymphocytes in man may have a life span of several years. Small lymphocytes obtained from individuals treated with high doses of irradiation several years previously, when stimulated in vitro with PHA, disclosed chromosome aberrations, suggesting that these cells existed in the circulation since initial X-ray therapy (284). It is of course tempting to speculate that the long lived small lymphocytes carry immunological memory. Memory can persist for several years in man. The follow-up studies of persons actively immunized during the second World War disclosed that a booster dose of tetanus toxoid led to strong secondary immune responses 6 to 19 years after active immunization, regardless of how much, if any, antitoxin was still evident in the circulation (209). Recently, Celada has presented evidence that the booster response could be explained on the basis of a persisting line of memory cells (285).

3.6 ANATOMICAL STRUCTURE, RECIRCULATION AND FUNCTION

Parrott et al (44) have described two anatomical zones in spleen and lymph nodes of mice, which have come to be known as

"thymus dependent areas": these are the pariarteriolar lymphoid sheaths of the spleen and the deep zone of the cortex of lymph nodes. Neonatal thymectomy causes a deficit of small lymphocytes in these two areas (44) whereas the red pulp of the spleen and the superficial cortex of lymph node are not affected. We have already mentioned the effect of neonatal thymectomy on the immune functions of mice. It may be worth mentioning that Kretschmer et al recently studied three cases of the DiGeorge syndrome (congenital aplasia of the thymus gland) and noticed histological defects similar to those of thymectomized mice, i.e., well formed germinal centers in the presence of hypocellularity of the periarteriolar regions of the spleen and/or the deep cortical areas of the lymph nodes (41). The thymus dependent areas seem to be the main traffic areas of recirculating lymphocytes in spleen and lymph nodes: labelled thoracic duct lymphocytes localize prominently in these two areas (295) and depletion of the circulating pool of lymphocytes by thoracic duct drainage, causes a deficit in the lymphocyte content of these zones (222).

The functional aspect of lymphocyte recirculation has also been considered. Ford and Gowans (55) suggest that recirculation facilitates the induction of the immune response by enabling a large proportion of lymphocytes to make contact with a local deposit of antigen.

3.7 MIGRATION AND FUNCTION

The migration of lymphocytes is both a specific and selective process. The properties determining the preferential migration pattern and homing habits of lymphocytes most likely reside in specific components or structures on the outer membrane of the cell, for the migration pathways of lymphoid cells are affected by treatment with enzymes which alter cell surface components (286, 287). For example, pretreatment with trypsin abolishes the ability of lymphocytes to enter the lymph node but does not affect their migration into the spleen (286). Neuraminidase-treated lymphocytes become trapped in the liver and there is decrease in the early selective accumulation of these cells in both spleen and lymph nodes (287).

Cell membrane properties may also play a role in the following patterns of cell migration:

a. Only 1 out of 10 lymphocytes perfusing the sheep popliteal lymph node migrate across the post-capillary venules (288).
b. Bone marrow cells enter the thymus gland more readily than any other cell population (13). Thoracic duct cells do not enter the thymus (55).
c. Thymus lymphocytes labelled in vitro enter the spleen and lymph node but in lesser numbers than do spleen cells or thoracic duct cells (289, 290).

d. Labelled marrow cells show a marked predilection for spleen red pulp (290).

e. Finally, Lance and Taub (291) segregated subpopulations of lymphocytes labelled with chromium-51 according to their distributional tendencies in serial transfers. Lymph node cells, which in a small proportion sought out the lymph node in the first recipient were, much more likely to relocate following their transfer from the first recipient in the lymph node of a second host. On the other hand, thoracic duct cells appear to have a very high affinity for lymph nodes in the first recipient. An almost similar observation was made with the thymus cells which localized in the lymph nodes. Thymocytes which had migrated to the lymph node of lethally irradiated animals exhibited a greater capacity to cause graft-versus-host reactions compared to the original thymus cell preparation. The authors suggest that this method selectively isolates the subpopulation of thymus or lymph node cells capable of recirculating.

In summary, we may state that the different methods used to fractionate lymphocytes have contributed to the demonstration of the heterogeneity of lymphocytes and, to a certain extent, have aided in the identification of certain populations of cells displaying a given function.

4 MITOGENIC TRANSFORMATION AND LYMPHOCYTE HETEROGENEITY

In the preceding chapter we have seen that lymphocytes separated by different methods often display diverse functions. In this section we will review experimental data indicating a differing response of lymphocytes to some mitogens and determine whether these differences can further facilitate the assessment of lymphocyte heterogeneity.

Following the discovery by Hungerford (292) and Nowell (293) that a saline extract from beans of the genus Phaseolus, phytohemagglutinin (PHA), could transform small lymphocytes in human peripheral blood into large primitive cells (blast cells), an enormous amount of literature concerned with lymphocyte stimulation by PHA and other mitogens sprang up in a relatively short period of time. This makes any review of this aspect of lymphocyte studies an almost impossible task. I am tempted to say, as Andre Gide (296) once did when he was faced with the ungratifying prospect of reading the confused and lengthy mythological books of India which he thought would help him to understand the poems of Radindranath Tagore: "Si le temps ne me manquait pas c'est le coeur qui me manquerait." (Even if I had the time I would not have the fortitude.)

Numerous changes, both biochemical and morphological, take place within the lymphocyte stimulated by a mitogenic agent:

the studies of some of these changes, i.e., transformation to blast-like cells, mitosis, DNA and RNA synthesis, have served to characterize most of the mitogens. The blast cell induced by the numerous mitogens has retained the attention of immunologists mainly because it demonstrated that the small lymphocyte was not a dead end cell but could take on an "immunologically active" morphology. These blast cells resemble in size, staining characteristics and/or electron microscopic appearance, the large pyroninophylic cells which appear in vivo in the spleen during a graft-versus-host reaction (135), or during the early phase of the secondary immune response to an antigen (294), in regional lymph nodes draining sites of primary immunization (297) and in the lymph leaving these nodes (298).

This chapter consists of two sections:

a) the first section is concerned with the categorization of the different mitogens into certain subgroups. The main characteristics of each subgroup will be outlined;
b) the second section deals with some studies demonstrating the differing responses of lymphocyte to several mitogens.

4.1 CLASSIFICATION OF THE MITOGENS

The mitogens can be divided into 4 categories: non-specific mitogens, specific mitogens, anti-immunoglobulin antisera and allogeneic and xenogeneic leucocytes.

4.1.1 THE NON-SPECIFIC MITOGENS

The non-specific mitogens are presumed to stimulate lymphocytes from immune and non-immune animals and man (305-307). These are PHA (292, 293), pokeweed mitogen (PWM) (309, 310), staphylococcal filtrate (311), streptolysin S (312), and anti-lymphocyte serum (ALS) (313).

The non-specific mitogens stimulate a large proportion of lymphocytes (306-308). There is limited information on the mechanism of action of these mitogens (306) and it is not known if they all stimulate the same population of lymphocytes. It is generally assumed that they trigger off the lymphocytes by interacting with a cell membrane receptor (307). Attempts to directly identify the receptor site for PHA, using radioactively-labelled or fluorescein-conjugated materials, has so far been unsuccessful. PHA has alternatively been localized to the cell membrane (314, 315), the nucleus (316, 317) or the cytoplasm of the cell (318, 319).

Anti-lymphocyte serum appears to have specificity for membrane determinants shared by lymphocytes but not found on other tissues. This specificity does not involve cell bound gamma globulin (320). Some indirect results suggest that the lymphocyte-surface reactive sites for PHA and anti-lymphocyte serum are closely related or identical (321): anti-lymphocyte sera at concentrations insufficient to induce blastogenesis can completely abolish the stimulatory effect

of PHA for human or mouse lymphocytes (321-323). ALS can also suppress PHA-induced cytotoxicity of lymphocytes for homologous fibroblast monolayer cultures (323).

A recent finding, indicating that pretreatment of mice with PHA during the neonatal period does not alter the subsequent PHA response of their lymphocytes, suggests that PHA is not acting as a specific primary antigenic stimulus (324).

4.1.2 SPECIFIC MITOGENS: ANTIGENS AND ALLERGENS

Antigens and allergens can stimulate the lymphocytes obtained from a donor previously sensitized to the specific antigen or allergen (252-255, 325-328). A relatively small proportion of lymphocytes are induced to undergo blastogenesis and mitosis when incubated with the specific mitogens as compared to the large number of blast-like cells induced by PHA (306, 307). The cell membrane receptor interacting with the antigen is assumed to be an antibody-like molecule. We have already reviewed the evidence in favor of the antibody nature of the cell surface receptor (Chapter 2.3.1).

There has been some controversy as to whether the specific transformation by antigen is a reflexion of cellular or humoral immunity. Lymphocytes of animals exhibiting delayed hypersensitivity in the absence of circulating antibodies can be stimulated with the specific antigen in vitro (327, 329). Mills (327) also reported

that lymphocytes obtained from guinea pigs immunized intravenously and which did not subsequently show delayed skin reactivity did not react with blastogenesis in the presence of the antigen in vitro. On the other hand, several investigators (162, 328) have demonstrated that spleen cells from rabbits immunized intravenously are responsive to the antigen in vitro.

4.1.3 ANTI-IMMUNOGLOBULIN ANTISERA

Antisera to rabbit allotypes (244) - induced by immunizing rabbits lacking a given allotypic specificity with immunoglobulin carrying this allotype - as well as heterologous antisera to rabbit immunoglobulins (245, 246) may stimulate blast transformation and DNA synthesis when added to lymphocytes in vitro. Anti-immunoglobulin antisera can also stimulate lymphocytes of a number of animal species, i.e., guinea pigs (320), chickens (330), mice (331), and man (332-333). Although anti-immunoglobulin antisera, like PHA or ALS, can stimulate normal and sensitized lymphocytes, it deserves to be considered in a special category because the cell membrane receptor for this reagent is considered to be a cell constituent with a well characterized structure, i.e., an immunoglobulin molecule. The activity of anti-immunoglobulin sera can be suppressed following absorption of the antiserum with IgG (334).

The observation by Sell that the univalent Fab fragment of the anti-immunoglobulin molecule can stimulate rabbit lymphocytes to transform, indicates that only interaction of sites with a complementary configuration is required to trigger this reaction (335). A high percentage of rabbit lymphocytes are transformed by sheep antisera to rabbit IgG or IgM (246) (about 80%); however, a much smaller proportion of chicken lymphocytes undergo blastogenesis following incubation with rabbit anti-chicken immunoglobulin sera (330).

4.1.4 ALLOGENEIC AND XENOGENEIC LEUCOCYTES

Lymphoid cells of a number of animal species can respond with blastogenesis and DNA synthesis when incubated in vitro with allogeneic (336-340) and xenogeneic cells (341). This reaction most likely represents a cellular response to a histocompatibility difference at a major locus (338, 342). Although the mixed leucocyte reaction is enhanced by previous contact of one donor with the other (343, 344) by way of a skin graft, this sensitization is not a prerequisite for the reaction to occur. Nevertheless, the mixed leucocyte culture reaction (MRC) has definite immunologic characteristics. It cannot be induced by incubation of lymphocytes from identical monozygotic twins or syngeneic animals (336, 337), nor by the incubation of lymphocytes from genetically dissimilar donors when one of the donors has been made tolerant to the cells of the

other donor (345). Furthermore, in mixed leucocytes cultures consisting of leucocytes of parental and F 1 hybrid, all the dividing cells are of parental origin (345).

A one way stimulation in MLC can be obtained by inhibiting the proliferation of one donor cell population by treatment with mitomycin-C (a DNA inhibitor) (346) or X irradiation (347, 348). These treatments do not abolish the "stimulating" capacity of the cells toward genetically different "responding" cell populations. It seems, however, that one cannot ascribe the stimulating capacity of these "inactive lymphocytes" only to their transplantation antigens. Despite a few reports indicating that disrupted leucocytes can stimulate homologous lymphocytes as well as intact cells (342, 344), the majority of investigators have shown that cell disruption destroys their stimulatory activity (341, 348-351). It seems likely, therefore, that direct interaction with an actively metabolizing, although "inactivated" cell is necessary for a maximal response. The mixed leucocyte reaction is sensitive to the action of anti-lymphocytic serum which presumably modifies the cell membrane and prevents optimal cell to cell interaction (349, 352, 353).

Two recent studies have tried to estimate the proportion of lymphocyte in a human (262) or rat (261) capable of responding to an allogeneic stimulus in the one way stimulation test. Estimates of the frequency of the initially responding cells varied around 1%.

However, up to 30 percent of human lymphocytes in culture after 7 days were transformed (262).

4.2 THE DIFFERING RESPONSES OF LYMPHOCYTES TO DIFFERENT MITOGENS

4.2.1 MORPHOLOGICAL HETEROGENEITY OF CELLS TRANSFORMED BY PHA OR PWM

Several investigators have compared the electron microscopic appearance of lymphocytes stimulated with PWM and PHA (309, 354). In a 72 hours culture, PWM-stimulated cells exhibit a better-developed rough surfaced endoplasmic reticulum (309, 354); in late cultures of lymphocytes incubated with PWM, further development of the endoplasmic reticulum is observed with the appearance of cells resembling mature plasma cells (309). The same is not true in long term cultures of lymphocytes stimulated with PHA.

4.2.2 KINETICS OF STIMULATION; ADDITIVE EFFECT OF MITOGENS AND LYMPHOCYTE HETEROGENEITY

Considerable differences have been observed in the duration of incubation necessary to allow for maximum stimulation by different mitogens (307, 355). We have already indicated that the maximum responses to non-specific mitogens are considerably higher than those evoked by the specific antigen. Furthermore, the response of

sensitized lymphocytes to antigen proceeds in graded fashion over a wide range of concentrations as compared to the very steep dose response curve obtained with PHA and ALS (320). However, the variation in response may simply reflect differences between the strength of the reagents used to stimulate the cells and does not imply that the populations of lymphoid cells affected by these reagents, or the reactive sites which serve as target for the mitogenic stimulus, are not the same.

The finding that lymphocytes which had been previously exposed to staphylococcal filtrate have an earlier blastogenic response to purified protein derivative than do cells not previously stimulated has been interpreted as evidence that the same cell population is responsive to both reagents (355). On the other hand, on the basis of the additive effects of PHA or PWM and antigen on DNA synthesis, it was suggested that different populations of cells were stimulated by the non-specific and specific stimuli: in two different studies, the simultaneous addition of PHA or PWM and SRBC to cultures of lymphocytes obtained from rabbits previously immunized with SRBC increased the uptake of thymidine above that observed in cultures treated with one stimulant alone (356, 357).

4.2.3 LYMPHOCYTE STIMULANTS AND ANTIBODY SYNTHESIS IN VITRO

4.2.3.1 NON-SPECIFIC MITOGENS AND IMMUNOGLOBULIN AND/OR ANTIBODY SYNTHESIS

Several workers using a variety of techniques claim to have demonstrated immunoglobulin synthesis in vitro by PHA-stimulated lymphocytes, but these results lack confirmation (307). A recent study indicates that cells responding to PWM are also capable of synthesizing immunoglobulin which can be detected by immunofluorescence (358).

The ability of non-specific mitogens to elicit a specific anamnestic response has also been investigated. Forbes (359) claimed that PHA stimulated the synthesis of anti-thyroglobulin antibodies in cultures of lymphocytes from Hashimoto patients. Similarly, Tao (114) incubated lymph node fragments from hyperimmunized rabbits with PHA and observed, in a few instances, the production of antibody to the specific antigen. However, using the hemolytic plaque technique it was demonstrated that neither PHA (356, 357, 360) nor PWM (357) could induce a secondary response to sheep erythrocytes in sensitized rabbit spleen cell suspensions in vitro.

4.2.3.2 ANTIGEN-INDUCED BLASTOGENESIS AND ANTIBODY PRODUCTION

In a preceding chapter (2.1.1) we referred to several studies indicating that antigen can induce a specific anamnestic response in vitro. PHA or PWM did not enhance the capacity of antigen to elicit a secondary antibody response in vitro (356, 357). It now remains to be established whether the antigen-induced blast cell is actually the one which synthesizes antibodies. This point is of particular interest in view of the work of Davies (16, 32) indicating that thymus derived cells proliferate in response to antigen but do not engage in antibody production. Greaves and Roitt have found that a certain percentage of PPD-stimulated cells react with fluorescein-conjugated PPD (358). Similarly, Lamvik (360) demonstrated lytic activity around blastoid cells in cultures of rabbit immune peripheral lymphocytes incubated with the specific antigen (SRBC) in vitro. On the other hand, two recent studies found no correlation between antigen activated blast cells and specific antibodies. Utilizing the technique of bacterial adherence, Simons and Fitzgerald (361) found no evidence of antibody production by antigen stimulated lymphoblastoid cells. Gery et al (362) found no correlation between the number of cells forming or carrying antibody against SRBC (plaque or rosette forming cells) and those stimulated to transform in vitro by this antigen. They realized

that these results could have been influenced by the greater sensitivity of one method (namely the lymphocyte stimulation) over the others. It was also suggested that unsensitized lymphocytes can be induced to transform by a stimulus released from other reacting cells.

4.2.4 LYMPHOCYTE LIFE SPAN AND MITOGENIC TRANSFORMATION

Short and long lived small lymphocytes from various lymphoid tissues of the rat were labelled and cultured by Metcalf and Osmond who reported that members of both groups enlarge when stimulated with PHA (363). In a recent study, Rieke and Schwarz (364) confirmed these findings and noted that while both long and short lived thoracic duct lymphocytes enlarge when stimulated with PHA, the short lived population is proportionately more responsive. Similarly, in the MLC, just as in PHA stimulated cultures, the short-lived small lymphocytes were found to be proportionately more responsive (365). We have mentioned earlier (Chapter 3.5) that there are indications that some small lymphocytes of man having a life span of several years may respond to PHA (284). Nowell (366) studied the lymphocytes of x-irradiated patients presenting chromosome aberrations at different intervals after irradiation. The cells with unstable aberrations were not detected in cultures

stimulated with tuberculin, if obtained from the donor eleven months after irradiation, whereas their frequency was only slightly reduced in PHA cultures.

4.2.5 LYMPHOCYTE ORIGIN AND/OR DEPENDENCE (THYMIC OR BURSAL) AND ITS CAPACITY TO RESPOND TO MITOGENIC STIMULATION

Several experiments indicate that thymus derived or dependent lymphocytes may be the ones responding to PHA and/or allogeneic leucocyte stimulation. The response of the lymphocytes of neonatally thymectomized mice (367), rats (368, 369) and chickens (370) to stimulation with PHA, PWM or mitomycin C treated allogeneic cells is markedly reduced. Lymphocytes obtained from rats thymectomized at birth exhibit a markedly and sometimes totally diminished reactivity against homologous lymphocytes (345). A recent study utilizing thymectomized, irradiated mice, injected with syngeneic bone marrow and reconstituted with a chromosomally marked thymus graft, indicate that 89 percent of the peripheral blood lymphocytes of these animals which respond to PHA are of thymic origin (17).

However, human lymphocytes obtained from a child thymectomized at birth tested for their ability to undergo blastogenesis when the donor was 2 1/2 years old were found to respond normally

to stimulation with PHA (371), thus demonstrating that the situation in man may not be comparable to that in the rodent.

On the other hand, lymphocytes of hypogammaglobulemic bursectomized chickens fail to respond to anti-immunoglobulin antiserum (334) although the response to PHA is normal (334, 369, 370), thus supporting the concept of a thymic origin of the PHA-responsive cells.

4.2.6 ANTIMETABOLITES AND MITOGENIC STIMULATION

A recent study (372) dealt with the question whether antimetabolites and prednisone have a similar action on the in vitro transformation of immune lymphocytes by specific antigen or PHA. Low concentrations of methotrexate, 5-fluorouracil and prednisolone completely prevented the blastoid transformation induced by antigen. Higher concentrations of these materials were needed to block the transformation induced by PHA. Methotrexate did not produce complete inhibition of PHA transformation even at high concentration.

4.2.7 "PURIFIED" LYMPHOCYTES AND MITOGENIC STIMULATION

Several authors have compared the responses to mitogens of "pure" lymphocyte suspensions, obtained by passage of leucocytes through glass bead columns, with that of the original or reconstituted

(purified lymphocytes plus effluent cells) cell suspension.

McFarland (373) observed that in both mixed leucocyte and antigen-stimulated cell cultures, native or reconstituted cell populations produced 3 to 10 times higher responses than "pure" lymphocyte populations. Hersh and Harris (374) also reported that glass bead purified lymphocyte suspensions have reduced blastogenesis responses to antigen when compared to unseparated leucocytes suspensions. The response to PHA was unaffected by column purification. Cultures of purified lymphocytes on macrophage monolayers generally restore the cells response to antigen. Oppenheim et al (375) made a similar observation but also noted that purification could affect stimulation by homologous leucocytes or low doses of PHA. These results suggest that the phagocytic cells, presumably removed by purification, are important for the in vitro transformation with antigen and may also facilitate the response to non-specific stimuli.

4.2.8 THE RELATION BETWEEN ANATOMIC LOCALIZATION AND THE ABILITY TO RESPOND TO STIMULATION WITH MITOGENIC AGENTS

There has been no systematic study of the response of all the anatomically segregated lymphoid cell populations to one or more of the mitogens classified above. The studies so far

published have used a limited number of cell populations and/or very few mitogens (306, 307). It would almost be an impossible task to cite all these studies. However, several patterns of responses can be outlined which may help to categorize some cell populations and contribute to the study of lymphocyte heterogeneity.

In general, lymphocytes from peripheral blood, thoracic duct, spleen and lymph nodes of man and a number of animal species can be stimulated in vitro to undergo blastogenesis and mitosis upon stimulation with non-specific and specific mitogens as well as allogeneic and xenogeneic lymphocytes (306, 307).

However, there is still some controversy as to whether bone marrow, appendix, bursa of Fabricius and thymus lymphocytes can respond to mitogenic stimulation and whether these cells are, in fact, immunocompetent (see Chapter 2.1.2 and 2.1.3). The reactivity of these cells with respect to their response to a number of stimuli, will now be discussed.

4.2.8.1 THYMOCYTES AND MITOGENIC TRANSFORMATION

The study of thymocyte response to PHA has given rise to contradictory reports. Some workers (376) failed to detect a response to PHA under culture conditions which permitted a good response with peripheral lymphocytes. Others (377-379) have

observed proliferation of thymocytes, which was however, less marked than that of peripheral cells. Two recent studies (380, 381) indicate, however, that the response of thymocytes to staphylococcal filtrate and/or PHA is markedly improved by culturing them at higher cell concentrations.

Human thymocytes have been found to undergo transformation when incubated in vitro with the gamma globulin fraction of a horse anti-human splenocyte antiserum (382). Rabbit thymocytes respond poorly when stimulated with allogeneic cells in vitro (307); however, rat thymocytes can be stimulated in the mixed leucocyte reaction (383, 384).

4.2.8.2 BURSA OF FABRICIUS CELLS AND MITOGENIC TRANSFORMATION

In one study, the lymphocytes from the bursa of Fabricius of the chicken failed to respond to PHA (385).

4.2.8.3 APPENDIX CELLS AND MITOGENIC TRANSFORMATION

Although lymphocytes from rabbit appendix were reported to be PHA-non-responsive by Schrek and Batra (387) two recent studies indicate that both rabbit (379) and human (386) appendix cells can respond to PHA. Only a small fraction of rabbit appendix lymphocyte respond to PHA (379), but in culture of

lymphocytes from human appendix, the number of transformed cells in the presence of PHA is comparable to that obtained with peripheral blood lymphocytes stimulated under similar conditions (386).

4.2.8.4 BONE MARROW CELLS AND MITOGENIC TRANSFORMATION

Human bone marrow cells separated through a column of glass microspherules have been studied for their capacity to respond to PHA in vitro: tritiated thymidine uptake was low in the initial suspensions and was not increased by the addition of PHA and tritiated uridine uptake was increased in only four of 41 cultures stimulated with PHA (388).

On the other hand, Singhal and Richter (162) stimulated normal rabbit bone marrow cells to incorporate increased amounts of tritiated thymidine following incubation with PHA and numerous antigens to which the animals had not previously been sensitized. Separation of the bone marrow cells on a sucrose gradient increased several fold the concentration of cells capable of responding to antigens (162).

In summary, we may state that the study of lymphocyte transformation has contributed to the demonstration of lymphocyte heterogeneity.

We will now turn our attention toward the "immunologic deficiency diseases," to see to what extent the in vitro lymphocyte studies in these clearly delineated "experiments of nature" have contributed to unravel lymphocyte diversity and function.

5 LYMPHOCYTE STUDIES IN IMMUNOLOGIC DEFICIENCY STATES

It was seen earlier that the study of the immunologic deficiencies has contributed to the acceptance of two separate lines of immunocytes responsible for humoral and cellular immunity, respectively, and originating from a common precursor (Chapter 1.2; see also Fig. 1 and Table 1). The studies to be reviewed in this section are concerned with the in vitro response of the immunologically deficient lymphocytes of man to PHA and other mitogens. They have helped to categorize the different immunologic deficiencies but have often revealed a wide variety of defects in the lymphocytes of patients presenting with identical syndromes.

5.1 CELLULAR IMMUNE DEFECT ASSOCIATED WITH THYMIC APLASIA (DIGEORGE SYNDROME)

By and large the lymphocytes of patients displaying thymic aplasia do not respond to PHA (40-42). Following implantation of a foetal thymus in two patients, the peripheral lymphocytes were found to transform normally in culture when stimulated with PHA (42, 43).

5.2 COMBINED CELLULAR AND HUMORAL DEFECTS. THYMIC DYSPLASIA AND SWISS TYPE AGAMMAGLOBULINEMIA

In vitro culture of lymphocytes has been attempted in a few cases of Swiss type agammaglobulinemia and/or congenital thymic dysplasia. Generally, no proliferation has been obtained with either PHA, specific antigens or allogeneic leucocytes as stimulants of blastogenesis (50, 371, 389, 390).

However, cells of one patient with the sex-linked form of congenital thymic dysplasia has been found to consistently respond to stimulation with allogeneic lymphocytes although the response to PHA was usually negative (389). A similar dissociation of responsiveness to PHA, on the one hand, and allogeneic cells, on the other hand, has also been observed with lymphocytes of a patient who lost the capacity to manifest cellular and humoral immunity in adult life (390).

5.3 HUMORAL IMMUNE DEFECTS. CONGENITAL AND ACQUIRED AGAMMAGLOBULINEMIA

Tormey et al (391) found a decreased synthesis of RNA and DNA in PHA-stimulated agammaglobulinemic lymphocytes. The majority of investigators, however, have reported (371, 390, 392-397) that the peripheral lymphocytes obtained from agammaglobulinemia

patients incubated in vitro with PHA show a degree of blast transformation and mitosis comparable to normals.

The proliferative response of agammaglobulinemia lymphocytes to specific antigens has been the subject of numerous and contradictory reports. Fudenberg and Hirschhorn (392) studied the lymphocytes of 5 agammaglobulinemia patients (two had "typical" sex-linked agammaglobulinemia and three the "acquired" form of the disease): the lymphocytes of all the patients failed to differentiate or produce γ globulin when challenged in the culture by the immunizing antigen. Similarly, Bach et al (390) challenged the lymphocytes of numerous agammaglobulinemic patients with antigen in vitro: out of 7 Bruton type cases, 5 did not respond and one gave a border-line response; the cells of three patients with late onset (acquired) agammaglobulinemia responded to at least some of the antigens. On the other hand, recent studies (371, 397) indicate that circulating lymphocytes from patients with congenital X-linked or acquired agammaglobulinemia proliferate in a normal manner following stimulation with a specific antigen. Furthermore, the agammaglobulinemic lymphocytes studied by Cooperband et al (397) produced a small quantity of IgG similar in amount to that found in normal cell cultures. The quantity of IgG produced was increased by stimulation with PHA.

In two different studies (390, 397), the lymphocytes of patients with congenital or acquired agammaglobulinemia were found to respond to stimulation with allogeneic cells (MLC). Lieber et al (396) studied 3 cases of sex-linked agammaglobulinemia and 9 cases of acquired agammaglobulinemia (5 males and 4 females): the three congenital cases failed to respond in MLC; the male acquired cases responded to variable degrees or not at all, while the female patients gave normal responses.

It is therefore obvious that, within each category of immunoglobulin deficiencies, there exist a variety of lymphocyte abnormalities which can be detected by in vitro cultures and mitogenic stumulation.

6 CONCLUSIONS AND OBJECTIVES OF PROPOSED WORK

The various investigations reviewed above have unequivocally demonstrated that the lymphocytes constitute a heterogeneous population of cells. The studies in which attempts were made to identify or isolate distinct populations of lymphoid cells and to ascribe particular functions to them were also discussed. Special emphasis was placed on the assessment of functional heterogeneity on the basis of the differing blastogenic responses of lymphocytes to different mitogens. However, there is no clear indication whether the same population or different populations of cells respond to the different mitogens in the normal or immune animal. Also, very little data have been uncovered concerning the nature of the sites on the cell with which these mitogenic agents interact. The majority of the studies reviewed above utilized only a few mitogens and/or the lymphocytes of only one or, at best, several of the lymphoid organs.

The experiments carried out in this investigation were designed to enable the investigator to test, simultaneously, the responsiveness of the lymphocytes of the different lymphoid organs of the rabbit (lymph node, spleen, thymus, bone marrow, sacculus rotundus, appendix, peripheral blood) toward the different mitogenic stimuli. It was anticipated that the patterns of response which would emerge would facilitate a categorization of the lymphoid cells on a functional basis and might also permit a better understanding of the conditions which decide whether a cell will participate in humoral or cellular immunity.

CHAPTER III

MATERIALS, METHODS AND EXPERIMENTAL PROTOCOLS

1 MATERIALS

1.1 PATIENTS

The subjects dealt with here include one with congenital agammaglobulinemia (No. 1) and two with 'primary' acquired hypogammaglobulinemia (Nos. 2 and 3).

The diagnosis was made in the first subject at the age of three when a serum protein electrophoretic pattern showed complete absence of γ-globulins. From the age of 9 months, he had recurrent respiratory infections and otitis media which necessitated almost constant antibiotic therapy.

The diagnosis of agammaglobulinemia was made in subjects Nos. 2 and 3 at the ages of 15 and 38, respectively. Subject No. 2 had recurrent infections for 9 years prior to diagnosis. Subject No. 3 had experienced her first significant infection at age 30 and since then has had severe pulmonary infections three or four times a year. Two years before the diagnosis was made, she gave birth to her third and last child, an Rh positive baby, who required an

exchange transfusion for erythroblastosis fetalis. The baby had a positive indirect Coombs test and the patient had elevated anti-D antibodies, indicating that she was then capable of producing isoagglutinins.

All three patients were unable to produce circulating antibodies to a variety of antigens after the diagnosis of agammaglobulinemia had been made. They also disclosed varying degrees of impairment in delayed-type dermal hypersensitivity. These observations are summarized in Tables 2 and 3.

All the patients were receiving injections of gamma globulin at 3- or 4-week intervals at the time of the current studies. Peripheral lymphocytes were obtained both before and after administration of γ-globulin to the patients. The cells were incubated in medium containing autologous plasma and challenged with either anti-immunoglobulin antiserum or PHA.

Peripheral blood lymphocytes were obtained from normal volunteers and these served as controls.

1.2 ANIMALS

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

1.3 ANTIGENS

1) Human serum albumin (HSA) - (Hyland Laboratories, Los Angeles, Calif.)
2) Bovine serum albumin (BSA) - (Pentex Incorp., Kankakee, Ill.)
3) S. typhi 0 901, acetone killed and dried (For preparation of this material, see ref. 398).

1.4 COMPLEMENT

The complement (C') used was fresh whole guinea pig serum obtained by repeated cardiac puncture of normal, adult guinea pigs. The pooled serum was absorbed with rabbit red cells and kept frozen at -20°C until used.

1.5 MITOMYCIN-C

Mitomycin-C was obtained from Nutritional Biochemicals, Clevaland, Ohio. Each vial was dissolved in medium 199 to give a final concentration of 100 µg per ml.

1.6 PHYTOHEMAGGLUTININ

The phytohemagglutinin-M (PHA-M) used in this study was obtained from Difco Laboratories, Detroit, Mich, USA. The contents

of a vial were dissolved in 5 ml of medium 199 and this solution, referred to as undiluted PHA, was diluted several times in medium 199. The PHA solutions were kept at -10°C.

1.7 RADIOACTIVE COMPOUNDS

Tritiated thymidine (thymidine-H^3) was obtained from Schwarz Bio-research Incorporation, Orangeburg, N.J., USA.

Goat gamma globulin (GGG) and goat anti rabbit immuno-globulin (GARIG) were labelled with Iodine I^{125} by Charles E. Frosst and Co., Montreal.

1.8 MEDIA

Medium 199 with bicarbonate (Microbiological Associates, Bethesda, Md., USA) to which is added penicillin (100 units per ml of medium) and streptomycin (100 µg per ml of medium) is referred to in the text as Med-PS. The penicillin and streptomycin stock solutions were obtained from Microbiological Associates, Bethesda, Md., USA.

For in vitro cultures, normal or gamma globulin-free homologous serum was added to Med-PS to yield a final concentration of 15 percent. In cultures of human lymphocytes, autologous plasma

was sometimes used. Normal rabbit serum (NRS) was purchased from Microbiological Associates.

Gamma globulin-free human or rabbit serum was prepared by precipitating the gamma globulin at 50 percent saturation with ammonium sulphate. The supernatant, essentially free of gamma globulin by electrophoresis on cellulose acetate strips and immunoelectrophoresis, was dialysed extensively against distilled water, then lyophilized and dissolved in a volume of medium 199 equal to that of the original serum. The pH was adjusted to 7 with sodium bicarbonate.

1.9 ANTI-HUMAN IMMUNOGLOBULIN ANTISERUM

Rabbit anti-human immunoglobulin antiserum was prepared by injecting 5 to 15 mg of purified immunoglobulin or Bence Jones protein dissolved in saline and emulsified in complete Freund's adjuvant into adult New Zealand white rabbits. Booster injections of 5 mg were made at 1 and 2 months after the initial immunization. The rabbits were bled approximately three times monthly and pools were made of bleedings which were shown to have specific antibodies in adequate amounts by immunoelectrophoresis. The same antiserum pools were used in all tests. A few antisera had antibodies in low titre to one or two contaminating serum proteins. Preliminary

experiments using lymphocytes from five normal subjects demonstrated that absorption of the contaminating antibodies either increased or did not significantly alter the blastogenic activity of the antisera. Therefore, the antisera used were not absorbed in order to avoid the production of soluble antigen-antibody complexes which, in themselves, have been found in this laboratory to have blastogenic activity. In all instances in which whole immunoglobulin molecules were used for immunization, the antibodies were directed predominantly to the corresponding immunoglobulin heavy chain. The specificity of the antisera was established both by immunoelectrophoretic and agar gel (Ouchterlony) analysis using purified myeloma or macroglobulinemia proteins as well as pooled normal serum as antigens.

1.10 GOAT ANTI-RABBIT IMMUNOGLOBULIN ANTISERA

The preparation and characteristics of the goat anti-rabbit immunoglobulin antiserum has been previously described (398). The goats were given 3 intramuscular injections of rabbit immunoglobulins (100 mg) in complete Freund's adjuvant at 7 day intervals: the two first injections consisted of rabbit IgM and the third injection consisted of both IgG and IgM. The specificity of the antisera was established by immunoelectrophoresis (Fig. 2).

The purified IgM and IgG rabbit immunoglobulins which were

used to immunize the goats were prepared by elution and purification of antibody molecules specifically adsorbed to S typhi O antigen. The method used was essentially that described by Robbins et al (399). Specific absorption of aliquots of immune rabbit sera averaging 200 to 300 ml was carried out by adding about 0.5 g of dried S typhi O 901 organisms to each 25 ml aliquot of serum. Elution of the specific antibodies was accomplished at low pH. Following elution, the proteins were concentrated on carbowax to give a final protein concentration of 10 to 15 mg/ml. Two- or three-milliliter samples of this material were chromatographed on a 2.5 by 100 cm Sephadex G-200 column (Pharmacia, Uppsala, Sweden (Fig. 3). Before chromatography, the identity of the material was assessed by immunoelectrophoresis (Fig. 2). Complete separation of the 19 S and 7 S globulins was assured by re-chromatography of the first peak (19 S antibodies). The second peak (7 S) was further purified by diethylaminoethyl (DEAE) cellulose chromatography. The antibodies under the first peak had the characteristics of 19 S antibodies, i.e., relatively high electrophoretic mobility and sensitivity to 2-mercaptoethanol (2-ME). The antibodies under the second peak had a slow electrophoretic mobility and were resistant to 2-ME (Figs. 2 and 3)

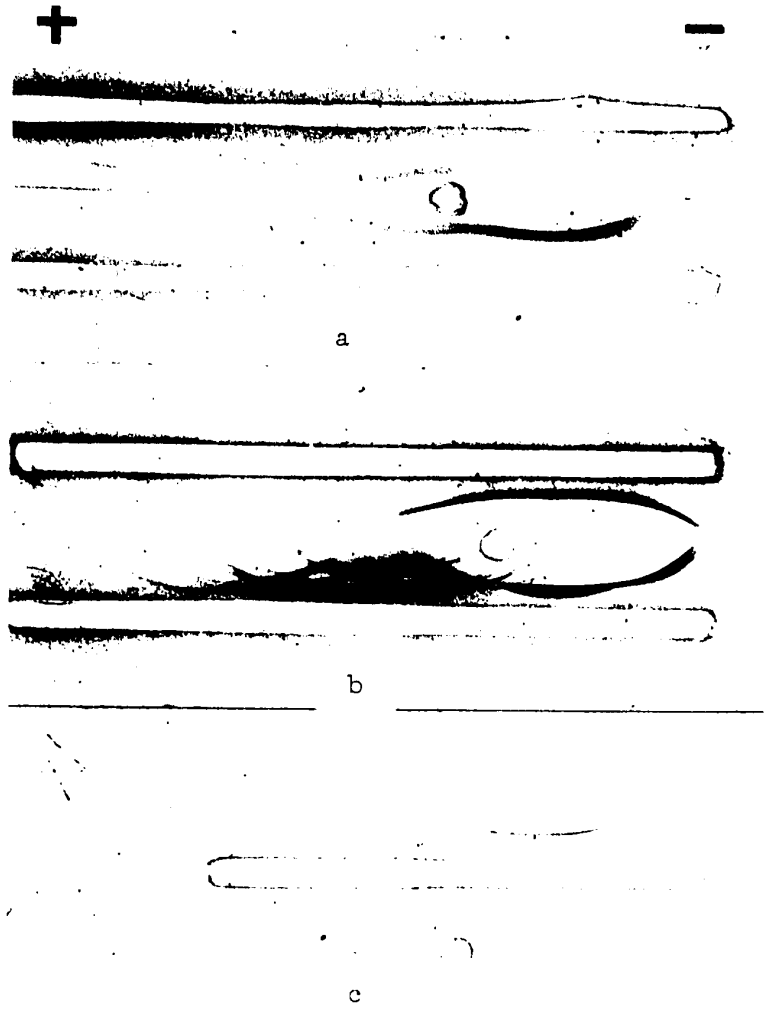


FIGURE 2

IMMUNOELECTROPHORESIS OF RABBIT PURIFIED ANTIBODIES AND GOAT ANTI RABBIT IMMUNOGLOBULIN ANTISERUM.

a) Immunoelectrophoretic pattern of rabbit proteins adsorbed to and eluted from S. typhi developed with specific goat anti rabbit IgM (upper trough) and goat anti rabbit serum, Hyland Corp. (lower trough).

b) Immunoelectrophoretic pattern of normal rabbit serum developed with goat anti serum to specific rabbit proteins adsorbed to and eluted from bacterial antigens (upper trough) and goat anti rabbit serum, Hyland Corp. (lower trough).

c) Immunoelectrophoretic pattern of Sephadex G-200 separated rabbit antibodies developed with goat antiserum to specific rabbit proteins adsorbed to and eluted from bacterial antigens. Upper well, 2nd peak (IgG); lower well, 1st peak (IgM).

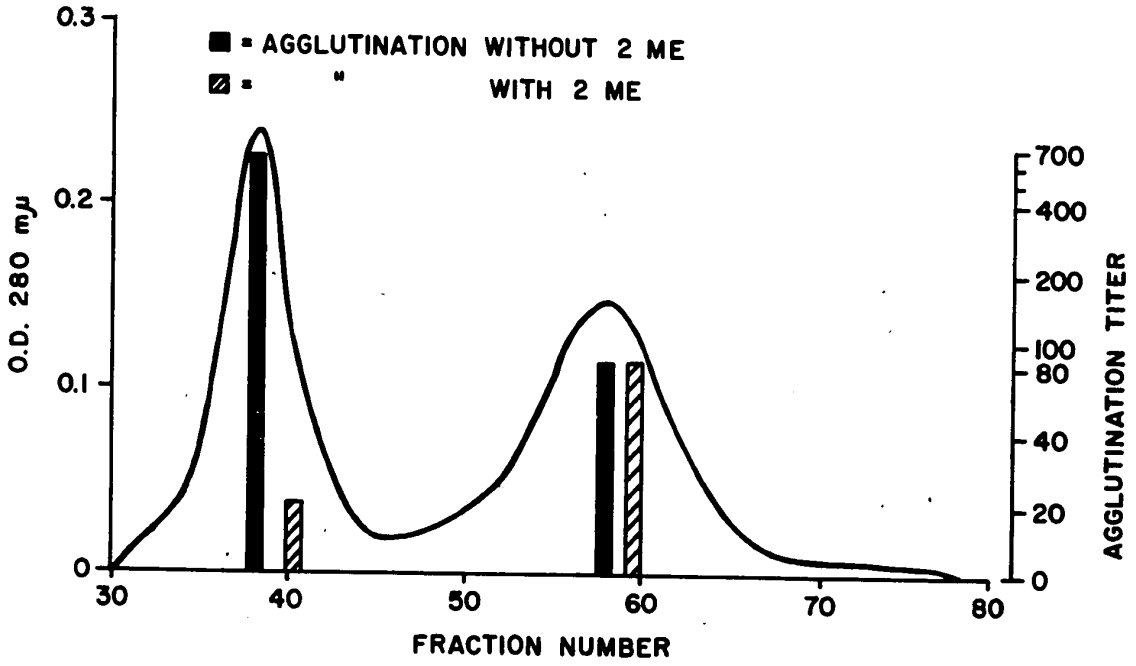


FIGURE 3

SEPHADEX G-200 CHROMATOGRAPHY OF PROTEINS ADSORBED TO AND ELUTED FROM S. TYPHOSA O 901.

2 GENERAL METHODS

2.1 IRRADIATION PROCEDURE

The rabbits were subjected to doses of 800 r whole body irradiation, using a Cobalt 60 source under the following conditions: skin source distance 200 cm, field size 50 x 50 cm, colarimeter size 20 x 20 cm and output 6.97 r per minute. The rabbits were then handled according to the experimental plan. Usually the rabbits were irradiated in batches of six.

2.2 IMMUNIZATION PROCEDURES

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

2.3 DYE EXCLUSION TEST

The viability of the various cells was determined by the dye exclusion test using 0.1 percent trypan blue. A drop of the dye

was added to one ml of the cell suspension and the latter was then analyzed in a hemocytometer. Cells that took up the dye are considered to be dead cells. The viability of the cells, on the basis of 200 cells counted, was recorded as percent dead cells.

2.4 PREPARATION OF NORMAL GOAT IgG OR GOAT ANTI-RABBIT IMMUNOGLOBULIN IgG

Pure IgG fractions of goat sera were prepared in the following manner: the gamma globulin fraction of an aliquot of serum (20-30 ml) was precipitated out of solution by ammonium sulphate precipitation according to the method of Stelos (400). The precipitated globulins were dissolved in a small volume of saline and dialysed against several changes, first of saline and then phosphate-buffered saline pH 8.0, 0.0175 M. The IgG portion of this gamma globulin preparation was isolated by chromatography on a DEAE-cellulose column containing 1-2 gm dry weight of the anion per mg of protein to be fractionated. The protein recovered following elution with phosphate-buffered saline (pH 8, 0.0175 M) had the immunoelectrophoretic characteristics of IgG.

2.5 IMMUNOELECTROPHORESIS

Immunoelectrophoresis was performed according to the method of Scheidegger (401) in 1 percent agar prepared in 0.05 M barbiturate buffer, pH 8.6. A potential gradient of 60 v was maintained across the slides for 40 min.

2.6 PROTEIN QUANTITATION

The amount of protein was estimated by the Lowry method (402).

2.7 PRECIPITIN CURVES

Determinations of anti-gamma-globulin antibodies in goat anti-rabbit-immunoglobulin antiserum were made by the quantitative precipitin technique according to the method of Campbell et al (403).

2.8 ABSORPTION OF GOAT ANTI-RABBIT-IMMUNOGLOBULIN (GARIG) WITH RABBIT GAMMA-GLOBULIN

The antibodies to rabbit gamma-globulin were adsorbed by mixing similar amounts of antiserum and antigen solutions in the zone of equivalence as determined by the precipitin curve: in brief, 2 ml of GARIG diluted two fold were added to 2 ml of

rabbit gamma globulin, Cohn fraction II (Pentex Incorp., Kankakee, Ill., USA) containing 125 μg protein per ml. The mixture was maintained at 37°C for 2 hr and at 4°C for 72 hr, after which time it was spun in the cold. The supernatant was filtered through a Millipore filter and stored at -10°C until used.

2.9 ABSORPTION OF GOAT ANTI-RABBIT-IMMUNOGLOBULIN (GARIG) WITH RABBIT THYMUS OR LYMPH NODE CELLS

Normal and immune thymus or lymph node cells, washed several times, were resuspended in Med-PS in concentration of 100-500 x 10^6 cells per ml. Immune thymus and lymph node cells were obtained from rabbits previously immunized as indicated above. One ml of the cell suspensions was added to one ml of the goat anti-rabbit immunoglobulin antiserum and the mixture was rotated slowly for one hour at 4°C with a multi-purpose rotator (Scientific Industries, Inc., Springfield, Mass.). The cell suspension was then spun in the cold and the sterile supernatant was used as a stimulant in lymph node cell culture using cells obtained from the same animal.

2.10 TANNED CELL HEMAGGLUTINATION TECHNIQUE

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

The hemagglutination test was performed in 13 x 100 mm lipped round bottom test tubes which were held in plastic frames containing 14 tubes to a row. The antiserum to be tested was diluted 10-fold with saline and doubling dilutions were prepared, using one ml volumes. One-tenth ml of the sensitized cells was added to each tube and the tubes were shaken well until the cells were evenly dispersed. The following controls were performed with each experiment: (i) incubation of sensitized cells with diluent

only and (ii) incubation of unsensitized cells with the antiserum. The tubes were allowed to stand at room temperature and usually read 16 - 20 hours later. In control tubes, the cells settled to the bottom to form a compact button. This is considered to be a negative result. In the presence of antibodies, the red cells formed a gelatinous-like layer covering the entire bottom surface of the tube and this disposition of the sensitized cells constitutes a positive hemagglutination reaction. The titer of the antiserum is expressed as the maximum dilution of the antiserum capable of effecting agglutination of the sensitized erythrocytes.

2.11 RADIOAUTOGRAPHY

The radioautographic procedure followed for the analysis of the cell suspension following incubation with the mitogens was essentially that described by Kopriwa and Leblond (404). The cells were spread onto gelatin-coated glass slides (0.5 gm gelatin and 0.05 gm chrome alum, made up to 100 ml with distilled water) which were then air dried. The coating procedure was carried out in a photographic dark room. A safelight filter (Kodak, Wratten Series 2) was used with a 15 Watt lamp at a distance of 3 feet above the working area. Kodak NTB-2 Liquid Nuclear Track Emulsion was heated to 37°C in a water bath and kept at this temperature throughout the procedure. The slides were held vertically by the

marked end and dipped into the emulsion. The excess emulsion on the back of the slide was wiped off with Kleenex tissue. The coated slides, maintained in a vertical position, were allowed to dry for 2-3 hours in complete darkness. During this period, the excess emulsion that drained was absorbed onto gauze. The slides were then stored in light-tight slide boxes containing a dessicating agent (Drierite pellets). The boxes were kept at 4°C for 2-4 days and then developed with Kodak D-19 developer for 3 minutes, followed by passage through a water rinse for one minute and Kodak fixer for 5 minutes. These solutions and the water used were maintained at room temperature. After washing, the slides were stained with Jenner's stain.

2.12 PREPARATION OF CELL SUSPENSIONS

2.12.1 PREPARATION OF HUMAN PERIPHERAL BLOOD LEUCOCYTES FROM NORMAL OR AGAMMAGLOBULINEMIC SUBJECTS

A suspension rich in leucocytes was obtained by gravity sedimentation of heparinized venous blood at 37°C for 45-60 min. The leucocyte-rich plasma layer was then centrifuged at 1000 rpm for 10 min and the cell sediment was washed twice with Med-PS. The cells were diluted to a concentration of 10^6 cells per milliliter

with Med-PS containing 15% autologous, normal or γ globulin-free homologous serum.

2.12.2 PREPARATION OF NORMAL RABBIT PERIPHERAL BLOOD LEUCOCYTES

The rabbit was bled from the heart with a heparinized syringe and the blood immediately diluted with 6 percent dextran in saline (M.W. 250,000, Pharmacia, Uppsala, Sweden) in a ratio 2:1, v/v (blood:dextran). The mixture was introduced into sterile disposable plastic tubes (Falcon Plastics, Los Angeles, Calif.) which were placed in an incubator at 37°C and allowed to sediment at a 60 degree angle for 45 minutes. The leucocyte-rich plasma-dextran layer was then carefully decanted, diluted 10-fold with medium 199 and centrifuged at 800 rpm for 10 minutes. The cells were resuspended in medium 199 and washed once before use.

2.12.3 PREPARATION OF NORMAL RABBIT BONE MARROW CELL SUSPENSIONS

The bone marrow cell suspensions were prepared by flushing the bone marrow from the head and upper shaft of the tibia with several aliquots of normal rabbit serum (NRS) into sterile plastic tubes. We have observed that the stability of the bone marrow cell suspension is enhanced if NRS is used in place of medium 199 and heparin. The cell mass was gently shaken

in the plastic tubes and centrifuged at 500 rpm for 5 minutes. The fatty upper layer was decanted and the cell button was suspended in Med-PS containing 15 percent NRS.

2.12.4 PREPARATION OF CELL SUSPENSIONS FROM RABBIT POPLITEAL LYMPH NODE, SPLEEN, THYMUS, APPENDIX AND SACCULUS ROTUNDUS

Normal or immune rabbits were sacrificed by the intravenous administration of nembutal (50 mg/kg body wt.). The lymph node, spleen, thymus, appendix and sacculus rotundus were removed in rapid order, the entire procedure taking no longer than several minutes. The organs were cut into small fragments and teased through a sterile wire mesh (50 mesh) into Med-PS by the application of slight pressure. The suspensions of dissociated cells were then washed once and diluted in Med-PS containing 15 percent NRS.

3 EXPERIMENTAL PROTOCOLS

3.1 CELL CULTURE PROCEDURES

3.1.1 STIMULATION OF HUMAN PERIPHERAL LYMPHOCYTES IN VITRO WITH PHA AND RABBIT ANTI-HUMAN IMMUNOGLOBULIN ANTISERUM

Four milliliter aliquots of the leucocyte suspension containing 10^6 cells per ml were introduced into individual sterile Falcon plastic tubes and 0.1 ml of the appropriate antiserum or normal rabbit serum was added. In each experiment two tubes received 0.25 ml PHA-M diluted 1:10. The tests were prepared in duplicate and the tubes were sealed and incubated at 37°C for 7 days. Since maximum stimulation by antiserum is observed only after 7 days in culture, those tubes to which PHA was added received this reagent on the 4th day of culture and were harvested on the 7th day as well.

Tritiated thymidine ($T\text{-}H^3$) (2 uc, spec. activity 1C/mM) (Schwarz Bio-Research Inc., Orangeburg, N.J.) was added to the tubes approximately 16 hours prior to the termination of culture at which point the tubes were centrifuged at 1000 rpm for 10-15 minutes, the supernatants were discarded and the cell buttons resuspended in two ml of five percent trichloracetic acid.

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

3.1.2 SIMULATION OF RABBIT LYMPHOID CELLS IN VITRO WITH PHA OR GOAT ANTI-RABBIT IMMUNOGLOBULIN ANTISERUM (GARIG)

The cells were suspended in medium 199 containing penicillin (100 units per ml) and streptomycin (100 µg per ml) (Med-PS) to which

was added either NRS or gamma-globulin-free NRS. Four ml aliquots of each of the cell suspensions (10^6 to 10^7 cells per ml) were transferred to sterile disposable (Falcon) plastic tubes. One quarter ml of the PHA solution or 0.1 or 0.2 ml of GARIG was added to each tube. Control tubes received 0.1 ml of normal goat serum or gamma globulin. The tubes were sealed and incubated at 37°C for 3 days and processed as described above under section 3.1.1. This procedure is illustrated in Fig. 4.

3.1.3 THE EFFECT OF PREINCUBATION OF RABBIT LYMPHOID CELLS WITH GARIG AND COMPLEMENT OR PHA AND COMPLEMENT ON THE SUBSEQUENT RESPONSE TO GARIG AND PHA

Cells of six of the organs previously enumerated (appendix, bone marrow, lymph node, spleen, peripheral blood, sacculus rotundus) were prepared in suspensions containing 4 to 8 x 10^6 lymphocytes per ml. The thymus cells were prepared as suspensions of 20 to 40 x 10^6 cells/ml. One ml aliquots of each cell suspension were dispensed into Falcon plastic tubes and sets of 6 tubes were incubated for one hour at 37°C with one of the following reagents: (a) complement, 0.1 or 0.25 ml; (b) PHA, 0.25 ml; (c) GARIG, 0.2 ml; (d) GARIG, 0.2 ml, plus complement, 0.25 ml; or (e) PHA, 0.25 ml, plus complement, 0.25 ml.

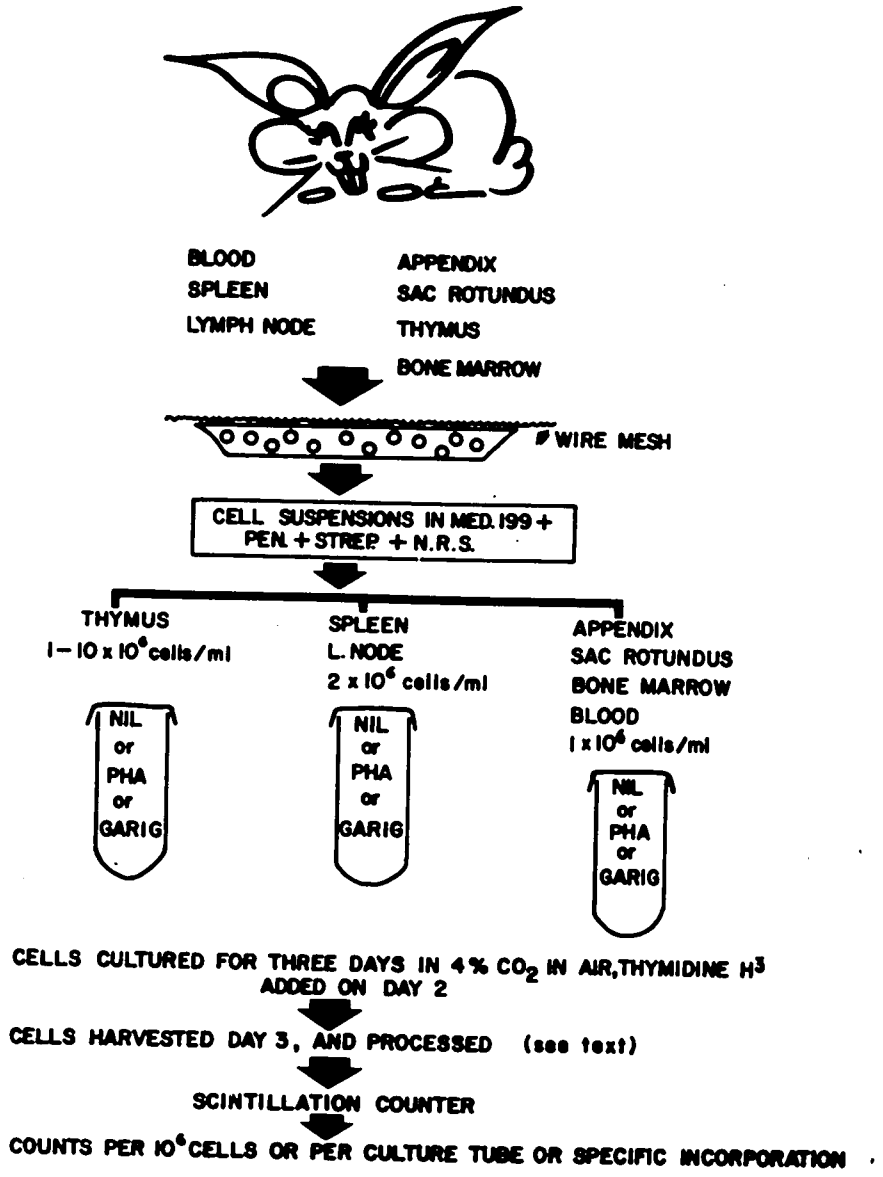


FIGURE 4

PROTOCOL FOR THE STUDY OF THE STIMULATION OF NORMAL RABBIT LYMPHOID CELLS WITH PHA OR GARIG.

Following incubation, all the cell suspensions were washed twice in medium 199 and that of each tube was resuspended in 4 ml of culture medium containing 15 percent gamma globulin-depleted NRS to give final cell concentrations of 5 to 10 x 10^6 cell/ml in the case of the thymus or 1 to 2 x 10^6 cells/ml for all the other organs. Two tubes of each original set of six received PHA (dil. 10-fold, 0.25 ml), two received GARIG (0.1 ml), and two were kept as control. All the tubes were cultured for 3 days and processed as described above under Section 3.1.1.

3.1.4 STIMULATION OF RABBIT LYMPHOID CELLS WITH MITOMYCIN-C INACTIVATED ALLOGENEIC AND XENOGENEIC CELLS

Human and rabbit peripheral leucocytes were used as stimulating cells. Human cells were obtained by gravity sedimentation of heparinized blood at 37°C for 45-60 minutes. As stated above, rabbit blood was mixed with dextran before sedimentation. The leucocytes recovered by centrifugation of the buffy coat were washed twice in medium 199 and resuspended in medium 199 containing 15 percent normal rabbit serum in a concentration of 2 x 10^6 cells per ml. The one-way stimulation test was carried out by the method of Bach and Woynow (346). The leucocytes were incubated with 25 gamma Mitomycin-C (Nutritional Biochemicals, Cleveland, Ohio)

per ml of cell suspension for 20 minutes at 37°C followed by 3 washes to remove the drug. The cells of the lymphoid organs of the rabbits, referred to as the responding cells, were suspended in Med-PS-NRS in a cell concentration of 2×10^6 cells per ml and tested for their ability to respond to the mitomycin-C treated rabbit (allogeneic) and human (xenogeneic) leucocytes. The stimulating cells were mixed with an equal number of responding cells to give a final combined cell concentration of 2×10^6 cells/ml in a total volume of 4 ml. Thymus cells were generally used in higher concentrations (2 to 10 x 10^6 cells/ml) for reasons discussed below. The mixed leucocyte cultures were maintained for 5 days at 37°C. Tritiated thymidine (2 uc) was added to each tube about sixteen hours prior to the termination of culture and the extent of radioactive incorporation by the responding cells was determined by the method described in Section 3.1.1.

3.1.5 THE EFFECT OF PREINCUBATION OF RABBIT IMMUNE SPLEEN AND LYMPH NODE CELLS WITH GARIG OR GARIG AND COMPLEMENT ON THEIR SUBSEQUENT RESPONSE TO HSA OR PHA

Cells of spleen and lymph nodes obtained from animals immunized with HSA or BSA 1 to 10 months previously were prepared in suspensions containing 8×10^6 lymphocytes per ml. One ml

aliquots of each cell suspension were dispensed into Falcon plastic tubes and sets of 10 tubes were incubated for one hour at 37°C with either (a) GARIG, 0.2 ml, (b) GARIG, 0.2 ml, plus complement 0.25 ml or (c) NGGG. Following incubation, all the cell suspensions were washed twice in Med-PS and that of each tube was resuspended in 4 ml of culture medium containing 15 percent NRS to give a final concentration of 2×10^6 cells/ml. Two tubes of each original set of ten were kept as control, two received PHA (dil. 10-fold, 0.25 ml), and the remaining six received the specific antigen in concentrations varying from 10 mg to 100 µg. The tubes were cultured for 3, 5 or 7 days and processed as described under Section 3.1.1.

3.1.6 STIMULATION OF RABBIT IMMUNE SPLEEN OR LYMPH NODE CELLS WITH PHA, GARIG, ANTIGEN (HSA), PHA PLUS ANTIGEN OR GARIG PLUS ANTIGEN

Cells of spleen and lymph nodes obtained from animals immunized with HSA 3 to 4 weeks previously were suspended in Med-PS to which was added 15 percent gamma globulin-free NRS. Four ml aliquots of each of the cell suspensions containing 2×10^6 cells per ml were transferred to sterile tubes. PHA, GARIG or HSA was added to each tube. Some tubes received PHA and HSA or GARIG and HSA simultaneously. Control tubes received 0.1 ml of normal goat serum. The tubes were sealed, incubated at 37°C for 3 or 7 days and processed as described under Section 3.1.1.

3.2 THE ABILITY OF RABBIT IMMUNE SPLEEN OR LYMPH NODE CELLS, PRE-INCUBATED WITH GARIG, NGGG OR ANTIGEN (HSA), TO CONFER ANTIBODY FORMING CAPACITY, WITH RESPECT TO HSA, TO IRRADIATED (800 R) IMMUNOINCOMPETENT RABBITS

Cells of the spleen and lymph nodes obtained from animals immunized with HSA 3 to 4 weeks previously were prepared in suspensions containing 3 to 20 x 10^6 lymphocytes per ml. One ml aliquots of each cell suspension was dispensed into Falcon plastic tubes and sets of 20 tubes were incubated for one hour at $37^{\circ}C$ with either GARIG 0.2 ml, NGGG 0.2 ml, HSA 1 mg or nil. At the end of the incubation period, the tubes which had been incubated with GARIG or NGGG each received 1 mg of HSA and the tubes which had been incubated with HSA each received 0.2 ml of GARIG. The tubes which had been incubated with medium only received no stimulant. After several minutes in the incubator, the content of the tubes were withdrawn and divided equally between four irradiated (800r) rabbits. The recipient rabbits were irradiated 1 to 3 hours before receiving the cells. The rabbits were bled at intervals of time and the circulating antibody titers were determined by the passive hemagglutination technique. This procedure is illustrated in Figure 5.

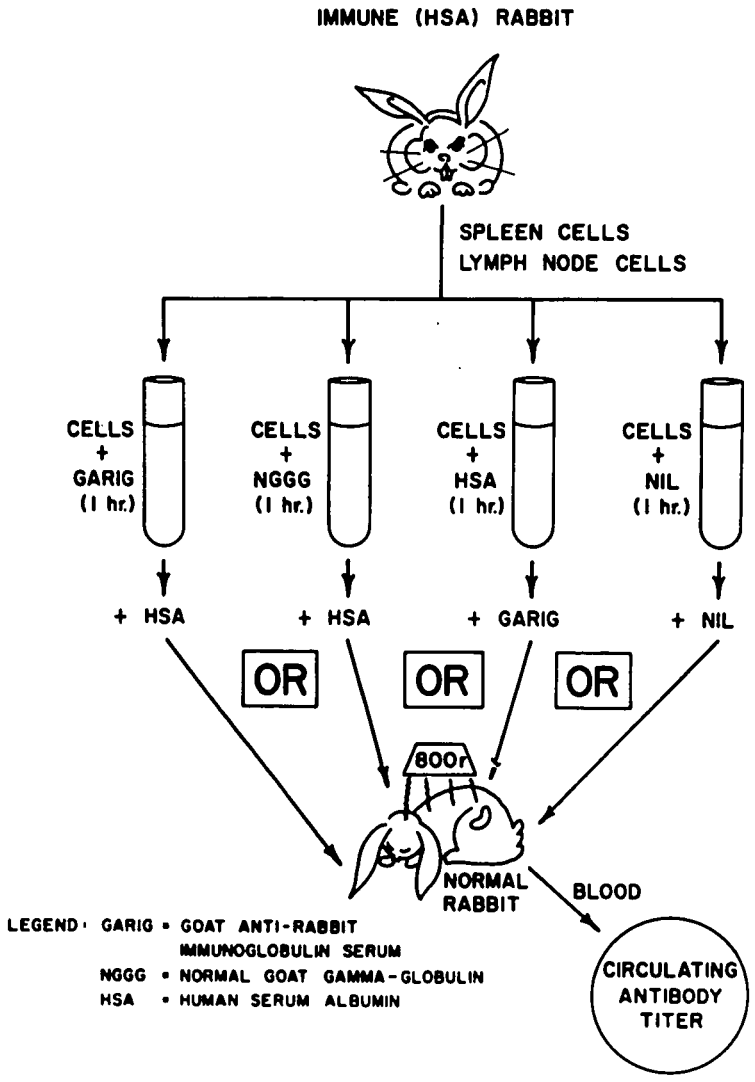


FIGURE 5

PROTOCOL FOR THE STUDY OF ANTIBODY FORMATION IN IRRADIATED UNIMMUNIZED RABBITS INJECTED WITH LYMPHOID CELLS PRE-INCUBATED FOR ONE HOUR WITH EITHER NGGG, GARIG OR HSA, FOLLOWED BY THE ADDITION OF HSA OR GARIG TO THE CULTURES PRIOR TO THEIR INJECTION INTO IRRADIATED HOSTS.

3.3 THE UPTAKE OF GARIG-I^{125} AND NGGG-I^{125} BY NORMAL RABBIT LYMPHOID CELLS AND ERYTHROCYTES

The IgG fractions of GARIG and NGGG were prepared as described in Section 2.4. They were labelled with I^{125} at the rate of 1 atom of iodine per molecule of protein by the iodine monochloride method of McFarlane (405). The starting solutions contained each 30 mg of protein per ml. They were diluted about 5-6 times during the labelling procedure, and exhibited a final specific activity of about 150 μc/mg. One ml aliquots of each of the lymphoid cell suspensions (10^6 cells per ml) prepared from the various lymphoid organs as well as an equal number of rabbit erythrocytes were incubated with either the I^{125} labelled gamma globulin fraction of GARIG (referred to as GARIG-I^{125}) or NGGG-I^{125} for 1 hour at $4^{\circ}C$. The GARIG-I^{125} and NGGG-I^{125} were used in equal protein concentration, giving almost identical specific activity. The cell suspensions were then washed 5 times with 10 ml of medium each time. The washed cells and the last washes were analyzed for their radioactive content using a Packard gamma counter. The results are presented as counts per minute per 10^6 cells or as specific activity.

CHAPTER IV

EXPERIMENTAL PROCEDURES AND RESULTS

1 THE RESPONSE OF NORMAL AND AGAMMAGLOBULINEMIC LYMPHOCYTES TO PHA AND ANTI-IMMUNOGLOBULIN ANTISERUM

PROCEDURE

Peripheral lymphocytes of normal and agammaglobulinemic subjects (Tables 2 and 3) were cultured as described in Section 3.1.1 of Chapter III at a cell concentration of 1×10^6 cells/ml. They were tested for their capacity to respond to stimulation with PHA or anti-immunoglobulin antiserum by an increased incorporation of tritiated thymidine. Preliminary studies indicated that several pools of rabbit antisera directed predominantly against one of the light or heavy chains of human immunoglobulins could stimulate the lymphocytes of normal individuals. Antisera showing the highest capacity to transform lymphocytes were selected for this study. They were used at the concentration found to stimulate the maximum uptake of tritiated thymidine: generally 0.05 to 0.2 ml per culture tube.

RESULTS

The uptake of tritiated thymidine by the cells stimulated

by antiserum was maximum after 7 days in culture, whereas it was maximum in cultures stimulated with PHA after 3 days (Table 4). For this reason, in all subsequent experiments, the tubes to which PHA was to be added received this reagent on the 4th day of culture, and all cultures were harvested on day 7. In general, the uptake of tritiated thymidine by normal lymphocytes was enhanced when cultured in medium containing gamma-globulin-free homologous serum rather than autologous or normal homologous serum (Table 5).

Incorporation of label by the lymphocytes of agammaglobulinemic subjects Nos. 2 and 3 following stimulation with antiserum was consistently low and often was not higher than that incorporated by control lymphocytes to which no antiserum was added. Cells of subject No. 1 gave a very high response with each of the anti-immunoglobulin antisera but not with normal rabbit serum or antiserum to human serum albumin. This response is comparable to that of lymphocytes of normal individuals (Table 6).

Incorporation of label by the cells of a normal control stimulated with anti-immunoglobulin antiserum was enhanced when they were cultured in Med-PS containing plasma obtained from any of the three agammaglobulinemic subjects (Table 7).

Cells of patients Nos. 1 and 3 gave a normal PHA response. The response of cells of patient No. 2 to PHA was reduced compared to that of the normal controls (Table 6).

The therapeutic administration of gamma-globulin to these patients did not result in any detectable alteration of the blastogenic response of their peripheral lymphocytes.

COMMENTS

These studies demonstrate, on the basis of the lymphocyte response to PHA and anti-immunoglobulin antiserum, a difference in responsiveness to stimuli of lymphocytes of patients presenting with an almost identical syndrome. The defect resulting in the failure of the lymphocytes of two patients to respond to anti-immunoglobulin antiserum cannot be attributed to inhibiting factors in their sera since the whole sera of these patients manifested no inhibitory activity when added to normal lymphocytes in the presence of anti-immunoglobulin antiserum.

It is particularly interesting to note the dissociation between the responses to PHA and anti-immunoglobulin antiserum, readily apparent in the response patterns of the lymphocytes of patients 2 and 3 but not found with the lymphocytes of normal individuals and patient No. 1. This dissociation is best illustrated in Table 8.

The response of the cells of patient No. 1 to anti-immunoglobulin antiserum indicates that immunoglobulin or immunoglobulin-like material is an integral part of these cells. If these

structures must be present at the cell surface for antigen recognition to ensue, the normal response of patient No. 1 to anti-immunoglobulin antiserum would indicate that the defect preventing the production of immunoglobulin in this patient is situated beyond the stage of antigen recognition.

TABLE 2

CLINICAL SUMMARY OF THREE PATIENTS WITH AGAMMAGLOBULINEMIA

Case No.	Sex	Age (yr)	Age onset of symptoms	Type of Agammaglobulinemia	Complications and associated abnormalities
1	M	6	9 mos.	Congenital, no family history	Dermatomyositis, recurrent aspetic meningitis and mental retardation.
2	M	24	6 yr.	Acquired, Primary	Advanced bronchiectasis with pulmonary insufficiency. Anaphylactoid reactions to injection of γ globulin.
3	F	46	30 yr.	Acquired, primary	Recurrent diarrhea over five year period, now cleared. Frequent URI's.

TABLE 3

IMMUNOLOGICAL STATUS OF THREE PATIENTS WITH AGAMMAGLOBULINEMIA

Case No.	Serum Immuno-globulin (mg%)*	Circulating Antibody resp.	Periph. lympho. per mm^3	Histology	Delayed Hypersensitivity
1	γG: 20-300 γA: 1 γM: 1 γD: 1	None to typhoid-para-typhoid Tetanus, polio	2520-3500	No plasma cells in marrow No lymphoid follicles	Skin test**: positive for Mumps DNCB: Not done
2	γG: 34-180 γA: 1 γM: 1 γD: 1	None to diphtheria, tetanus polio	987-3048	Follicles seen in Lymph nodes	Skin test: negative DNCB: Negative
3	γG: 180-200 γA: 12-20 γM: 7-15 γD: 1	None to typhoid-para typhoid B Diphtheria, tetanus	728-2040	Few plasma cells in marrow	Skin test: negative DNCB: Negative

* Average values of 10 controls. γG: 1300, γA:320, γM: 110, γD: 6.

** The following antigens were used: Old tuberculin, P.P.D., Candida Albincans, Mumps, Trichophyton, Histoplasma capsulotum, Steptokinase - Streptodornase

TABLE 4

INCORPORATION OF TRITIATED THYMIDINE BY NORMAL HUMAN CIRCULATING LYMPHOCYTES, CULTURED IN MED-PS CONTAINING 15 PER CENT AUTOLOGOUS PLASMA, INCUBATED FOR DIFFERENT PERIODS OF TIME WITH PHA OR ANTI-IMMUNOGLOBULIN SERUM

Stimulant added to culture	Day of addition of stimulant	Incorporation of tritiated thymidine (counts per minute) by cultures on day:		
		3	5	7
None or NRS	0	744	732	356
PHA	0	112,359	---	---
PHA	2	---	59,171	---
PHA	4	---	---	46,231
R 135 Anti-IgG	0	1,206	1,688	4,378
R 125 Anti-IgG	0	854	12,215	38,610

TABLE 5

INCORPORATION OF TRITIATED THYMIDINE BY NORMAL HUMAN PERIPHERAL LYMPHOCYTES CULTURED IN MED-PS CONTAINING NORMAL OR GAMMA-GLOBULIN-FREE HOMOLOGOUS SERUM

Stimulant added to culture	Incorporation of tritiated thymidine (counts/min/culture) by cells incubated in medium enriched with:		
	Autologous serum	Homologous serum	Gamma-globulin-free serum
Nil	5,172	4,648	2,398
PHA	80,621	92,715	87,683
R 125 Anti-IgG	16,340	12,330	25,875

TABLE 6

IN VITRO INCORPORATION OF TRITIATED THYMIDINE

BY AGAMMAGLOBULINEMIC PERIPHERAL LYMPHOCYTES*

STIMULANT	UPTAKE OF TRITIATED THYMIDINE (counts per min.)					
	PATIENT 1		PATIENT 2		PATIENT 3	
	Before γ glob.	After γ glob.	Before γ glob.	After γ glob.	Before γ glob.	After γ glob.
None	619	458	88	390	376	252
PHA	58,479	49,806	7,684	6,894	38,283	33,242
R 82 Anti γG	20,836	22,687	77	219	374	130
R 136 Anti γG	14,833	19,810	151	89	410	190
R 130 Anti γM	17,475	26,059	156	93	986	420
R 75 Anti γA	28,227	not done	91	193	572	369
R 170 Anti γD	11,396	18,756	113	85	1,628	1,553
R 83 Anti-λ light chain	3,136	4,439	60	130	327	200
R 132 anti-κ light chain	2,943	not done	275	135	717	316

* The lymphocytes were stimulated in medium containing 15% autologous plasma before and 2 days after administration of gamma globulin to the patients.

TABLE 7

IN VITRO INCORPORATION OF TRITIATED THYMIDINE BY NORMAL LYMPHOCYTES IN NORMAL AND PATIENT'S PLASMA

	Uptake of tritiated thymidine (counts per min) by the lymphocytes incubated in:				
Stimulant	Autologous Plasma	γ glob. depleted normal plasma	Agammaglobulinemic Plasma 1	2	3
None	1066	349	1214	1183	1375
PHA	101010	81967	90909	103092	112965
R 125 Anti γG	8442	15600	10869	10098	8933
R 131 Anti γA	4753	7693	8708	6841	6346
R 83 Anti-λ	12222	19417	12300	12769	14079

TABLE 8

DISSOCIATION OF THE RESPONSES TO PHA AND ANTI-IMMUNOGLOBULIN ANTISERUM OF THE CIRCULATING LYMPHOCYTES OBTAINED FROM THREE PATIENTS WITH AGAMMAGLOBULINEMIA

Patient No.	Relative blastogenic response of circulating lymphocytes incubated with: PHA	Anti-immunoglobulin antiserum
1	++++	++++
2	+++	+
3	+++	+

2 RESPONSE OF NORMAL RABBIT LYMPHOCYTES TO PHA AND GOAT ANTI-RABBIT IMMUNOGLOBULIN ANTISERUM (GARIG)

PROCEDURE

Lymphoid cells from peripheral blood, bone marrow, appendix, lymph node, spleen, sacculus rotundus and thymus were prepared in suspensions and cultured as indicated in Chapter III, Section 3.1.2. They were tested for their ability to respond to PHA or GARIG. The incorporation of tritiated thymidine by the cell suspensions was determined according to the procedure detailed in Chapter III, Section 3.1.1.

For radioautographic analysis, the cell suspensions at the termination of culture were smeared onto gelatin-coated glass slides which were then dried, coated with the photographic emulsion, stored at $4^{\circ}C$ and developed and stained in the manner described in Chapter III, Section 2.11. The goat anti-rabbit immunoglobulin preparation used in all the experiments reported in this section contained 500 µg of antibody per ml directed against rabbit gamma-globulin, as determined by the quantitative precipitation technique. Preliminary experiments indicated that 0.1 to 0.2 ml of the antiserum, equivalent to 50 - 100 µg of antibody protein, exhibited the optimal stimulatory effect toward rabbit lymphocytes.

RESULTS

The time for optimal and maximum stimulation of rabbit spleen cells by both PHA and GARIG was three days in our test system (Table 9). Cells incubated with NGGG for three days did not incorporate more tritiated thymidine than did control cultures.

The stimulation with GARIG was greater when the cells were cultured in medium fortified with NRS depleted of gamma-globulin, as compared to medium contining whole NRS (Table 10).

PHA and GARIG varied greatly with respect to the period of time necessary for incubation with the lymphocytes in order to get maximum stimulation. Only one hour of incubation with PHA, followed by incubation for three days without PHA, was sufficient to permit optimum stimulation of the normal rabbit spleen cells (Table 11). Similar results were obtained with the lymphoid cells of the other organs tested. Such was not the case with GARIG, however. Incubation of the lymphocytes with GARIG for 1, 6, or 24 hours followed by incubation for 3, 2.75 or 2 days without GARIG, respectively, resulted in no significant stimulation. Optimum stimulation with GARIG was observed only if the cells were incubated with this stimulant for the entire culture period (Table 11).

The cells of all the lymphoid organs tested, with the exception of the thymus, gave an optimum response to PHA and GARIG when incubated in a concentration of 1 to 2 x 10^6 cells/ml. This is

demonstrated in Table 12 where it can be seen that the amount of tritiated thymidine incorporated by rabbit splenocytes increased as the concentration of the cultured cells was increased beyond 2×10^6 cells/ml. However, the maximum specific incorporation of thymidine, i.e., the ratio of thymidine uptake by the stimulated culture to that taken up in the control culture, was obtained when the cultures contained 1 or 2×10^6 cells/ml. Almost identical results were obtained with cells from the peripheral blood, bone marrow, appendix, sacculus rotundus and lymph nodes.

The thymus cells reacted optimally to PHA at a cell concentration of 10^7 per ml (Table 13). Although the thymus lymphocytes at times responded to PHA when cultured at a lower cell concentration (1 to 2×10^6 cells/ml), their response to PHA could be markedly improved by increasing the cell concentration to 5 to 10×10^6 cells/ml (Table 13).

The lymphoid cells of all the organs tested responded to both PHA and GARIG, except the thymus cells which did not respond to GARIG (Tables 13 and 14). The responses of the different lymphoid cell suspensions varied markedly, however, being only marginal for the bone marrow cells, which displayed a specific activity of no more than 2 to 3 (Table 14).

The results of some radioautographic analyses are presented in Fig. 6. The majority of the labelled cells following incubation

with GARIG or PHA were distinguished as blast cells. There appeared to be a correlation between the uptake of tritiated thymidine by the cell suspensions, as determined by scintillation counting, and the number of labelled cells seen in radioautographs. Control specimens incubated in the absence of mitogens, as well as thymocytes incubated with GARIG, contained only very few labelled cells on day 3. Thymocytes incubated with PHA, as well as all the other cell populations incubated with either GARIG or PHA, showed a significant increase in labelling when compared with control cultures.

COMMENTS

These studies demonstrate that the cells of six of the seven lymphoid organs of the normal rabbit were stimulated to synthesize new DNA and incorporate tritiated thymidine following in vitro incubation with PHA or GARIG. However, there were great differences in the responses of the different cell populations to each mitogen. The bone marrow responded moderately to both stimulants; the appendix cells responded very markedly to GARIG, but much less to PHA, whereas the reverse was true for the sacculus rotundus cells, which responded more to PHA than to GARIG. When cultured at the optimum cell concentration, the thymocytes responded to PHA better than most of the other cell populations. However, at no time did the thymocytes give a significant response to GARIG.

These studies demonstrate that, in the normal rabbit, a population of cells (thymocytes) exists which responds to one mitogen (PHA) and not to another (GARIG).

TABLE 9

TRITIATED THYMIDINE UPTAKE OF NORMAL RABBIT SPLEEN LYMPHOCYTES INCUBATED WITH PHA OR GARIG FOR 3 OR 5 DAYS IN CELL CULTURE IN VITRO

Stimulant Added to Cultures	Day of Addition of Stimulant	Tritiated Thymidine Uptake by Cultures on Day. (Counts per Minute)*	
		3	5
None	0**	1,259	594
NGGG	0	1,360	670
PHA	0	14,623	723
PHA	2	--	4,819
GARIG	0	6,074	1,384

* Each value represents the mean of triplicate determinations.

** Day 0 represents the beginning of incubation.

TABLE 10

INCORPORATION OF TRITIATED THYMIDINE BY NORMAL RABBIT SPLEEN CELLS CULTURED IN VITRO FOR 3 DAYS IN MEDIUM CONTAINING NORMAL OR GAMMA-GLOBULIN DEPLETED HOMOLOGOUS SERUM

Stimulant Used	Incorporation of Tritiated Thymidine (Counts/Min/Culture)* by Cells Incubated in Medium Enriched with	
	Normal Rabbit Serum	Gamma-globulin Depleted Rabbit Serum
None	927	1,218
PHA	4,257	4,463
GARIG	2,368	5,959

* Each value represents the mean of triplicate determinations.

TABLE 11

THE EFFECT OF VARYING THE INCUBATION TIME OF NORMAL RABBIT SPLEEN CELLS WITH PHA AND GARIG ON THE SUBSEQUENT INCORPORATION OF TRIAIATED THYMIDINE BY THE CELLS INCUBATED IN CULTURE FOR THREE DAYS

Stimulant Added	Duration of Incubation with Stimulant	Duration of Incubation in Absence of Stimulant	Incorporation of Tritiated Thymidine by the Cells after 3 Days of Culture (Counts per Minute per Culture*)
None	Nil	3.00 Days	490
PHA	1 Hour	3.00 Days	2,725
PHA	6 Hours	2.75 Days	3,075
PHA	3 Days	Nil	3,150
GARIG	1 Hour	3.00 Days	500
GARIG	1 Day	2.00 Days	550
GARIG	2 Days	1.00 Days	1,374
GARIG	3 Days	Nil	2,430

* Each value represents the mean of triplicate determinations.

TABLE 12

THE IN VITRO RESPONSE TO PHA AND GARIG OF NORMAL RABBIT SPLEEN CELLS INCUBATED AT DIFFERENT CELL CONCENTRATIONS

Cell conc/ml	Incorporation of tritiated thymidine (count per minute and mean specific activity) by cells incubated with the following:				
	NIL (Control)	PHA		GARIG	
	c/p/m*	c/p/m	MSA**	c/p/m	MSA
1×10^6	2035	20,872	10	7,612	3.7
2×10^6	2719	40,816	15	15,723	5.7
4×10^6	5615	52,309	9.3	16,501	2.9

* c/p/m = Counts per minute. Each value represents the mean of triplicate determinations.

** Mean Specific Activity, which is defined as the ratio of thymidine uptake by the stimulated culture to that taken up by the control.

TABLE 13

THE IN VITRO RESPONSE TO PHA AND GARIG OF NORMAL RABBIT THYMOCYTES INCUBATED AT DIFFERENT CELL CONCENTRATIONS

Cell Conc/Ml	Uptake of Tritiated Thymidine by the Thymus Cells (counts/min/culture)* Incubated in the Presence of		
	No Stimulant Added	PHA	GARIG
1×10^6	323	188	267
1×10^6	125	460	230
1×10^6	752	1,463	765
5×10^6	708	5,030	1,102
5×10^6	530	6,482	531
10×10^6	466	3,733	1,006
10×10^6	530	15,251	364
10×10^6	311	2,198	303
10×10^6	1,292	14,509	1,938
10×10^6	607	5,858	1,229

* Each value represents the mean of triplicate determinations.

TABLE 14

PROLIFERATIVE RESPONSE OF NORMAL RABBIT LYMPHOCYTES

TO PHA AND GARIG

Cells of Organ Tested	Cell Conc/ml (4 ml)	Stimulant Used (Counts/Min/Culture)*			Mean Specific Activity**	
		Control	GARIG	PHA	GARIG	PHA
Thymus	10×10^6	1,292	1,938	14,509	1.5	13.0
Bone Marrow	1×10^6	31,250	83,457	85,443	2.4	2.2
Spleen	2×10^6	2,460	13,755	11,105	6.2	8.3
Lymph Nodes	2×10^6	693	6,838	5,981	9.5	7.3
Sacculus Rotundus	1×10^6	205	4,612	13,192	11.0	44.0
Peripheral Lymphocytes	1×10^6	1,204	16,513	11,412	22.0	7.0
Appendix	1×10^6	94	3,019	615	30.0	5.6

* Based on a single experiment. Mean value of triplicate determinations.

** Ratio of thymidine uptake by stimulated culture to that taken up in control culture. Based on five different experiments.

a

b

c

FIGURE 6

RADIOAUTOGRAPHS OF RABBIT LYMPH NODE CELLS STIMULATED IN VITRO FOR THREE DAYS WITH a) PHA b) GARIG c) NGGG.

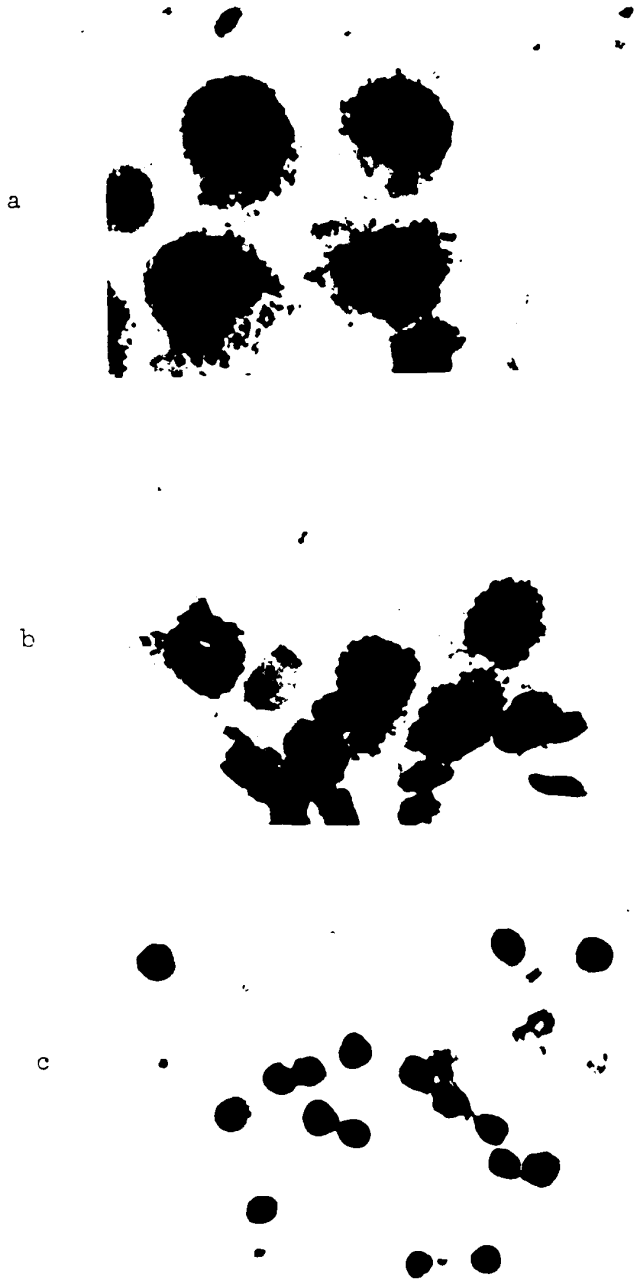


FIGURE 6

RADIOAUTOGRAPHS OF RABBIT LYMPH NODE CELLS STIMULATED IN VITRO FOR THREE DAYS WITH a) PHA b) GARIG c) NGGG.

3 THE EFFECT OF PREINCUBATION OF RABBIT LYMPHOID CELLS WITH GARIG AND COMPLEMENT OR PHA AND COMPLEMENT ON THE SUBSEQUENT RESPONSE TO GARIG AND PHA

PROCEDURE

The procedure used in the following experiment has been described under METHODS (Chapter III, Section 3.1.3). In brief, the cells were preincubated with either complement, PHA, GARIG, PHA plus complement (C') or GARIG plus complement, washed free of reagents and subsequently cultured in vitro at the optimum cell concentrations indicated in EXPERIMENT II. They were tested for their ability to respond to PHA and GARIG.

RESULTS

As can be seen in Table 15, pretreatment of the spleen cells with PHA for one hour followed by culture for three days in the absence of PHA still resulted in almost maximum blastogenesis as compared to cultures incubated in the presence of PHA for the entire 3 day period. Interestingly enough, the addition of GARIG to the cell suspension which has been pre-incubated with PHA or PHA and C' for only one hour induced a further increase in the blastogenic response of these cells, as compared to the cell incubated with either GARIG or PHA alone, indicating an additive effect. This effect of PHA and GARIG could be

demonstrated for the cells of all the lymphoid organs investigated, except for the thymus (Table 19). Pre-incubation of the cells with PHA and C' did not alter the response of the cells as compared to cells pre-treated with PHA only. Cells preincubated with C' or GARIG for one hour subsequently behaved in the same manner as the control cells (Table 15). On the other hand, pre-treatment of the cells for one hour with GARIG and C' resulted in complete suppression of the ability of the cells to respond to GARIG. However, the response to PHA was, if anything, enhanced (Table 15). This effect of pre-incubation of lymphoid cells with GARIG and C' was consistently found for cells of all the lymphoid organs evaluated (Tables 16, 17, 18) and required a finite amount of complement (0.25 ml). A lesser amount of C' had a less marked effect or no effect at all.

The antisera were tested for complement-dependent cytotoxicity by the dye exclusion test. The antisera showed great variability in their complement dependent cytotoxic effect. Generally, the number of cells from the peripheral blood, bone marrow, lymph node, spleen, sacculus rotundus and appendix killed by GARIG plus complement varied between 5 and 12 percent. In only two tests was the number of dead lymph node cells found to be much higher, reaching levels of 23 and 27 percent. In the absence of complement, the number of dead cells seldom exceeded 1 percent. Pre-incubation of thymocytes with GARIG in the presence or absence of complement resulted in 3 to 5 percent dead cells (Table 20).

COMMENTS

These studies demonstrate that the population of lymphocytes capable of responding to GARIG can be selectively inactivated by pre-incubation of the lymphoid cells with GARIG plus complement. The capacity of the cells to respond to PHA remained unimpaired following this treatment.

TABLE 15

THE INCORPORATION OF TRITIATED THYMIDINE BY NORMAL RABBIT SPLEEN CELLS STIMULATED WITH PHA AND GARIG FOLLOWING PREINCUBATION OF THE CELLS FOR ONE HOUR WITH GARIG AND COMPLEMENT (C'), PHA AND C', C', PHA OR GARIG

Cells Preincubated for one hour with	Incorporation of Tritiated Thymidine by the Preincubated cells following Incubation for Three More Days in the Presence of (Counts per Minute per Culture)*		
	NIL	PHA	GARIG
NIL	336	3,693	1,458
C'	387	2,941	1,002
PHA	2,729	2,892	5,285
PHA + C'	2,701	2,403	4,118
GARIG	458	4,569	1,245
GARIG + C'	334	4,141	302

* Each value represents the mean of triplicate determinations.

TABLE 16

PROLIFERATIVE RESPONSE OF NORMAL RABBIT APPENDIX AND BONE MARROW LYMPHOID CELLS TO PHA AND GARIG AFTER PRETREATMENT FOR ONE HOUR WITH GARIG AND COMPLEMENT OR PHA AND COMPLEMENT (C')

Cells of Organ Tested (4 ml)	Stimulant Added	Uptake of Tritiated Thymidine by the Cells Pretreated with (Counts/Min/Culture)*			(Mean Specific Activity)**		
		NIL	PHA + C'	GARIG + C'	NIL	PHA + C'	GARIG + C'
Appendix	None	131	943	153		8.0	1.2
1×10^6/ml	PHA	1,256	1,275	1,728	8.7	8.1	8.0
	GARIG	818	1,627	201	7.8	12.0	1.1
Bone Marrow	None	7,826	12,754	7,681		2.0	0.9
1×10^6/ml	PHA	23,764	15,767	17,731	2.4	2.2	2.2
	GARIG	16,446	23,789	7,416	2.2	2.4	0.9

* Based on a single experiment. Mean value of triplicate determinations.
** Ratio of thymidine uptake by stimulated culture to that taken up in control culture. Based on five different experiments.

TABLE 17

PROLIFERATIVE RESPONSE OF NORMAL RABBIT LYMPH NODE AND PERIPHERAL BLOOD LYMPHOCYTES TO PHA AND GARIG AFTER PRETREATMENT FOR ONE HOUR WITH GARIG AND COMPLEMENT OR PHA AND COMPLEMENT (C')

Cells of Organ Tested (4 ml)	Stimulant Added	Uptake of Tritiated Thymidine by Cells Pretreated With (Counts/Min/Culture)*			(Mean Specific Activity)**		
		Nil	PHA + C'	GARIG + C'	Nil	PHA + C'	GARIG + C'
Lymph node	none	323	2,506	729		10.0	1.2
2×10^6/ml	PHA	4,609	3,413	6,771	12.0	12.4	10.0
	GARIG	1,553	5,220	751	8.0	16.0	1.2
Peripheral lymphocytes	none	770	2,952	428		6.0	1.1
1×10^6/ml	PHA	3,500	3,433	2,508	6.0	8.1	6.3
	GARIG	2,590	5,843	504	6.2	11.7	1.2

* Each value represents the mean of triplicate determinations. Values are representative of a single experiment.

** Ratio of thymidine uptake by stimulated culture to that taken up by control culture. Values based on five different experiments.

TABLE 18

PROLIFERATIVE RESPONSE OF NORMAL RABBIT SPLEEN AND SACCULUS ROTUNDUS LYMPHOID CELLS TO PHA AND GARIG AFTER PRETREATMENT FOR ONE HOUR WITH GARIG AND COMPLEMENT OR PHA AND COMPLEMENT (C')

Cells of Organ Tested (4 ml)	Stimulant Added	Uptake of Tritiated Thymidine by Cells Pretreated with (Counts/Min/Culture)*			(Mean Specific Activity)**		
		Nil	PHA + C'	GARIG + C'	Nil	PHA + C'	GARIG + C'
Spleen	None	305	4,066	444		10.0	1.1
2×10^6/ml	PHA	5,593	4,858	6.053	12.0	12.5	15.0
	GARIG	6,502	9,099	569	9.1	19.0	1.1
Sacculus Rotundus	None	238	2,686	211		17.0	0.9
	PHA	4,576	3,761	3,272	20.0	17.7	15.0
1×10^6/ml	GARIG	1,607	4,022	312	7.1	22.0	1.8

* Each value represents the mean of triplicate determinations. Values are representative of a single experiment.

** Ratio of thymidine uptake by stimulated culture to that taken up by control culture. Values based on five different experiments.

TABLE 19

PROLIFERATIVE RESPONSE OF NORMAL RABBIT THYMOCYTES TO PHA AND GARIG AFTER PRETREATMENT FOR ONE HOUR WITH GARIG AND COMPLEMENT OR PHA AND COMPLEMENT (C')

Cells/ml (4 ml)	Stimulant Added	Uptake of Tritiated Thymidine by the Cells Pretreated with (Counts/Min/Culture)*			(Mean Specific Activity)**		
		Nil	PHA + C'	GARIG + C'	Nil	PHA + C'	GARIG + C'
2×10^6/ml	none	150	450	122		2.6	0.9
	PHA	512	609	566	2.5	3	2.7
	GARIG	112	415	147	1	2.2	1.3
5×10^6/ml	none	170	1,118	215		10.0	1.0
	PHA	1,713	1,384	1,757	10.0	8.7	11.0
	GARIG	247	1,048	260	1.2	9.0	1.2

* Each value represents the mean of triplicate determinations. Values are representative of a single experiment.

** Ratio of thymidine uptake by stimulated culture to that taken up by control culture. Values based on five different experiments.

TABLE 20

THE CYTOTOXIC EFFECT OF GARIG AND COMPLEMENT

DETERMINED BY THE DYE EXCLUSION TEST

Cells of Organ Tested	Percentage of Dead Cells Following Incubation with GARIG*	
	No Complement Added	Complement Added
Appendix	2%	6%
Bone Marrow	1	4
Lymph Node	1	12
Peripheral lymphocytes	0	5
Sacculus rotundus	3	8
Spleen	0.5	10
Thymus	3	5

* Average results of two experiments

4 THE EFFECT OF PRE-INCUBATION OF GARIG WITH RABBIT GAMMA-GLOBULIN OR RABBIT LYMPHOID CELLS ON THE CAPACITY OF GARIG TO STIMULATE NORMAL RABBIT LYMPHOID CELLS IN VITRO

PROCEDURE

GARIG was incubated with either rabbit gamma-globulin, at the point of equivalence, or with large numbers of normal or immune rabbit lymphoid cells as described in sections 2.8 and 2.9 of Chapter III.

RESULTS

The absorption of GARIG with rabbit immunoglobulins at the point of equivalence completely abolished its capacity to stimulate rabbit lymphocytes (Table 21). When GARIG was incubated with 100 or 500 x 10^6 normal or immune cells from the thymus or lymph nodes only an insignificant and/or inconsistent decrease in the stimulating capacity was noted (Table 22).

COMMENTS

These studies demonstrate that the mitogenic activity of GARIG can be directly attributed to the specific anti-rabbit immunoglobulin antibodies present in this antiserum, since the activity

was lost following absorption with rabbit gamma-globulin. These findings suggest, therefore, the existence of immunoglobulins or immunoglobulin-like receptors on the lymphocyte surface.

The failure of lymphoid cells to absorb the mitogenic agent in GARIG may be attributed to an insufficient number of immunoglobulin-like receptors on the cell surface. This concept will be discussed in greater detail in the General Discussion.

TABLE 21

THE STIMULATORY CAPACITY OF GARIG FOLLOWING ABSORPTION WITH RABBIT GAMMA-GLOBULIN

Cells of Organ Tested	Specific Incorporation of Tritiated Thymidine by Cells Stimulated with:		
	PHA	GARIG	ABS-GARIG*
Lymph node	8	7.5	1.7
Spleen	11	9	1.2
Thymus	9.2	1.3	1.1

* GARIG absorbed with rabbit gamma-globulin at the point of equivalence.

TABLE 22

THE STIMULATORY CAPACITY OF GARIG FOLLOWING ABSORPTION WITH RABBIT THYMUS OR LYMPH NODE CELLS

Stimulant added to culture	Mean specific activity of cultures of lymph node cells stimulated in vitro for three days
PHA	8
GARIG	7.5
GARIG-T *	8.2
GARIG-LN **	6.8

* GARIG absorbed with thymus cells

** GARIG absorbed with lymph node cells

5 THE RESPONSE OF RABBIT LYMPHOCYTES TO MITOMYCIN-C-TREATED ALLOGENEIC AND XENOGENEIC LEUCOCYTES

PROCEDURE

The procedure was essentially that described in Chapter III, Section 3.1.4. Rabbit cells from the different lymphoid organs (responding cells) were tested for their ability to respond to mitomycin-C-treated rabbit or human peripheral blood leucocytes (stimulating cells). Control tubes contained cells of the responding lymphoid cell populations, half of which had been treated with mitomycin-C. The mixed cultures and control tubes contained 2×10^6 cells per ml. Incubation was terminated on day 5 and the cells were processed in the manner described in Chapter III, Section 3.1.1.

RESULTS

The response of rabbit lymphoid cells stimulated with mitomycin-C-treated allogeneic and xenogeneic cells was optimum at day 5, although at times a slightly greater response in terms of absolute counts could be obtained at day 7. All cultures were therefore terminated on day 5.

All the rabbit lymphoid cells responded to the inactivated xenogeneic cell stimulus (human peripheral lymphocytes inactivated with mitomycin-C) (Table 23). However, the response was generally

diminished when inactivated allogeneic cells (mitomycin-C-treated rabbit peripheral leucocytes) were used as stimulants. The rabbit bone marrow and spleen cells responded only slightly (Table 23). The response obtained with thymus lymphocytes was comparable with that obtained with the other lymphoid cells.

COMMENT

Each population of rabbit lymphoid cells showed a distinct pattern in its response to stimulation with either the allogeneic or xenogeneic leucocytes. The thymocytes consistently responded to stimulation with inactivated leucocytes even at a cell concentration of 10^6 cells per ml, a condition which permitted only a very low and inconsistent response to PHA, at best.

TABLE 23

RESPONSE OF RABBIT LYMPHOCYTES TO XENOGENEIC AND ALLOGENEIC MITOMYCIN-C-TREATED-LEUCOCYTES

Cells of Organs Tested	Cell Conc/ml (4 ml)	Stimulant Used (Counts/Min/Culture)*			Mean Specific Activity+	
		Control	H_m**	R_m***	H_m	R_m
Appendix	1×10^6	104	3,232	642	22.0	4.5
Bone marrow	1×10^6	3,100	7,286	1,638	3.0	1.2
Sacculus Rotundus	1×10^6	160	6,171	1,817	11.0	6.0
Peripheral Lymphocytes	1×10^6	212	1,015	1,700	4.3	3.9
Lymph Nodes	1×10^6	82	1,426	7,145	18.0	40.0
Spleen	1×10^6	773	2,178	1,647	5.7	1.6
Thymus	1×10^6	20	271	51	10.8	2.6
Thymus	5×10^6	33	917	57	26.6	2.5

* Based on a single experiment. Mean value of triplicate determinations.
** H_m - Mitomycin-treated human peripheral leucocytes.
*** R_m - Mitomycin-treated rabbit peripheral leucocytes.
\+ Ratio of thymidine uptake by stimulated culture to that taken up in control culture. Based on five different experiments.

6 THE UPTAKE OF GARIG-I^{125} AND NGGG-I^{125} BY NORMAL RABBIT LYMPHOID CELLS

PROCEDURE

The procedure to determine the uptake of GARIG-I^{125} or NGGG-I^{125} by rabbit lymphoid cells or erythrocytes has been described in detail in Section 3.3 of Chapter III. In preliminary experiments, 10^6 lymph node or thymus cells were incubated with various amounts of GARIG-I^{125} or NGGG-I^{125}. The uptake of both materials by the cells increased with increasing concentrations of GARIG-I^{125} or NGGG-I^{125}. The highest concentration of labelled material used in these experiments was 100 µg/ml.

RESULTS

At all concentrations of labelled materials (from 0.01 µg up to 100 µg/ml) the thymocytes incorporated almost identical amounts of GARIG-I^{125} and NGGG-I^{125}. Lymph node cells always took up 5 times more GARIG-I^{125} than NGGG-I^{125} when incubated with concentrations varying from 100 to 0.1 µg/ml. At lower concentrations the differences tended to diminish.

The uptake of radioactivity by the different lymphoid cell populations incubated with identical amounts of NGGG-I^{125} (1 µg/10^6 cells) was about the same for all the organs studied and was not much greater

than that taken up by the erythrocytes (Table 24).

On the other hand, the uptake of GARIG-I^{125} by lymphoid cells was much greater than that taken up by the red blood cells, with the sole exception of the thymus cells which adsorbed a lesser amount of radioactivity, equal to that taken up by the rabbit erythrocytes. The concentration of GARIG-I^{125} incubated with the cells was 1 $\mu g/10^6$ cells (Table 25).

COMMENT

In their ability to bind GARIG-I^{125} or NGGG-I^{125}, the thymocytes behaved almost like the erythrocytes in that they took up an almost similar amount of either material. All the other lymphoid cell populations took up a much greater amount of GARIG-I^{125} than NGGG-I^{125}. This would suggest that thymocytes, just like erythrocytes, lack the immunoglobulin sites which specifically bind GARIG.

TABLE 24

UPTAKE OF RADIOACTIVITY BY NORMAL RABBIT RED BLOOD CELLS AND LYMPHOID CELLS FOLLOWING INCUBATION FOR TWO HOURS WITH NGGG-I^{125}

Cells of Organ Incubated	Uptake of Radioactivity by the Incubated Cells (Counts per Minute per 10^6 Cells)*	Specific Activity**
Appendix	2,852	1.1
Bone Marrow	5,371	2.1
Lymph Node	2,706	1.1
Peripheral lymphocytes	6,023	2.4
Spleen	6,550	2.7
Sacculus Rotundus	2,635	1.1
Thymus	2,271	0.9
Red Blood Cells	2,520	-

* Each value represents the mean of triplicate determinations.

** The specific activity is defined as the ratio of the uptake of NGGG-I^{125} by the lymphoid cell suspension to that taken up by the red blood cells.

TABLE 25

UPTAKE OF RADIOACTIVITY BY NORMAL RABBIT RED BLOOD CELLS AND LYMPHOID CELLS FOLLOWING INCUBATION FOR TWO HOURS WITH GARIG-I^{125}

Cells of Organ Incubated	Uptake of Radioactivity by the Incubated Cells (Counts per Minute per 10^6 Cells)*	Specific Activity**
Appendix	12,446	4.9
Bone Marrow	13,350	5.3
Lymph Node	18,710	7.3
Peripheral Lymphocytes	14,500	5.9
Spleen	35,053	13.7
Sacculus Rotundus	10,855	4.2
Thymus	3,662	1.4
Red Blood Cells	2,550	-

* Each value represents the mean of triplicate determinations.

** The specific activity is defined as the ratio of the uptake of GARIG-I^{125} by the lymphoid cell suspension to that taken up by the red blood cells.

7 THE EFFECT OF PRE-INCUBATION OF RABBIT IMMUNE SPLEEN AND LYMPH NODE CELLS WITH GARIG OR GARIG AND COMPLEMENT ON THEIR SUBSEQUENT RESPONSE TO HSA OR PHA

PROCEDURE

The procedure was essentially the one described in Chapter III, Section 3.1.5. Spleen and lymph node cells obtained from rabbits immunized with HSA 4 weeks to 10 months previously were incubated with NGGG, GARIG or GARIG plus complement for one hour, washed free of these reagents and tested for their ability to respond to PHA and HSA in vitro by the incorporation of tritiated thymidine. Preliminary experiments indicated that the optimal or maximal response to antigen by immune spleen or lymph node cells occurred between day 3 and day 7 of incubation. Therefore all cultures were terminated on day 3, 5 or 7. In each experiment the immune cells were incubated with various concentrations of antigen. The stimulation obtained with the antigen concentration exhibiting the optimum activity is reported here.

RESULTS

Pre-incubation of immune cells with NGGG did not affect their subsequent response to antigen or PHA. On the other hand, pre-incubation of immune cells with GARIG or GARIG plus complement reduced the specific uptake of tritiated thymidine by the cells in the presence

of antigen (HSA) without affecting their capacity to respond to PHA (Figs. 7 - 10). The inhibition of the capacity to respond to antigen was almost complete for immune spleen and lymph node cells at day 3. Lymph node cells of some rabbits immunized 4 weeks before had a tendency to overcome the inhibitory effect of GARIG by day 5 or 7 of culture. However at no time did their mitogenic response reach the levels attained by control cells incubated with NGGG (Figs. 9 and 10).

COMMENTS

These studies demonstrate that the interaction between antigens and immune cells, which leads to blastogenesis and mitosis can be blocked by pre-incubation of these cells with anti-immunoglobulin antiserum, thereby supporting the concept that a specific antigen interacts with immune cells through antibody-like cell membrane receptors.

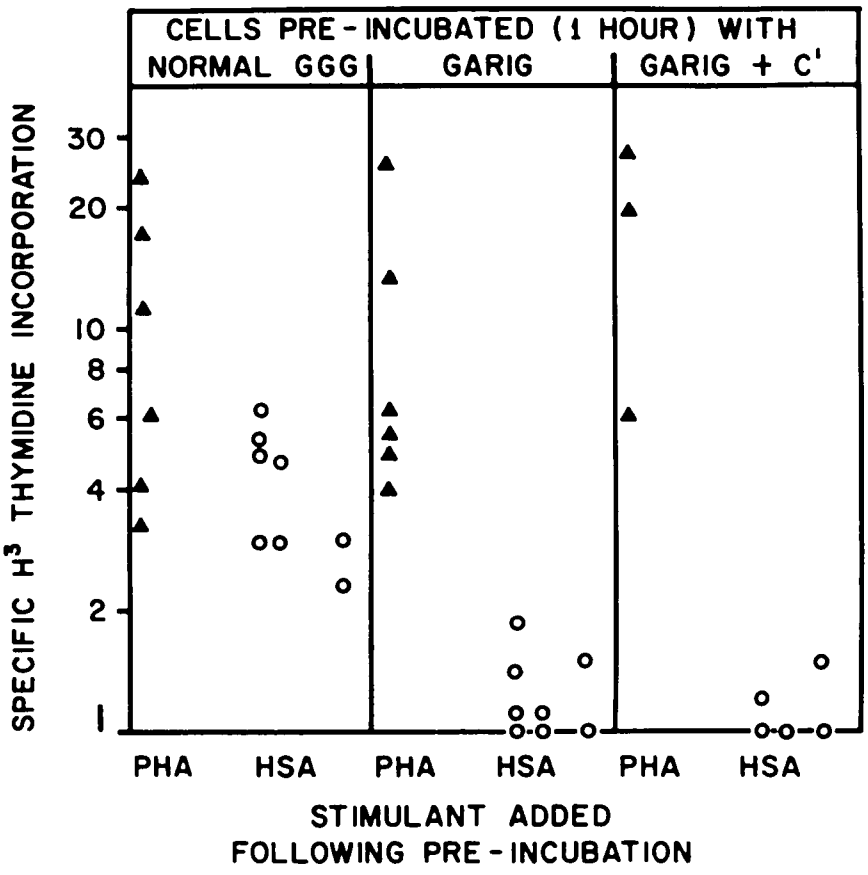


FIGURE 7

THE INHIBITION OF ANTIGEN-INDUCED SPECIFIC BLASTOGENESIS (DAY 3) OF IMMUNE RABBIT SPLEEN LYMPHOCYTES BY PRIOR INCUBATION OF THESE CELLS WITH GARIG OR GARIG AND COMPLEMENT.

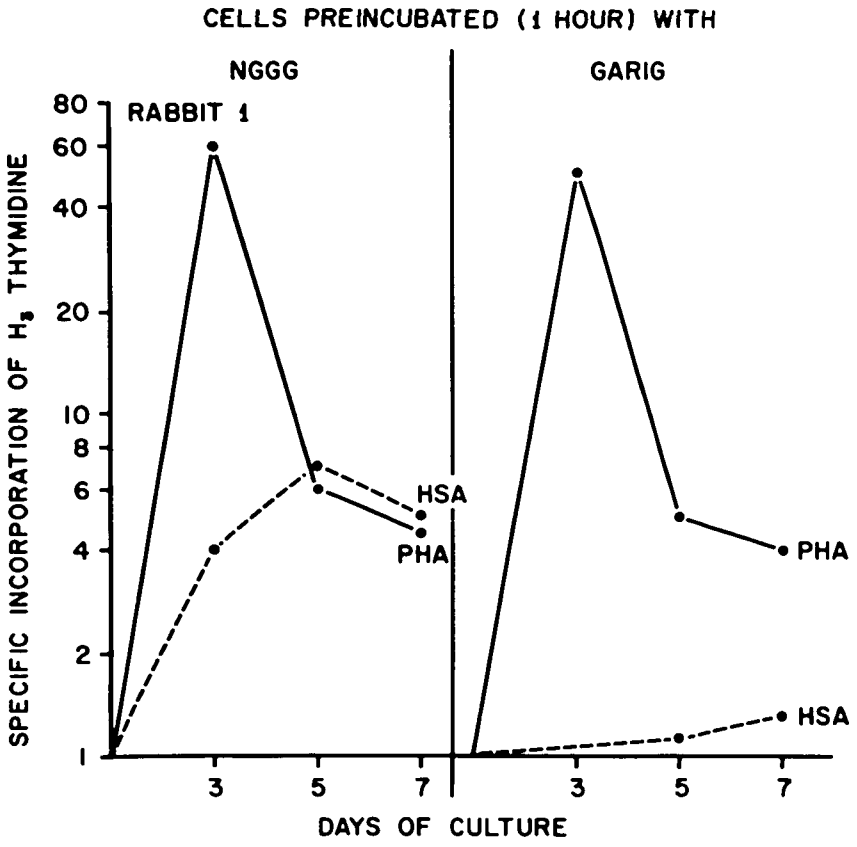


FIGURE 8

SPECIFIC INCORPORATION OF H_3 THYMIDINE BY LYMPH NODE CELLS OF HSA IMMUNIZED RABBITS STIMULATED WITH PHA OR HSA FOLLOWING PRE-INCUBATION FOR ONE HOUR WITH NGGG OR GARIG.

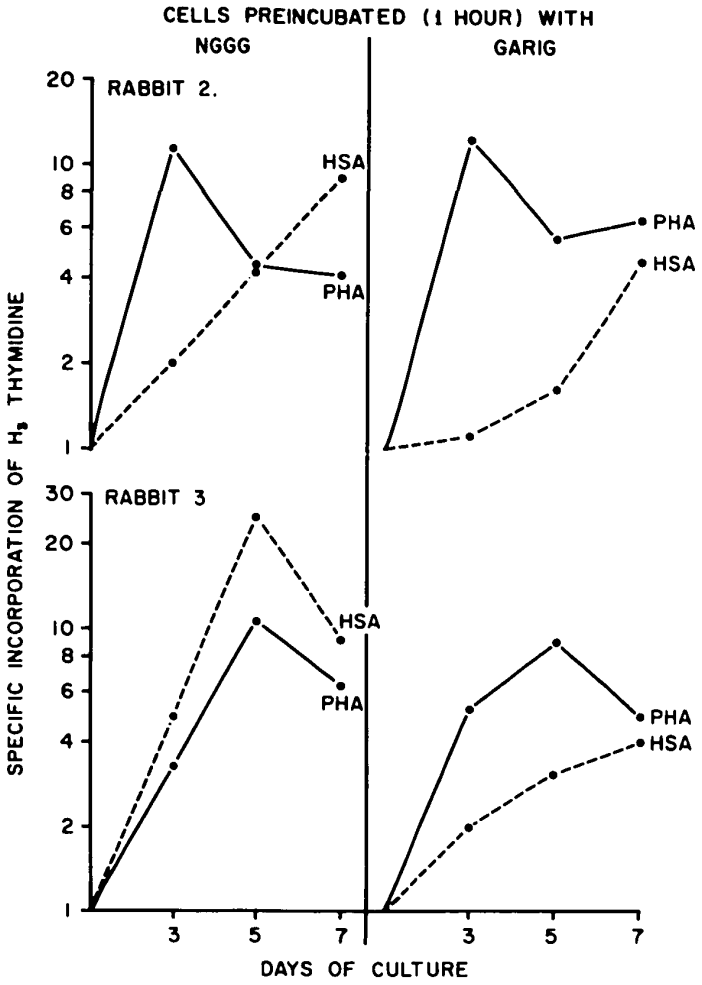


FIGURE 9

SPECIFIC INCORPORATION OF H_3 THYMIDINE BY LYMPH NODE CELLS OF HSA IMMUNIZED RABBITS STIMULATED WITH PHA OR HSA FOLLOWING PRE-INCUBATION FOR ONE HOUR WITH NGGG OR GARIG.

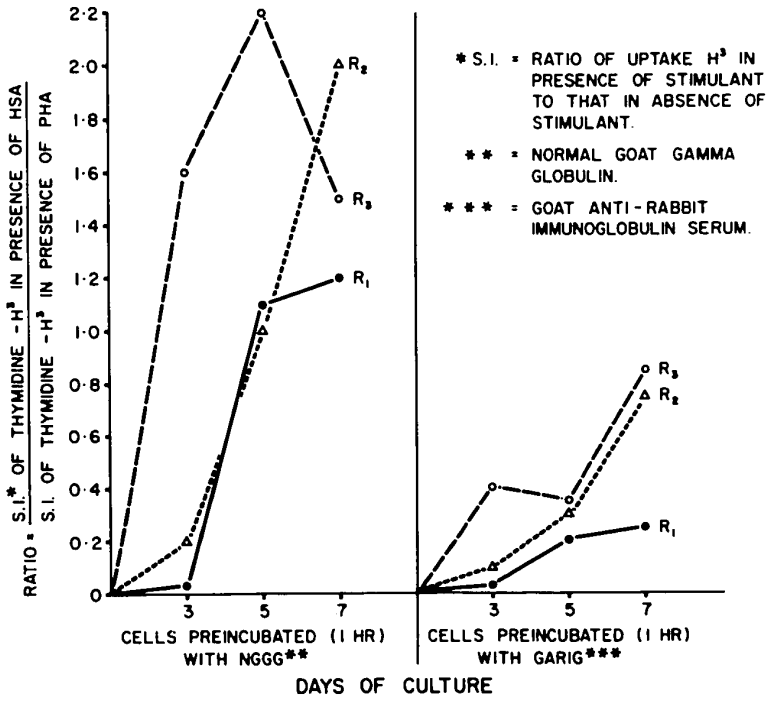


FIGURE 10

UPTAKE OF THYMIDINE H^3 BY IMMUNIZED (HSA) RABBIT LYMPH NODE CELLS STIMULATED WITH PHA OR HSA FOLLOWING PRE-INCUBATION FOR ONE HOUR WITH NGGG OR GARIG.

8 STIMULATION OF RABBIT IMMUNE SPLEEN OR LYMPH NODE CELLS WITH PHA, GARIG, ANTIGEN (HSA), PHA PLUS ANTIGEN OR GARIG PLUS ANTIGEN

PROCEDURE

The procedure used in these experiments has been described in Chapter III, Section 3.1.6. In brief, lymph node cells of rabbits immunized 4 weeks previously with HSA were tested for their ability to respond to PHA, GARIG, HSA, PHA plus HSA or GARIG plus HSA. The cultures were terminated at day 3 or 7 and the amount of tritiated thymidine incorporated by the cells was estimated by the technique described in Chapter III, Section 3.1.1.

RESULTS

The incorporation of tritiated thymidine was always greater when immune cells were incubated with PHA and HSA together than with either stimulant alone. Figure 11 shows the results of a typical experiment demonstrating a truly additive effect between PHA and HSA. Table 26 shows the mean specific activity of cultures stimulated according to the procedure just described. There was no additive effect between GARIG and HSA and moreover, when the cultures were maintained for 7 days, the presence of GARIG completely abolished the stimulatory effect of HSA.

COMMENT

These experiments suggest that PHA and antigen trigger two different populations of lymphocytes, in view of the additive effect of these two stimulants for immune cells. The same does not seem to be true for GARIG and antigen, which showed no additive effect in vitro. The latter two reagents would therefore appear to interact with identical sites on the same cells.

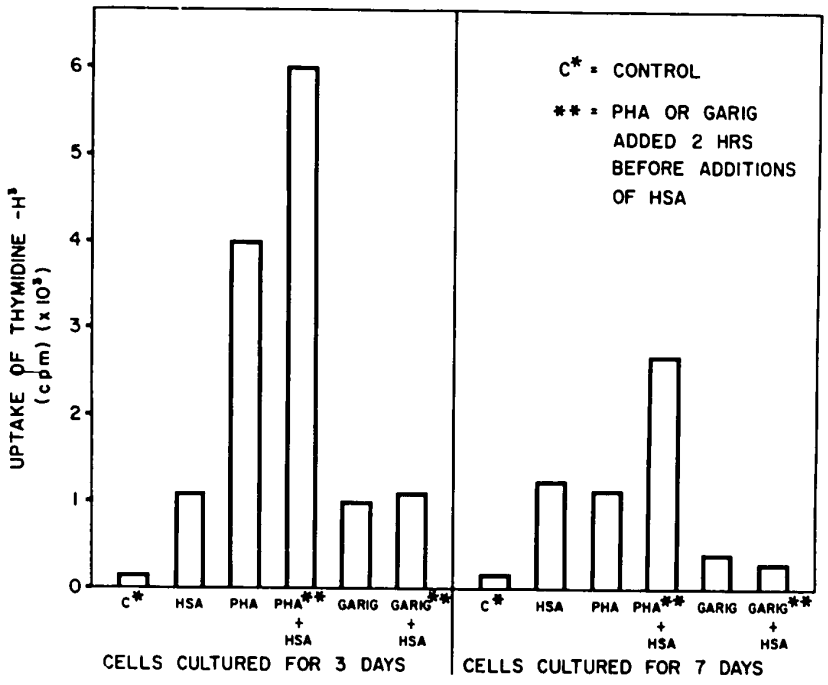


FIGURE 11

INCORPORATION OF THYMIDINE H^3 BY IMMUNE (HSA) RABBIT LYMPH NODE CELLS STIMULATED IN VITRO WITH HSA, PHA, GARIG, PHA PLUS HSA OR GARIG PLUS HSA.

TABLE 26

INCORPORATION OF TRITIATED THYMIDINE BY RABBIT IMMUNE (HSA) LYMPH NODE CELLS STIMULATED WITH PHA, HSA, GARIG, PHA PLUS HSA OR GARIG PLUS HSA

Stimulant added to culture	Mean specific activity* of cultures of immune lymph node cells, stimulated in vitro at day:	
	3	7
PHA	7	2.5
GARIG	4.7	1.2
HSA	3.4	5
PHA+HSA	9.3	7
GARIG+HSA	5	0.9

* Based on 5 different experiments

9 THE ABILITY OF IMMUNE RABBIT SPLEEN OR LYMPH NODE CELLS, PRE-INCUBATED WITH GARIG, NGGG OR ANTIGEN (HSA), TO CONFER ANTIBODY FORMING CAPACITY WITH RESPECT TO HSA TO IRRADIATED (800 R) IMMUNO-INCOMPETENT RABBITS

PROCEDURE

The procedure used in these experiments is described in Chapter III, Section 3.2. Lymph node and spleen cells from immunized rabbits pre-incubated with GARIG (1 mg of antibody) or NGGG were tested for their ability to transfer to irradiated recipients the capacity to form antibody against HSA. HSA was added to the pre-incubated cells prior to their transfer. Cells pre-incubated with HSA received GARIG prior to transfer to irradiated animals. Control irradiated rabbits received only incubated immune cells without antigen. The rabbits were bled at intervals of 5 to 7 days after cell transfer and the circulating antibody titers were determined by the passive hemagglutination technique (Chapter III, Section 2.10).

In preliminary experiments, it was established that the 800 R irradiated rabbits did not produce antibodies when challenged with 25 mg of HSA for at least 4 - 6 weeks following irradiation

RESULTS

Control animals which received immune cells without antigen

as well as those animals which received 15 to 50 x 10^6 immune cells pre-incubated with GARIG but subsequently mixed with antigen (5 - 10 mg of HSA) produced very little or no antibody against HSA. On the other hand, pre-incubation with NGGG did not prevent the production of antibody in recipient rabbits (Tables 27-29 and Fig. 12). The inhibitory effect of GARIG could be partly overcome when a larger number of cells (100 x 10^6) were transferred and the antigen challenge increased to 20 mg. However, the antibody titers of rabbits receiving cells pre-incubated with GARIG were less than those of animals which were injected with immune cells pre-incubated with NGGG (Table 30).

COMMENTS

These studies demonstrate that the ability of immune cells to transfer the capacity to form humoral antibodies to irradiated recipients upon challenge with the specific antigen can be blocked by the pre-incubation of these cells with anti-immunoglobulin antisera. The mechanism whereby antigen stimulates immune cells to form antibody seems to involve the interaction of the antigen with cell surface antibody-like receptors.

TABLE 27

ANTIBODY FORMATION IN IRRADIATED (800r) RABBITS INJECTED WITH ALLOGENEIC IMMUNE SPLEEN CELLS AND ANTIGEN (HSA). THE EFFECT OF PRE-INCUBATION OF THE SPLEEN CELLS IN VITRO WITH GARIG, NGGG OR HSA

Cells (15×10^6) Pre-Incubated with the Following for 1 hour Prior to Cell Transfer	Reagent Added to Pre-Incubated Cells Immediately Prior to Cell Transfer	Hemagglutinating Antibody Titer* on Following days Subsequent to cell transfer to Irradiated Recipient Rabbits				
		5	10	14	21	30
NIL	NIL	20	20	0	0	0
GARIG	HSA (5 mg)	0	0	0	20	20
NGGG	HSA (5 mg)	0	0	40	640	80
HSA (5 mg)	GARIG	0	0	80	640	320

* Each value represents the mean of the titers of four different rabbits

TABLE 28

ANTIBODY FORMATION IN IRRADIATED (800r) RABBITS INJECTED WITH ALLOGENEIC IMMUNE LYMPH NODE CELLS AND ANTIGEN (HSA). THE EFFECT OF PRE-INCUBATION OF THE CELLS IN VITRO WITH GARIG OR NGGG

Cells (50×10^6) Pre-Incubated with the Following for 1 Hour Prior to Cell Transfer	Reagent Added to Pre-Incubated Cells Immediately Prior to Cell Transfer	Hemagglutinating Antibody Titer* Subsequent to Cell Transfer to Irradiated Recipient Rabbits				
		5	10	14	21	30
Nil	Nil	0	0	20	20	20
GARIG	HSA (10 mg)	0	0	0	20	0
NGGG	HSA (10 mg)	0	80	160	320	80

* Each value represents the mean of the titers of four different rabbits.

TABLE 29

ANTIBODY FORMATION IN IRRADIATED (800r) RABBITS INJECTED WITH ALLOGENEIC IMMUNE LYMPH NODE CELLS AND ANTIGEN (HSA). THE EFFECT OF PRE-INCUBATION OF THE LYMPH NODE CELLS IN VITRO WITH GARIG OR NGGG

Cells (50×10^6) Pre-Incubated with the Following for 1 Hour Prior to Cell Transfer	Reagent Added to Pre-Incubated Cell Immediately Prior To Cell Transfer	Hemagglutinating Antibody Titer* Subsequent to Cell Transfer to Irradiated Recipient Rabbits				
		5	10	14	21	30
Nil	Nil	0	0	0	0	0
GARIG	HSA (10 mg)	0	0	0	0	0
NGGG	HSA (10 mg)	0	640	2560	640	160

* Each value represents the mean of four different rabbits.

TABLE 30

ANTIBODY FORMATION IN IRRADIATED (800r) RABBITS INJECTED WITH ALLOGENEIC IMMUNE LYMPH NODE CELLS AND ANTIGEN (HSA). THE EFFECT OF PRE-INCUBATION OF THE CELLS IN VITRO WITH GARIG OR NGGG

Cells (100×10^6) Pre-incubated with the Following for 1 Hour Prior to Cell Transfer	Reagent Added to Pre-Incubated Cells Immediately Prior to Cell Transfer	Hemagglutinating Antibody Titer* Subsequent to Cell Transfer to Irradiated Recipient Rabbits				
		5	10	14	21	30
Nil	Nil	0	0	0	0	0
GARIG	HSA (20 mg)	0	0	80	160	160
NGGG	HSA (20 mg)	0	160	640	1280	640

* Each value represents the mean of the titers of four different rabbits.

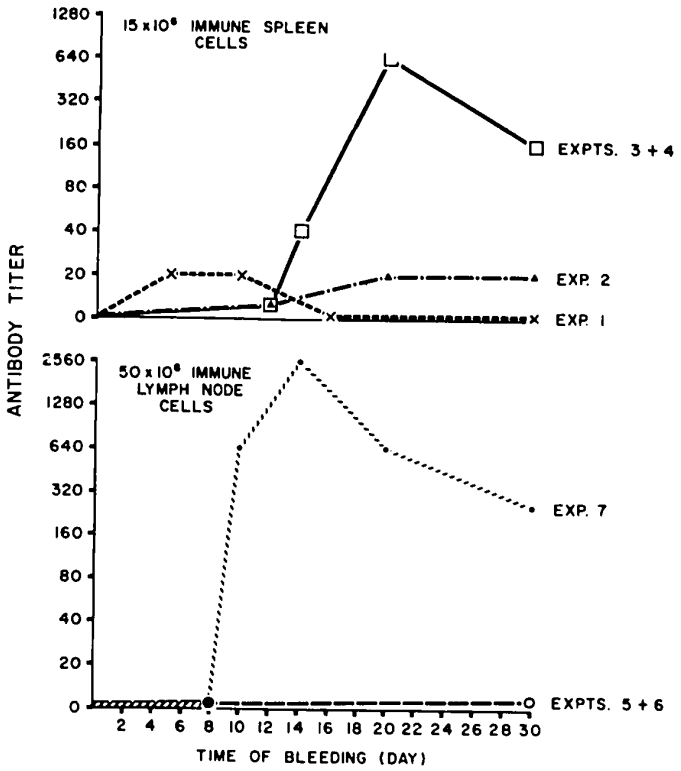


FIGURE 12

ANTIBODY FORMATION IN IRRADIATED (800r) RABBITS INJECTED WITH EITHER ALLOGENEIC IMMUNE SPLEEN CELLS (15×10^6) OR IMMUNE LYMPH NODE CELLS (50×10^6) AND ANTIGEN (HSA). THE EFFECT OF PRE-INCUBATION OF THE CELLS WITH GARIG, NGGG OR HSA.

Expts. 1 and 5 - Rabbits Injected with Cells Incubated in Medium Only
Expts. 2 and 6 - Rabbits Injected with HSA and Cells Pre-Incubated for One Hour with GARIG.
Expts. 3 and 7 - Rabbits Injected with HSA and Cells Pre-Incubated for One Hour with NGGG.
Expt. 4. - Rabbits Injected with GARIG and Cells Pre-Incubated for One Hour with HSA.

CHAPTER V

GENERAL DISCUSSION

The purpose of this investigation was to demonstrate the heterogeneous nature of the lymphoid cells on the basis of their in vitro responses to a number of mitogenic stimuli considered to possess immunologic significance and, if possible, to relate the responses to these mitogens to the capacity of the lymphoid cell to participate in humoral and/or cellular reactions. As was discussed at length in the Historical Review, lymphocytes can be separated from each other by a number of physico-chemical techniques. Attempts have also been made to differentiate the lymphocytes on the basis of their in vitro responses to mitogenic stimuli (307). However, one cannot state with any degree of certainty that the same lymphocytes can react to the different stimuli or that different populations of cells are so affected. Very few attempts have been made to relate the in vitro response of the lymphocyte to the immunocompetent state. Furthermore, little is known about the nature and composition of the receptor sites on the lymphocytes with which the different stimulating agents interact (306, 307). One reason for this dearth of knowledge may be that the majority of the studies reviewed above utilized only one or a few mitogens and/or lymphocytes from one or, at best, several

of the lymphoid organs. Any attempts to extrapolate from one study to the other would be exceedingly difficult in view of the fact that mitogenic agents do not all behave in the same way, are not derived from even similar sources and impart marked differences in the patterns of the induced responses (306).

The experiments carried out in this investigation utilized a minimum of one mitogen from each sub-group of mitogens enumerated in Chapter II, Section 4.1, as well as the lymphocytes of all the organs considered to be immunologically relevant.

The technique of cell culture was utilized here since it permits for a continuous sampling and manipulation of the cells which could not be performed in vivo. Furthermore, one can analyze the individual responses of lymphoid cells of a number of different organs simultaneously under identical conditions.

The initial series of experiments were carried out in order to establish the optimal conditions for the stimulation of the lymphoid cells by the different stimulants, i.e., cell concentration, period of incubation, etc. It is interesting to note that, upon culture of rabbit lymphoid cells with PHA and anti-immunoglobulin antiserum, the optimal period of incubation is three days with respect to both stimulants. However, human peripheral blood lymphocytes stimulated with PHA and anti-immunoglobulin antiserum respond optimally at days 3 and 7, respectively.

The response of both human and rabbit lymphocytes to anti-immunoglobulin antiserum was enhanced when the cells were cultured in medium fortified with gamma-globulin-depleted homologous serum rather than whole serum. The use of gamma-globulin-depleted serum also ruled out the possibility of stimulation by antigen-antibody complexes which would have resulted from interaction of normal gamma-globulin molecules in the medium with antibodies directed to them in the anti-immunoglobulin serum. Indeed, such complexes have been found to cause blastogenesis (406, 407).

Some of the parameters used for the demonstration of the functional heterogeneity of lymphoid cells in the different lymphoid organs of the normal rabbit are illustrated in Figure 13. Studies were also carried out with immune rabbit lymphoid cells and circulating lymphocytes from normal and agammaglobulinemic subjects. The main findings may be recapitulated as follows:

(a) There were present in the circulation of two of the three agammaglobulinemic patients lymphocytes which were capable of responding to PHA and not to anti-immunoglobulin antiserum. This contrasted with the situation found in normal individuals and a third patient with congenital agammaglobulinemia, whose lymphocytes could always respond to both PHA and anti-immunoglobulin antiserum.

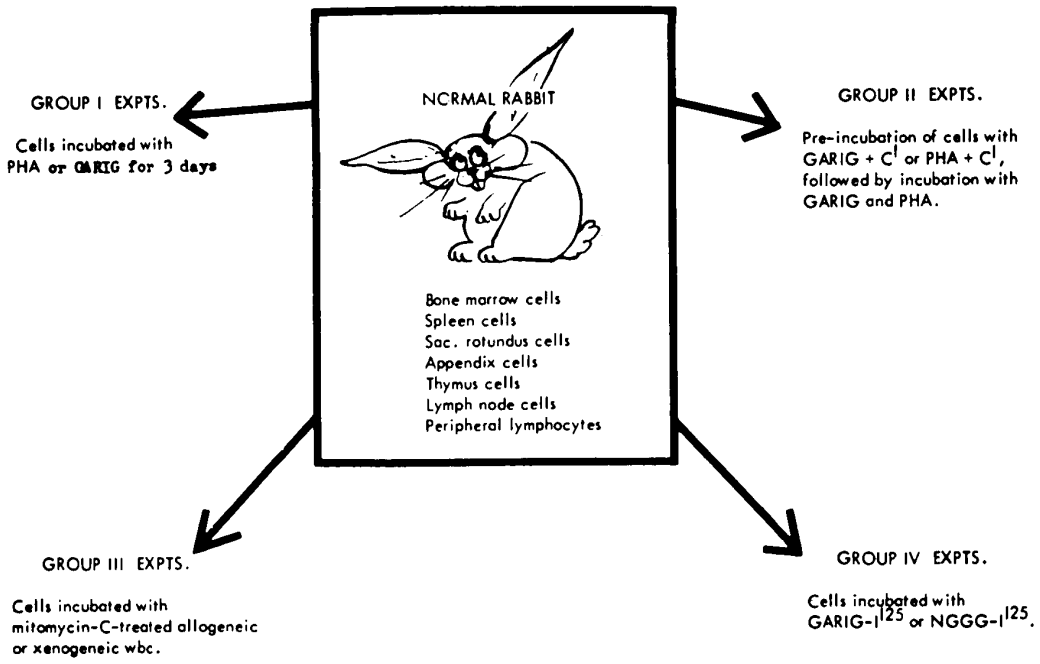


FIGURE 13

PROTOCOLS FOR THE DEMONSTRATION OF THE FUNCTIONAL HETEROGENEITY OF LYMPHOID CELLS IN THE DIFFERENT LYMPHOID ORGANS IN THE NORMAL RABBIT.

(b) The lymphocytes obtained from seven different lymphoid organs in the rabbit responded to PHA. Previous studies have left doubt as to the capacity of the cells in some of these organs to respond to PHA.

In one report in the literature, human bone marrow cells were found not to respond to PHA (388). In the present study rabbit bone marrow cells could consistently respond to PHA although to a lesser extent than did the cells of the other organs.

It was also found that rabbit appendix lymphocytes gave a response to PHA comparable to that obtained with spleen or lymph node cells notwithstanding two reports (379, 387) which indicate that rabbit appendix cells, unlike human appendix cells (386), give a weak or no response to PHA.

PHA at times failed to stimulate the thymic cells when the latter were incubated in a cell concentration of only one million cells per ml. However, the response to PHA by these cells incubated at a higher cell concentration was marked and in the range of the responses observed with the cells of the other lymphoid organs. Results of a similar nature, with regard to the relationship of thymic cell concentration and the response to PHA, have been previously observed (380, 381).

(c) The lymphoid cells of all the lymphoid organs tested responded to GARIG, with the sole exception of the thymocytes which

did not respond to GARIG even when tested in cell concentrations varying from one to ten millions per ml of culture fluid. Similarly the thymocytes adsorbed much less GARIG-I^{125} than did the cells of the other lymphoid organs.

(d) An additive effect was observed when GARIG was added to lymphoid cells which had been pre-incubated with PHA for one hour. The latter incubation was shown to be sufficient in most instances to result in optimal stimulation of the cells. The subsequent addition of GARIG to the cells for the duration of culture induced a proliferative effect significantly greater than that induced by PHA or GARIG alone.

(e) Pre-incubation of immune rabbit spleen and lymph node cells with GARIG for one hour was not sufficient to result in blastogenesis. However, this pre-incubation period selectively blocked the response of these cells to antigen without affecting their response to PHA.

(f) Pre-treatment of immune cells with GARIG abolished or decreased their capacity to form antibodies to a specific antigen when injected with that antigen into irradiated recipients.

(g) PHA and HSA showed a true additive effect in their ability to stimulate lymphocytes from HSA-immune rabbits in vitro.

A similar observation has been made with spleen cells of SRBC immune rabbits upon the addition of PHA and SRBC (356, 357). On the other hand, in the present study, there was no additive effect between GARIG and HSA. Moreover, the mere presence of GARIG, which by day 7 of culture no longer possessed the capacity to stimulate rabbit lymphocytes, prevented the blastogenic response to antigen.

(h) All the lymphoid cell populations tested responded to varying degrees, to stimulation with mitomycin-C inactivated leucocytes. Previous studies have shown that the lymphoid cells of various organs of a number of animal species could respond with blastogenesis and DNA synthesis when stimulated in vitro with allogeneic (336-340) or xenogeneic cells (341). However, in contrast to the easily demonstrable response of human lymphoid cells in mixed leucocyte cultures, rabbit lymphocytes are not easily stimulated by allogeneic cells (307), a finding confirmed in our present investigation. In all cases, the stimulus provided by the xenogeneic cells was consistently found to be superior, especially with respect to lymphoid cells which hardly responded to stimulation with the allogeneic stimulus.

(i) Pre-incubation of lymphocytes with GARIG and complement selectively impaired the capacity of the cells to respond to GARIG, but left intact their proliferative responsiveness to stimulation with PHA. On the other hand, pre-incubation of the cells with PHA and complement

or complement alone did not affect the capacity of the cells to respond to stimulation with either PHA or GARIG.

The selective inactivation of GARIG-responsive cells by GARIG and complement could be the result of a blocking effect which would be specific for GARIG, would require an optimum amount of complement and would not affect PHA responsive cells. Our findings suggest, however, that more likely incubation of the cells with GARIG and complement results in immune lysis of a select population of cells, those capable of reacting with the anti-rabbit immunoglobuin antibodies in GARIG. This complete suppression of responsiveness to GARIG is probably related to the fact that 8 to 12 percent of the cells had been killed by the treatment with GARIG and complement, utilizing the dye exclusion test. Whether the lysis of such a small percentage of cells can explain the specific inhibitory effect for GARIG is not certain. The response of several populations of cells to either GARIG or PHA were often of the same magnitude as determined by both radioactivity scintillation counting and radioautography. This would tend to indicate that a large population of lymphocytes at the term of a three day culture had been stimulated by GARIG and is in agreement with a previous finding of up to 80 percent of blast cells in the culture of rabbit cells stimulated by anti-immunoglobulin antiserum (246). These blast cells present at the termination of culture may not have all been stimulated at the start. The recent

work of Bach et al (262) supports this assumption in that they demonstrated that although 30 percent of the cells at the termination of a mixed leucocyte culture are transformed, the number of cells engaging in the reaction at the start of the culture does not exceed 1 percent. Besides proliferation, other mechanisms have been evoked to explain the presence of a large number of blast cells at the conclusion of a stimulated culture and some of these mechanisms could well operate in the stimulation of rabbit cells by antiserum. Cells can be recruited during the course of culture either by direct action of the stimulated cells (408) or by the release of subcellular growth promoting factors which induce the proliferation of otherwise non-reactive cells (350, 409). It is also possible, as recently proposed by Skamene and Ivanyi (330) that stimulation with one class of anti-immunoglobulin uncovers or activates receptors for other classes of antisera.

Since normal goat serum or gamma-globulin does not exhibit blastogenic activity, the stimulus provided by GARIG can only be attributed to its property as an antiserum directed to rabbit immunoglobulins. It may therefore be presumed that the blastogenic response to GARIG is dependent on the reaction of antibodies in the antiserum with immunoglobulin receptor sites on the surface of the normal rabbit lymphoid cells.

The evidence in favor of the receptor being an antibody-immunoglobulin on the surface of the lymphocyte, was summarized in Section 2.3.1 of the Historical Review. Merler and Janeway (243) were able to isolate, by mercaptoethanol treatment of human immune lymphocytes, material possessing properties of immunoglobulins and recognition sites for the antigens to which the donors had been immunized.

Several studies indicate that immune lymphocytes react with antigens through receptor sites having the binding properties and characteristics of an antibody: free hapten can block receptor sites and prevent blastogenesis of immune lymphocytes subsequently stimulated with hapten-protein conjugates (252, 253); the affinity of the receptor site for the antigen capable of triggering this reaction is closely related to the affinity of the antibody produced (254, 255). Similarly, free antigen can prevent the attachment of normal precommitted or immune cells to antigen coated glass bead columns (218-220). Finally, Mitchison (242) has shown that pre-treatment of immune mouse lymphocytes with anti-immunoglobulin antiserum could inhibit the synthesis of antibodies by these immune cells exposed to the specific antigen before their transfer to irradiated hosts. This finding was confirmed in the present study using a rabbit system.

The findings of Sell and Gell lend further support to the existence of an immunoglobulin recognition site on the normal rabbit lymphocyte. They demonstrated that homologous rabbit anti-allotype serum (244) and heterologous anti-immunoglobulin antiserum (245, 246) could stimulate normal rabbit lymphocytes to undergo blastogenesis and mitosis. The observation by Sell (335) that the univalent Fab fragment of the anti-immunoglobulin molecule could stimulate rabbit lymphocytes to transform, indicated that only interaction of sites with a complementary configuration is required to trigger this in vitro cell response. It has since been observed that heterologous anti-immunoglobulin antiserum can induce blastogenesis of lymphocytes of a number of other animal species, i.e., guinea pig (320), chickens (330), mice (331), and man (332, 333). It has also been demonstrated that anti-lymphocyte serum (ALS) can induce blastogenesis of human (313), rabbit (410), and guinea pig (320) lymphocytes. Since the blastogeneic activity of the anti-immunoglobulin antiserum can be suppressed following absorption of the antiserum with IgG whereas the blastogenic activity of the ALS is not diminished following absorption with serum proteins (320) but can be depressed following absorption with lymph node cells (320), it may be concluded that the sites on the surface of the lymphocyte to which the blastogenic factor in the ALS is directed is not an immunoglobulin but rather another cell membrane component.

Recent studies suggest that PHA may interact with the same site(s) on the lymphocyte to which ALS is directed (321). It has observed that human or mouse lymphocytes incubated with anti-lymphocyte sera used in concentrations insufficient to induce blastogenesis, failed to respond if subsequently stimulated with PHA (321-323). ALS can also suppress the PHA-induced cytotoxic effect of lymphocytes on homologous fibroblast monolayer cultures (323). Results of the present study would indicate that PHA does not interact with the sites on the lymphocyte to which GARIG is directed since pre-incubation of the cells with GARIG did not result in inhibition of responsiveness toward the stimulatory effect of PHA. On the other hand, pre-incubation with GARIG abolished the stimulatory effect of antigen on immune spleen and lymph node cells. It is probable, therefore, that ALS and PHA interact with sites which are quite similar or identical but different from the immunoglobulin receptors to which GARIG and antigen are directed. These findings facilitate an understanding of the mode of action of PHA and anti-immunoglobulin antisera. Unlike PHA, anti-immunoglobulin antiserum would stimulate only those cells carrying specific sites, i.e., immunoglobulins, capable of interacting with the anti-immunoglobulin antibodies.

In the first series of experiments it was demonstrated that peripheral lymphocytes obtained from patients with agammaglobulinemia could be stimulated to undergo blastogenesis upon stimulation with PHA

but not with anti-human immunoglobulin antiserum, whereas peripheral lymphocytes of normal individuals can respond to stimulation by both of these agents. This dissociation of responsiveness to PHA and anti-immunoglobulin serum in the cases of agammaglobulinemia as well as the results referred to above, imply that either (a) there normally exist two populations of lymphocytes, one capable of responding to PHA and the other to anti-immunoglobulin antiserum, with the latter population of cells absent in the case of agammaglobulinemia (Fig. 14) or (b) that the capacity to respond to these two mitogenic agents is normally inherent in the same lymphocyte but that in the case of a particular disease, such as agammaglobulinemia, the ability of the cell to respond to anti-immunoglobulin antiserum is lost. The findings presented in this investigation lend strong support to the former interpretation since the response of the population of normal rabbit lymphoid cells to PHA was not diminished if the cells capable of responding to anti-immunoglobulin antiserum (GARIG) were initially inactivated by pre-treatment of the cells with GARIG and guinea pig complement.

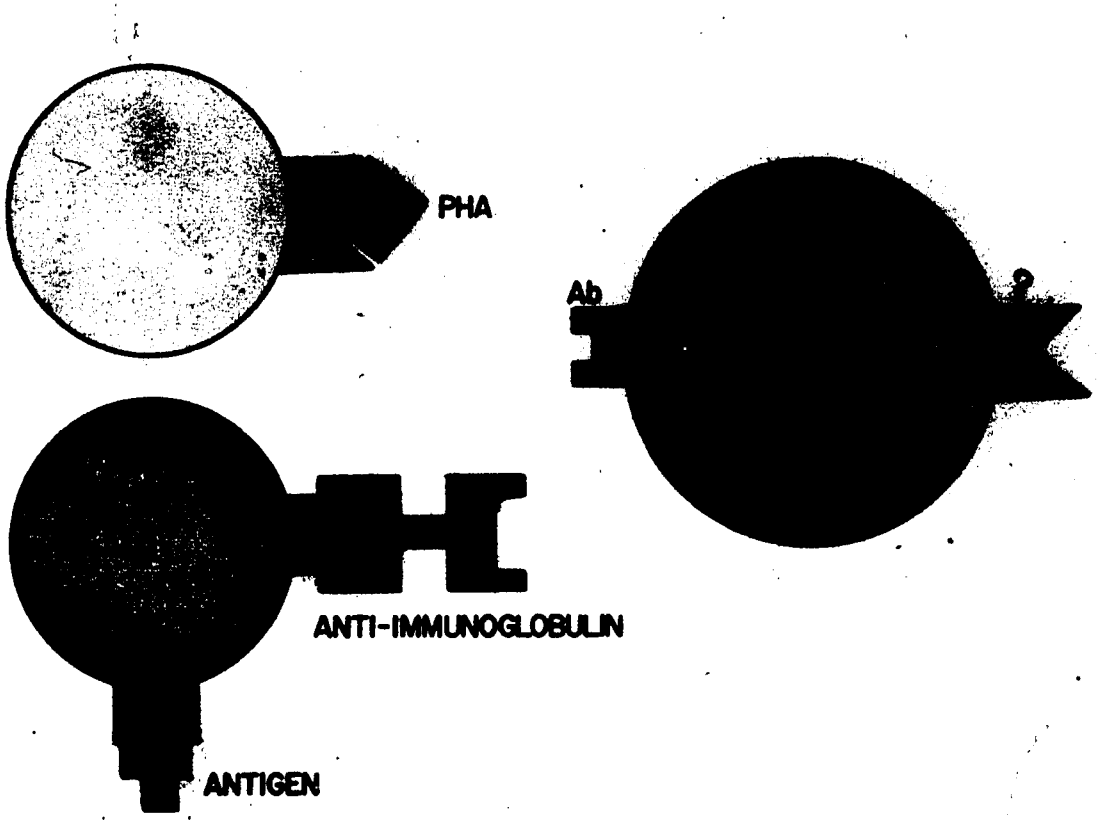
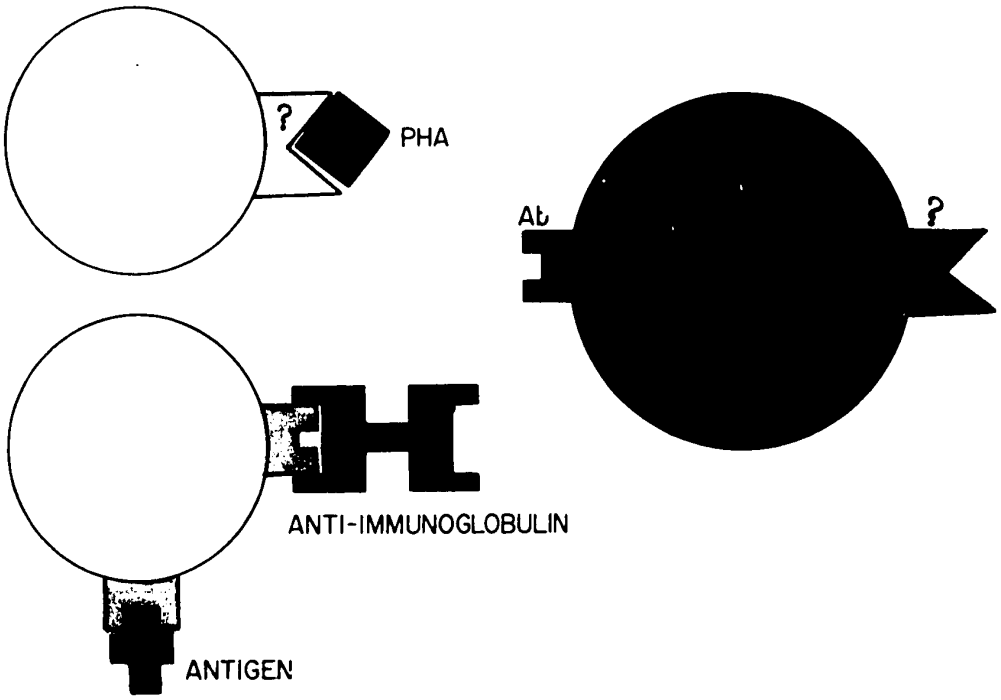


FIGURE 14

LYMPHOCYTE RECEPTORS AND MITOGENIC SUBSTANCES: THE EXISTENCE OF A SINGLE OR TWO POPULATIONS OF LYMPHOCYTES REACTIVE TOWARD PHA AND GARIG. A DIAGRAMATIC REPRESENTATION.


?
PHA
Ab
?
ANTI-IMMUNOGLOBULIN
ANTIGEN

A point that requires further clarification is the failure of the normal rabbit thymus cells to respond to GARIG under conditions where the response to PHA is optimal, This finding suggests that thymocytes lack the specific immunoglobulin receptor(s) possessed by lymphoid cells of the other lymphoid organs. The low uptake of radioactively-labelled GARIG by the thymocytes, compared to the uptake by the other lymphoid cells, provides further support in favor of the absence of immunoglobulins on the thymocyte surface. This is consonant with the failure of normal rabbit or mouse thymus cells to confer the capacity to form antibodies upon transfer to irradiated recipient animals (28, 35, 119, 146).

On the other hand, thymocytes are capable of mediating graft-versus-host reactions (153-160). In this study they were also capable of responding to stimulation by allogeneic and xenogeneic mitomycin-C inactivated lymphocytes (the one-way stimulation test). In fact, the thymocytes responded as well, and at times better, than cells of the other lymphoid organs to the xenogeneic stimulus. This response of lymphocytes in the mixed cell culture has definite immunologic characteristics. It cannot be induced by incubation of lymphocytes from identical monozygotic twins or syngeneic animals (336, 337) nor by the incubation of lymphocytes from genetically dissimilar donors when one of the donors had been made tolerant to the cells of the other donor (345). Furthermore, in mixed leucocyte

cultures consisting of leucocytes of parental and F 1 hybrid origin, all the dividing cells are of parental origin (345).

It has been reported that the response of lymphocytes of neonatally thymectomized mice (367), rats (368, 369) and chickens (370) to stimulation with PHA or mitomycin-C treated allogeneic cells is markedly reduced. It has also been demonstrated that lymphocytes obtained from two thymectomized rats are incapable of responding toward each other in the mixed leucocyte reaction (345). On the assumption that the mixed leucocyte response is representative of a cellular immune reaction, these findings would support the currently accepted view that the cells mediating cellular immunity are thymic or thymus-derived and that their response to PHA is also indicative of this immunologic function.

Lymphoid cells of hypogammaglobulinemic bursectomized chickens fail to respond to anti-immunoglobulin antiserum (334) but respond normally to PHA (334, 369, 370). Since immunocompetent cells capable of mediating humoral immunity are considered to be bursal derived or dependent, whereas cells which mediate cellular immunity are considered to be thymic derived or dependent, it would appear that responsiveness to PHA and/or allogeneic and xenogeneic cell stimuli may indeed provide a means of identifying those cells capable of mediating thymus-dependent cellular immunity. The presence of immunoglobulin sites on the surface of the cell, permitting it to

respond to stimulation with GARIG, may therefore serve as a means of detecting and identifying cells capable of mediating humoral immunity (Fig. 15).

The great percentage of cells capable of engaging in a cellular immune manifestation against a strong transplantation antigen (143, 256, 257, 261, 262), as opposed to the fewer number of progenitor cells capable of producing antibodies against SRBC (128, 258, 259), has led several authors (257, 262) to assume the existence of two different modes of antigen recognition: one pertaining to the cells mediating humoral immunity and the other to the cells mediating cellular immunity. The recognition of the antigen by cells mediating the humoral immune response would appear to require an immunoglobulin receptor site on the cell surface, capable of interacting with the antigen. Such a receptor appears not to exist on cells capable of mediating specific cellular immunity. The mechanism whereby these latter cells recognize an antigen and interact with it remains to be determined.

These experiments present further evidence in favor of the division of the immune system into separate humoral and cellular branches.

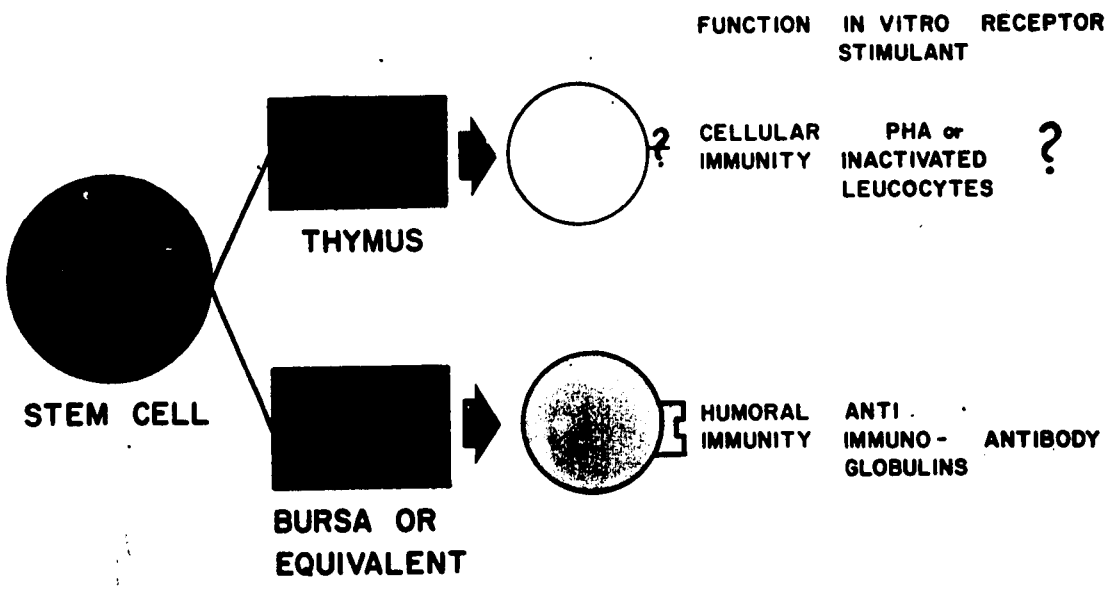


FIGURE 15

RELATION BETWEEN LYMPHOCYTE FUNCTION AND IN VITRO PROLIFERATIVE RESPONSE(S) FOLLOWING STIMULATION WITH PHA, ANTI-IMMUNOGLOBULIN SERUM AND INACTIVATED LEUCOCYTES.

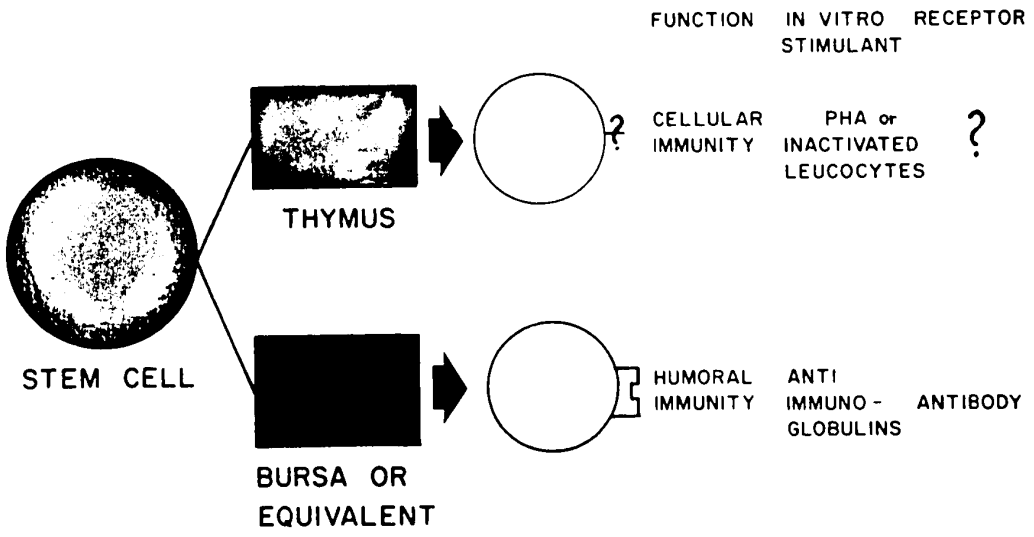


FIGURE 15

RELATION BETWEEN LYMPHOCYTE FUNCTION AND IN VITRO PROLIFERATIVE RESPONSE(S) FOLLOWING STIMULATION WITH PHA, ANTI-IMMUNOGLOBULIN SERUM AND INACTIVATED LEUCOCYTES.

SUMMARY AND CONCLUSIONS

Circulating lymphocytes of normal and agammaglobulinemic subjects as well as cell populations from different lymphoid organs of normal and immune rabbits were investigated for their capacity to respond in vitro to a number of stimuli, such as PHA, anti-immunoglobulin antiserum, antigen and allogeneic and xenogeneic leucocytes. Immune spleen or lymph node cells were also tested, following pretreatment with goat anti-rabbit immunoglobulin antiserum (GARIG) or normal goat gamma-globulin (NGGG), for their capacity to form antibodies when injected with antigen into irradiated recipients. Normal rabbit lymphoid cells were also tested for their capacity to adsorb radioactively-labelled anti-immunoglobulin antiserum. The data gathered from these investigations indicate that:

(a) There appear to be two different populations of lymphocytes, one capable of responding to anti-immunoglobulin serum and the other capable of responding to PHA. This statement is based on the finding that the circulating lymphocytes of two agammaglobulinemic patients responded to PHA but not to anti-immunoglobulin serum. PHA could stimulate lymphocytes from all the lymphoid organs of the normal rabbit, including thymocytes. GARIG did not stimulate thymocytes but stimulated the cells of all the other lymphoid organs.

There was a true additive effect between GARIG and PHA. Pre-treatment with GARIG and complement, of cells capable of responding to GARIG, selectively abolished the capacity of these cells to respond to GARIG but left intact their response to PHA.

(b) GARIG and antigen appear to react with identical sites on the surface of the lymphocytes. This statement is based on the finding that pre-incubation with GARIG selectively blocked the response of immune rabbit spleen and lymph node cells to antigen without affecting their response to PHA. Similarly, pre-treatment of these cells with GARIG abolished or decreased their capacity to form antibodies to a specific antigen when injected with the same antigen into irradiated recipients. There was no additive effect between GARIG and antigen although PHA and antigen showed a true additive effect.

(c) Thymocytes adsorbed much less GARIG-I^{125} than did cells of the other organs. This finding, as well as their non-responsiveness to GARIG in vitro, contrasted with their normal response to stimulation with inactivated leucocytes and PHA.

On the basis of the results presented and the findings of other investigators it is concluded that:

1. There exists a population of lymphoid cells carrying immunoglobulin or immunoglobulin-like receptors on their surface. These receptors interact with antigen and seem to mediate the recognition process leading to a humoral immune response.

2. There exists a population of cells capable of reacting with PHA and/or inactivated leucocytes. These responses indicate a potential capacity of the cells to mediate cellular immunity and do not seem to necessitate the presence of immunoglobulin-recognition sites on the cell surface.

3. The thymus in the normal adult rabbit appears to consist of cells capable of mediating cellular immunity only.

4. The other lymphoid organs appear to possess cells capable of mediating humoral and cellular immunity.

CLAIMS TO ORIGINALITY

1. It was demonstrated that the circulating lymphocytes of two agammaglobulinemic patients could be differentiated on the basis of their response to phytohemagglutinin (PHA) and anti-immunoglobulin serum. This contrasted with the situation found in normal individuals and in a third patient with congenital agammaglobulinemia whose lymphocytes could always respond to both stimuli.

2. It was demonstrated that lymphocytes obtained from seven different lymphoid organs in the rabbit responded to PHA. The lymphoid cells of all these organs responded to goat anti-rabbit immunoglobulin serum (GARIG), with the sole exception of the thymocytes, which did not respond to GARIG. Similarly, the thymocytes adsorbed much less GARIG-I^{125} than did the lymphoid cells of the other organs.

3. It was demonstrated that rabbit thymocytes, as well as the other lymphoid cell populations, could respond in varying degrees to stimulation with mitomycin-C inactivated allogeneic or xenogeneic leucocytes.

4. It was demonstrated that pre-treatment with GARIG and complement of rabbit lymphocytes, capable of responding to GARIG and PHA, selectively abolished the capacity of these cells to respond to GARIG but left intact their response to PHA.

5. It was demonstrated that pre-incubation with GARIG selectively blocked the in vitro response of immune rabbit spleen and lymph node cells to stimulation with the specific antigen without affecting their response to PHA.

6. It was also demonstrated that pre-treatment of immune rabbit lymphoid cells with GARIG abolished or decreased their capacity to form antibodies to a specific antigen when injected with this antigen into irradiated recipients.

7. It was demonstrated that, in their capacity to stimulate rabbit lymphocytes in vitro, there was a true additive effect between PHA and GARIG or PHA and antigen (HSA). There was no additive effect between GARIG and HSA.

8. These studies demonstrated the existence of a population of lymphocytes capable of responding to stimulation with GARIG and antigen and of a different population of cells capable of responding to stimulation with PHA.

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