MEMBRANE DYNAMICS OF MOUSE SPLEEN B AND T LYMPHOCYTES DURING THE PRIMARY IMMUNE RESPONSE

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TO MY WIFE, IVY AND MY PARENTS

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ABSTRACT

The reverse immune cytoadherence technique (RICA) which detects surface associated Ig on lymphocytes was used to study the number of Ig carrying B cells in normal as well as antigen stimulated mouse spleen cells. This technique involves the use of a 5S hybrid antibody with two specificities, one an anti-mouse Ig and the other an anti-protein. The hybrid antibody reacts with surface associated Ig through its anti-Ig site and with protein coated red blood cells (indicator cells) via the anti-protein site, thus forming a rosette which consist of a centrally located lymphocyte surrounded by a collar of red cells. This version is called single RICA.

A modification of RICA was devised in which two hybrid antibodies were used each with a different anti-Ig and anti-protein sites. The binding of each hybrid antibody was shown with the use of two different indicator cells. The anti-Ig site in each hybrid antibody was highly specific for one of the two major mouse Ig classes: IgG (7SIgG2a) and IgF (7SIgG1) globulins. A cell forming rosette with only one kind of indicator cells is called a single rosette and

indicates that the cell carries only one class of Ig. On the other hand, a cell forming a rosette with two different indicator cells is called a mixed rosette and demonstrates that the cell bears more than one Ig class on its surface.

Single RICA revealed that in the normal resting mouse spleen, the number of Ig carrying cells were about 330/1000 spleen cells. Of this, 300 cells carried IgF globulin (IgF cells) and 200 cells expressed IgG (IgG cells) on their surfaces and since the sum of the two is higher than the total number of Ig carrying cells some cells obviously carry both Ig classes.

Of the 300 cells carrying IgF and/or IgG, approximately 200 carried both IgG and IgF on the same cell ($\gamma\phi$ cells), while the other 100 cells expressed only IgF (ϕ cells). Few, if any, cells carried only IgG (γ cells).

Using anti-θ cytotoxicity tests, the T cell population in the mouse spleen was found to be 270 cells/1000 spleen cells. Thus, the total B and T cells in the spleen were about 600 cells/1000 spleen cells.

It has been demonstrated that elimination of T cells from a spleen cell suspension did not affect the detection of the number of B cells or vice versa. This indicated that Ig carrying B cells are distinct from θ carrying T cells.

Antigen (POL) injection induced large scale changes of B and T cells in terms of surface associated

Ig as shown by RICA and cytotoxicity. Six hours after antigenic stimulation, there was an overall increase of Ig carrying cells. Exposure in vitro of normal spleen cells to serum collected 6 hours after antigenic stimulation reproduced the increase of Ig carrying cells observed in vivo. The increase was shown to be due to acquisition of a cytophilic IgG globulin possibly in the form of antigen-Ig complex by a T cell. Evidence in support of this conclusion was derived from a variety of experimental approaches. Elimination of θ carrying cells from spleen cell suspension abolished the increase of Ig carrying cells induced by the 6 hour serum. Thymus but not bone marrow cells acquired the cytophilic Ig from 6 hour serum. Mice were irradiated and then reconstituted with either thymus or bone marrow cells. Only the spleen cells from the thymus reconstituted mice showed an increase of Ig carrying cells 6 hours after antigenic challenge. The number of T cells detected in the spleen with an anti-θ serum at 6 hours was found decreased by approximately 10%. It is conceivable that in T cells acquiring the cytophilic Ig, the θ antigen becomes undetectable by the anti-θ serum. Some of the cells which carry Ig prior to immunization have also been affected at 6 hours. This was shown by the decrease of the cells carrying IgF. The results with specific hybrid antibodies show that the cytophilic Ig belongs to the IgG class and results in the appearance of a large population

of cells carrying only IgG (γ cells) which are not detectable prior to immunization.

During the first two weeks the number of Ig carrying cells progressively decreases and reaches the lowest point (38% of pre-immunization level) at 15 days. Subsequently, the number of Ig carrying cells returns to normal by the end of the 4th to 5th week. The changes observed in vivo during the first two weeks were reproduced in vitro by treating normal spleen cells with sera collected during this period.

It was shown that 6 hour serum contains a cytophilic IgG which was taken up by both T cells (inducing an overall increase of Ig carrying cells) and by B cells (resulting in a decrease of the detection of IgF carrying cells). Furthermore, the 6 hour serum contains a dialysable factor which mobilizes the surface Ig from B cells. The surface Ig regenerates after removal of the 6 hour serum. The attachment of the cytophilic IgG and the mobilization of surface Ig by the dialysable factor, were temperature independent. However, the surface Ig did not regenerate at 4° C.

The sera at 24 hours, 7 days and 15 days also contained a dialysable factor, which could mobilize surface Ig, but no cytophilic Ig was detectable. Concanavalin A could not restrict the mobilization of surface Ig induced by the dialysable factor of the 6 hour or 7 day serum.

Thus, mobilization of surface Ig by this factor present several striking differences from that induced by heterologous anti-Ig. In conclusion, the results from the studies reported here indicate that:

- antigen affects a very large population of both T and B cells.
- T cells acquire a cytophilic IgG (possible in a form of complex with antigen) within 6 hours after immunization.
- B cells also take up such an Ig at the same time.
- soluble dialysable factors are detected in the serum of the animals which mobilize surface Ig from B cells.
- surface Ig mobilization may be an important factor resulting in significant decreases of the number of Ig carrying cells during the first two weeks detectable in vivo.
- the mechanism of such mobilization presents several striking differences from that induced by heterologous anti-Ig sera.

INTRODUCTION

I. CELLS INVOLVED IN THE IMMUNE RESPONSE.

Immunological responses are divided into those resulting in the production of humoral antibody and those characterized by the appearance of sensitized cells. Both depend upon the activity of small lymphocytes (Gowans and McGregor, 1965) which are ultimately derived from precursors in the bone marrow (Miller and Mitchell, 1969). The small lymphocytes are considered today as the immunologically competent cells (Medawar, 1961). They comprise a heterogeneous population of cells with respect to their origin (central vs peripheral) (Cooper et al, 1966), life span (long life vs short life) (Everett and Tyler, 1967), the differential migratory pathways (Lance and Taub , 1969) and their functional dependence on the central lymphoid source (thymic or bursal dependence) (Waksman et al, 1962).

The functional heterogeneity of lymphocyte populations which take part in the immune response was not realized until the demonstration by Miller (1961) that neonatal thymectomy in mice prevented the proper development of all cell mediated and some humoral responses to some antigens, particularly foreign serum proteins and heterologous erythrocytes. This finding represents one of the most important developments in cellular immunology. Neonatal thymectomy not only impaired the immunological capacity of the animal, but also prevented the development of an entire subpopulation of lymphocytes, including those in certain defined areas of the spleen

(periarteriolar) and lymph nodes (paracortical), the majority of thoracic duct lymphocytes and half of those in the peripheral blood (Miller, 1962; Parrott et al, 1966). Removal of the same organ in the adult did not influence immunological capacity, unless the pool of immunocompetent lymphoid cells was depleted as a result of age or following destruction by irradiation or by antilymphocytic serum (Miller,1965; Miller et al, 1963; Martin and Miller, 1968). This indicated that the presence of the thymus in the critical first few days of life was very important for the development of the immunological capacity. Apart from the impairment of all cell-mediated immune capacity and antibody response to some antigens after thymectomy, little or no impairment was seen in the antibody response to other antigens such as pneumococcal polysaccharide (Humphrey et al, 1964) polymerized flagellin (Armstrong et al, 1969), polyvinylpyrrolidone (PVP) and E. coli polysaccharide (Andersson and Blomgren, 1971). On the basis of the thymic dependence for eliciting an immune response, antigens are classified into "thymus dependent" and "thymus independent".

Miller's finding (1961) complemented those of Good and his colleagues (Good, 1955; Good et al, 1962) who noted that in human congenital immunological deficiency diseases, two systems of cells could be independently affected. In Bruton's type of agammaglobulinemia, the plasma cells are absent and the humoral responses abolished or diminished while the cellular responses are normal or only slightly impaired. In "Swiss" type of agammaglobulinemia the thymus

is hypoplastic while in the Di George syndrome it is totally absent (Di George, 1965). In both of these conditions the cellular immune responses are impaired but the immunoglobulin (Ig) and antibody production is normal. These studies, together with Miller's results of thymectomy in mice, clearly indicated that the small lymphocytes, although indistinguishable morphologically, can be functionally divided into "thymus dependent" and "thymus independent" cell populations. The former is mainly responsible for cell-mediated immunity and the latter for humoral antibody responses.

Although immunological capacity of an animal depends on the presence of the thymus in the first few days of life, the thymocytes themselves have been shown to be non-immunocompetent or only slightly so as compared to lymph node or spleen cells (Vos et al, 1959; Billingham and Silvers, 1961; Thorbecke and Cohen, 1964). Thus the thymus is known as a central lymphoid organ not itself engaged in immune responses but essential for the correct development of the peripheral organs such as spleen and lymph nodes which contain immunocompetent cells.

Much debate arose over whether the thymus actually produces the peripheral immunocompetent cells or simply contributes to their maturation by means of a humoral secretion. Some studies have shown that cells which could be identified by a chromosome marker, actually leave thymus grafts and settle in the spleen and lymph nodes (Ford, 1966; Davies, 1969).

Furthermore, cells which were carefully labeled in the intact thymus, were shown to peripheralize, especially around the time of birth (Weissman, 1967). However, evidence has also accumulated for a thymic hormone capable of promoting immunological competence of precursor cells from thymectomized mice (Osoba and Miller, 1963; Trainin et al, 1969; Goldstein et al, 1970). The importance of this humoral activity under normal circumstances is not clear at the present time.

A more clear-cut functional dichotomy of the lymphoid cell lines responsible for the two distinct immunological responses, emerged from the studies of the chicken lymphoid system. It has been known for several years that injection of eggs with testosterone would suppress the development of the bursa of Fabricius, a cloacal lymphoid organ and also impair antibody production in the adult bird (Glick et al, 1956). It has also been shown that removal of the bursa or the thymus had quite separate consequences. Bursectomy reduces the antibody response, depletes the lymphoid organs of plasma cells, blocks the formation of lymphoid follicles and suppresses the immunoglobulin production, but not homograft immunity or delayed hypersensitivity (DH). Thymectomy has exactly the reverse effect (Warner and Szenberg, 1964; Cooper et al, 1966; Ivanyi et al, 1969). Antibody forming cells were not found in the bursa (Dent and Good, 1965), though young bursal cells were shown to be capable of transferring

antibody production to irradiated birds (Gilmour et al, 1970). However, just as with the thymus and cell mediated immunity, the deficit after bursectomy is out of proportion to the modest content of immunocompetent cells in the organs. Thus the bursa in the chicken came to be considered as another central lymphoid organ. Through its influence, a sub-population of lymphoid cells in the peripheral organs, especially those in the lymphoid follicles, will mature and take part in the humoral immune response. This line of lymphoid cells as those derived from, or dependent on, the existence of, the thymus is called bursa-derived or bursa dependent lymphocytes.

In mammals a central organ equivalent to bursa has not yet been discovered, although some studies (Cooper et al, 1966; Good and Finstad, 1971) indicated that the gut associated lymphoid tissues might function as the bursa in chicken. Most investigators believe that the bone marrow, although serving as the ultimate source of stem cells in the adult animal (Ford, 1966; Miller and Mitchell, 1969), may also play a role as the bursal equivalent central lymphoid organ in mammals, since it has been shown that thymectomy does not affect the population of cells in the lymphoid follicles, and bone marrow can repopulate these areas in thymectomized irradiated mice. It is also thought that lymphoid tissues themselves might exert by a humoral secretion the stimulus for differentiation of the bone marrow-derived cell

in its local environment (Moore and Owen, 1967b). For simplicity, a thymus or thymus derived or dependent immunocompetent cell is called a "T" cell, and a bursa or bone marrow (bursal equivalent in mammal) or bursa dependent immunocompetent cell, is called a "B" cell (Roitt et al, 1969).

II. SYNERGISM OF "T" AND "B" CELLS IN THE IMMUNE RESPONSE.

Although the functional compartmentalization of immunocompetent lymphocytes into "T" and "B" classes allows them to perform efficiently separate immunological functions, in recent years it has been realized that these two distinct lymphoid cell lines interact synergistically with one another in the development of certain immune responses, notably the humoral immune response to some thymus dependent antigens such as sheep erythrocytes and foreign serum proteins (Miller 1961, 1962; Papermaster et al, 1962; Martinez et al, 1962). The fact that the immunological defect of neonatally thymectomized animals could be partially restored to normal by implantation of a thymus graft (Dalmasso et al, 1963; Taylor, 1963; Miller et al, 1964) and that the lethally irradiated mice, reconstituted with syngeneic bone marrow cells will recover full immunocompetence only if the thymus was present indicated that the "T" cell component was needed for the expression of immunocompetence.

The simplest and most elegant demonstration of the requirement for both "T" and "B" cell types in antibody responses was an experiment by Claman et al (1966) in which

lymphoid cells were injected into irradiated mice together with sheep erythrocytes (SRBC). Thymus cells alone produced no hemolytic foci or plaque formation (PFC), marrow cells made a few, but the mixture of both gave significantly more antibody producing cells. A variation of the above experiment was performed by Miller and Mitchell (1967) who injected first the thymus cells to an irradiated mouse, removed its spleen a week later and injected the cells together with fresh bone marrow cells into a second irradiated host. In this double "transfer system", it was found that antigen (SRBC) had to be given to both hosts in order for the second one to mount an antibody response. Instead of thymus, Miller and Mitchell (1968) also used thoracic duct cells to cooperate with marrow cells. However, the thoracic duct cells alone gave hemolytic foci, and the size but not the number of them was increased when marrow was added. Davies et al (1967) also demonstrated that thymus-derived and marrow-derived cells could be isolated from radiation chimeras and the cooperation of "B" and "T" cells was evaluated by transferring them into the secondary hosts: alone neither population made a full response but in combination they did.

The demonstration of "T" and "B" cell cooperation in vivo for the immune response provoked studies of the antibody response in vitro following the recent improvements in tissue culture techniques (Mishell and Dutton, 1967; Marbrook, 1967). The response in vitro of spleen cells was

remarkably similar to that in vivo, but the mixture of marrow and thymus cells were inactive. However, by culturing the thymus and marrow cells separately for a few days in the spleen of irradiated mice, cell suspensions were obtained which demonstrated cooperation when mixed together and cultured in vitro (Dutton et al, 1971b). Using the same technique as Dutton, Mosier et al (Mosier, 1967; Mosier et al, 1970) demonstrated that not only "T" and "B" cells were needed for the immune response in vitro, but a glass adherent cell, presumably a macrophage was required.

Cooperation between "B" and "T" cells so far has been successfully demonstrated only in a mouse system. This may be due to the unavailability of inbred strains in the other species. In chickens, although the thymus-bursa system might seem ideal for demonstrating cooperation, preliminary results have been unconvincing (Gilmour et al, 1970)

The demonstration of "T" and "B" cell interaction in the antibody response has created a challenge which not only led Immunologists to explore the nature of this response, but also forced them to investigate whether the cooperation between "B" and "T" cells might underline other known immunological phenomena such as memory, unresponsiveness, carrier effect and antigenic competition.

(1) Memory

That specific memory exists in the "T" cell population has been shown in double transfer experiments by

Miller and Mitchell (1967) in which thymus cells cultured for a week in the spleen of an irradiated mouse, cooperated with marrow cells only if they were stimulated with SRBC in both hosts and not if the first host received horse RBC instead. Similar experiments which demonstrated "T" cell memory were performed by others (Mitchell and Miller, 1968; Miller et al, 1971; Shearer and Cudkowicz, 1969). Chan et al (1970) and Hartman (1970, 1971) also demonstrated in an in vitro system that specific memory existed in "T" cells. Takahashi et al (1970a) demonstrated memory in "T" cells by specific elimination of "T" cells from the immune spleen which resulted in loss of the ability of such cells to transfer secondary response to SRBC.

Memory in the "B" cell population was first thought to be non-existent (Shearer and Cudkowicz, 1969; Cunningham 1969). However, by using as "T" and "B" cell donors, congenic mice differing genetically only at the loci coding for immunoglobulin allotype, Jacobson et al (1970) showed that all the IgG anti-SRBC plaque forming cells (PFC) produced in an adoptive transfer response to a second antigenic challenge were of the "B" cell allotype, thus indicating memory in "B" cells. Jehn and Karlin (1971) have shown that both thymocytes and bone marrow cells could adoptively transfer memory in the response to SRBC. More convincing results came from the study by Takahashi et al (1971b) who showed that the elimination of "B" cells in immune spleen resulted in definite

reduction of secondary responses. Responsiveness could only be reconstituted by immune "B" cells but not by normal spleen cells. Miller and Sprent (1971a) demonstrated that the response in irradiated recipients of primed "T" cells to fowl IgG could only be enhanced by addition of fowl IgG primed "B" cells and not with unprimed "B" cells, thus demonstrating the existence of memory in "B" cells.

(2) Immunological Tolerance

From early studies it was clear that "T" cells could manifest a specific tolerant state. Isakovic et al (1965) observed that thymus grafts from tolerant rats were able to transfer specific tolerance to thymectomized recipients. Gershon et al (1968b) found a reduced "T" cell mitotic response in SRBC tolerized animals. Taylor (1968, 1969) demonstrated that "T" cells of mice made tolerant to bovine serum albumin (BSA) were unable to cooperate with normal "B" cells in an adoptive transfer response. Miller and Mitchell (1970) induced tolerance to SRBC in mice with cyclophosphamide and observed that recirculating "T" cells in thoracic duct lymph were specifically tolerant.

While evidence for "T" cell tolerance is abundant, it is much more difficult to establish the existence of specific tolerance in "B" cells. Taylor (1968, 1969) and Miller and Mitchell (1970) in the above experiments showed that "B" cells from tolerant animals were able to cooperate with normal "T" cells in adoptive transfer responses.

Playfair (1969) used higher doses of SRBC plus cyclophosphamide to induce tolerance in mice and found "B" cells were only transiently tolerant. More positive results were obtained by Gershon and Kondo (1970) who observed that "B" cells could be rendered tolerant by repeated injections of SRBC. Perhaps the most elegant experiment demonstrating that both "T" and "B" cells can be made tolerant was the study by Chiller et al (1970, 1971) who observed the kinetic differences which existed in rendering "T" and "B" cells tolerant to human Ig (HGG). While "T" cells could be rendered tolerant easily (within 2 days), with low doses of tolerogen and maintained tolerance for much longer periods of time (77 days), "B" cells took longer to exhibit tolerance (11 days), and could be achieved only with high doses of tolerogen, and recovered from tolerance much earlier (49 days) as compared to "T" cells. These observations clearly demonstrated that both "T" and "B" cells could be rendered immunologically tolerant and also explained the apparent discripancies of earlier studies.

(3) Carrier Effect

The phenomenon of carrier effect which was observed by Benacerraf et al (1967) and Rajewsky and Rottlander (1967) was first found by Mitchison (1969a) to be due to an interaction of two cells. He showed that spleen cells from animals preimmunized with 4 hydroxy-5 iodo-3-nitrophenacetyl-ovalbumin (NIP-OVA) injected into irradiated recipients could make a good response if challenged with

NIP-OVA but not if challenged with heterologous NIP-Bovine serum albumin (NIP-BSA). However, when spleen cells from donors immunized with NIP-OVA were injected together with spleen cells from donors immunized with BSA, a perfectly good secondary response to NIP-BSA conjugate was obtained. This observation was further confirmed by many other investigators (Mitchison et al, 1970; Hamaoka et al, 1971; Mitchison, 1971; Kotiainen, 1971; Roelants and Askonas, 1971). Subsequently, Raff (1970b) showed that the carrier specific cooperating cells or "helper" cells were thymus-derived "T" cells, whereas the precursors of anti-hapten antibody forming cells were not.

(4) Antigenic Competition

Antigenic competition is a phenomenon which is observed when an animal is immunized with two antigens simultaneously or in close sequence resulting in a depressed antibody response to the second antigen (see review by Adler, 1964). This phenomenon contradicts Burnet's clonal selection theory. Studies by Radovich and Talmage (1967) and by Möller and Sjöberg (1970) suggested the existence of a soluble inhibitory factor active in antigenic competition. Some investigators suggested the existence of a multipotential lymphoid cell, occurring with limited frequency (Schecter, 1968; Albright et al, 1970) for which the antigens actually compete. Recent studies by Gershon and Kondo (1971a, 1971b) have indicated that antigenic competition was a thymus dependent phenomenon. They suggested that an inhibitory factor for

antigenic competition was released by "T" cells. However, Kerbel and Eidinger (1971), based on their finding that "T" cells decreased as the total spleen cells increased after antigenic stimulation, suggested that antigenic competition was caused by a general shift in the frequency of specific helper "T" cells, thus resulting in the lesser chance of interaction between a "T" and a "B" cell of the same specificity.

III. THE MECHANISM OF T AND B CELL INTERACTION: CURRENT HYPOTHESES

A number of working models have been proposed by various authors in recent years in order to explain the nature of "T" and "B" cell interaction. Since only "B" cells produce antibody (Davies et al, 1967; Mitchell and Miller, 1968) "T" cells are considered to act as helper cells.

(1) Antigen concentration

This theory was proposed by Mitchison (1969b) to explain the function of "T" cells in presenting antigen to "B" cells. It was postulated that the "T" cell concentrates the antigen on its surface and presents a large number of antigen molecules to a group of receptors on the surface of the "B" cell. It is assumed that the signals arising from conformational change in a large number of receptor immunoglobulins results in the triggering of a response. Alternately the wandering "T" cells collect antigen and bring it to the "B" cells. Since the "T" cells comprise the major portion of the recirculating small lymphocyte pool (Miller and Sprent,1971b)

such a proposal appears attractive.

(2) Carrier antibody

This hypothesis proposed by Bretscher and Cohn (1968, 1970) states that the induction of humoral antibody formation involves the obligatory recognition of two determinants on an antigen, one by receptor antibody on a "B" cell and the other by carrier antibody which may be free in the circulation or may be carried on a cell. The generation of carrier antibody is a thymus dependent process. Interaction of antigen with the receptor carrying "B" cell in the absence of carrier antibody results in specific "B" cell tolerance. The advantage of this hypothesis is that it provides a mechanism for the initiation of tolerance and the discrimination between self and non-self. Associated recognition of antigen by carrier and receptor antibodies results in the initiation of antibody response.

An alternative of this hypothesis is that the "T" cell elaborates a unique class of antibody (IgX) with specific binding capacity for certain determinants on the antigen which it then concentrates either onto the "B" cell itself or onto macrophages (Miller et al,1971;Mitchison,1969a).The recent finding described by Feldmann (1972) may be very relevant to this hypothesis.

(3) Thymus-derived mediators

In this hypothesis it was postulated that upon contact with antigen the "T" cell secretes a diffusable mediator which is not antigen specific and plays a regulatory role on "B" cells. Dutton et al (1971a) had postulated that the "B" cell

is only triggered when it receives two signals, one from the interaction of its immunoglobulin receptor with antigen and the second from a stimulated "T" cell which elaborates the mediator, which can trigger any "B" cell within reach. The only limitation is the effective distance of the diffusion gradient of the mediator.

Recent experimental evidence has accumulated which suggested that "T" cells can elaborate a soluble factor to enhance the immune response of B lymphocytes (Haskill et al, 1970; Doria et al, 1972; Gorczynski et al, 1972).

IV THE INTERACTION OF ANTIGEN WITH LYMPHOCYTES

The specific immune response is a result of the interaction of antigen with lymphocytes. Thymus-dependent antigens such as heterologous erythrocytes and foreign serum proteins, as previously discussed, require the presence of both "T" and "B" cell types to elicit the antibody response. Thus, it is important to understand the nature and the mechanisms of this interaction in order to understand whether immunological specificity towards a given antigen is dictated by one or both cell types.

(1) Antigen Binding Lymphocytes

The ability of antigen primed lymphocytes to undergo blastogenesis or to take up tritiated thymidine for DNA synthesis in vitro when cultured with the same antigen (Oppenheim et al, 1965; Pearmain et al, 1963; Elves et al, 1963), clearly suggested that initiation of immunological

events following the exposure of immunocompetent cells to antigen included as an early step combination between antigen and a receptor on lymphocytes.

Binding of antigen to lymphocytes, although noted by previous investigators (Kaplan et al, 1950; Coons et al, 1951) has not been investigated in any detail. Nossal and co-workers (1966) described the concentration of flagellin in spleen lymphoid follicles after intravenous injection into rats, but the cell types involved were not defined. Han and Johnson (1966) injected ferritin labelled with iodine ^{125} into the foot pads of rats and observed ferritin in lymphocytes. Direct evidence for the binding of antigen to lymphocytes came from a number of different studies. The most important ones include the binding of SRBC to the surface of a small number of normal lymphocytes to form rosettes (Biozzi et al, 1967; Möller and Greaves, 1971; Greaves and Hogg, 1971; Bach and Dardenne, 1972); the detection of radioactive antigen on the surface of lymphocytes by radioautography (Naor and Sulitzeanu, 1967; Byrt and Ada, 1969; Humphrey and Keller, 1970; Davie et al., 1971; Dwyer et al, 1971, 1972; Unanue, 1971a); the specific elimination of immunocompetent cells from normal and immune lymphoid cell populations by antigen-coated columns (Wigzell and Andersson, 1969; Wigzell and Mäkelä, 1970; Truffa-Bachi and Wofsy, 1970) and specific inactivation of immunocompetent cells from a lymphoid population by highly radioactive antigen

which would kill the antigen binding cells (Ada et al, 1970; Ada and Byrt, 1969; Humphrey et al,1971; Unanue, 1971b).

Although lymphocytes bind antigens, the question whether "T" or "B" or both cell types are involved is still controversial. Using rosette formation (Greaves and Möller, 1970; Greaves and Hogg, 1971; Möller and Greaves, 1971; Bach and Dardenne, 1972) and radioactive suicidal experiments (Basten et al, 1971), evidence has been presented that both "T" and "B" cells can bind antigens and can be specifically tolerized. However, using the same approaches, Schlesinger and Yron (1970), Brody (1970), Gorczynski et al (1971) and Unanue (1971a) found that only "B" cells bind antigens but "T" cells do not. In view of the fact that both "T" and "B" cells can carry specific memory and can be specifically tolerized (Section II), it is reasonable to expect that both cells can interact with antigen, unless other mechanisms exist in which interaction of antigen with one of the cells can profoundly affect the other in a specific way.

(2) <u>The Nature of Binding of Antigen to Lymphocytes</u>

Under the assumption that only antibody recognizes antigen (Mitchison, 1967), it is reasonable to believe that binding of antigen to lymphocytes is mediated by the same mechanism as antigen-antibody reactions. Mitchison demonstrated (1967) that induction of the secondary immune response by incubation of primed cells in vitro with antigen can be inhibited by pretreating the cells with anti-Ig antiserum.

Such antisera have been shown to block the uptake of radioactively labelled antigens by normal lymphoid cells (Byrt and Ada, 1969; Unanue, 1971a; Werner et al, 1970) to block the retention of immunocompetent cells by antigen-coated columns (Wigzell et al, 1971; Walters and Wigzell, 1971) and to inhibit rosette formation (Biozzi et al, 1967; McConnell et al, 1969; Greaves and Hogg, 1971). These results suggested that Ig is the site on the lymphocyte surface which binds antigen. Since anti-Ig sera inhibit both the "B" cell function for antibody production (Lawton et al, 1972; Sjoberg and Greaves, 1971; Manning and Jutila, 1972a; Greaves, 1971a; Kincade et al, 1970; Pierce et al, 1972) and the "T" cell function such as graft versus host reaction (Mason and Warner, 1970) and mixed lymphocyte reactions (Greaves et al, 1969; Horung et al, 1971) it is believed that both "T" and "B" cells bind antigens through Ig molecules on their surface.

(3) Immunoglobulin Structures on the Lymphocyte Surface

Binding of antigens to lymphocytes and the subsequent blocking by anti-Ig sera demand the existence of Ig structures on the lymphocyte surface. Even at the end of the last century, Ehrlich (1900) postulated that animals produce various types of cells with "receptors" which are complimentary to the injected or invading bacterial or cellular antigens. As a consequence of interaction between the antigens and closely fitting "receptors" on the cell surface, the cells are stimulated for the regeneration of tne homologous "receptors"

which later pass into the blood as antibodies. Jerne's (1955) natural selection theory may be considered the first to revive Ehrlich's theory of antibody formation. Shortly thereafter Burnet (1959) modified the Ehrlich-Jerne selective theory introducing his clonal selection theory of acquired immunity, in which he postulated the existence of preformed "receptors" on the antigen sensitive lymphocyte surface presenting the same complementarity to antigen as the circulating antibodies. A signal resulting from contact of antigenic determinants and receptors would initiate cellular events leading to antibody formation. In essence the theory postulates the existence of receptors of antibody nature, thus Ig on the normal non-stimulated lymphocytes.

As Burnet's clonal selection theory (1959) became known, investigations of receptors of Ig nature on the surface of cells of lymphoid tissues were vigorously pursued. Perhaps the earliest demonstration of such surface Ig was the blast transformation of rabbit lymphocytes in culture by anti-rabbit Ig sera, including anti-allotypic sera, as reported by Sell and Gell (1965) and subsequently by others (Daguillard and Richter, 1969; Oppenheim et al, 1969). More direct and quantitative evidence on the existence of Ig on the lymphocyte surface derived from the utilization of various techniques employing heterologous anti-Ig antisera. The most widely used ones are the fluorescent antibody technique utilizing fluorescein conjugated anti-Ig (Raff et al, 1970; Pernis et

al, 1970; Rabellino et al, 1970; Unanue et al, 1971); auto-radiography using radioactively labelled anti-Ig (Raff et al 1970; Davie et al, 1971b; Bankhurst and Warner, 1971) cyto-toxicity using anti-Ig sera and complement (Takahashi et al, 1971b); the mixed antiglobulin technique in which anti-Ig agglutinates the Ig carrying lymphocytes with Ig coated SRBC, resulting in the formation of rosettes (Coombs et al, 1970) and the enzymatic radio-iodination method in which membrane bound Ig on viable lymphocytes was iodinated by enzyme lactoperoxidase. Radiolabelled molecules on the cell membrane were found to precipitate with anti-Ig antisera (Baur et al, 1971). Finally the reverse immune cytoadherence technique utilizes a 5S hybrid antibody which carries an anti-Ig site to react with surface bound Ig on lymphocytes while with other site reacts with a protein coated indicator cell to form rosettes (Paraskevas et al, 1970, 1971a). These studies have shown that Ig existed on the surface of some but not all normal lymphocytes. They have also raised the question whether both B and T lymphocyte populations bear surface Ig.

The existence of Ig on the surface of T cells remains today controversial. In favor of the existence of surface Ig on T cells are reports that anti-light chain antiserum abrogated the ability of thymus cells to participate in a graft-versus-host reaction (Mason and Warner, 1970; Greaves et al, 1971) and inhibited thymic dependent reactions of peripheral lymphocytes such as mixed lymphocyte reaction

(Greaves et al, 1969). Other studies have shown that anti-light chain or anti-μ chain sera could interfere with a number of "T" cell functions such as helper function (Lesley et al, 1971), antigen binding property (Greaves and Hogg, 1971; Bach and Dardenne, 1972; Humphrey et al, 1971) and thymus and non-thymus cell collaboration (Basten et al, 1971). Small amounts of Ig on "T" cells have been demonstrated recently (Hammerling and Rajewsky, 1971; Nossal et al, 1972). Using the same approach to suppress "T" cell function by anti-light chain, anti-Ig or class specific antisera, Canty and Wunderlich (1970), Takahashi et al (1971a)and Manning and Jutila (1972a)however, obtained negative results. Using various other techniques as discussed above, it has been shown by most studies that Ig-carrying cells are consistently absent from the thymus of mouse (Takahashi et al, 1971b; Raff et al, 1970; Paraskevas et al, 1970; Rabellino et al, 1971), rabbit (Pernis et al, 1970; Daguillard and Richter, 1969; Coombs et al, 1970), guinea pig (Paraskevas et al, 1971b), chicken (Kincade et al, 1971; Rabellino and Grey, 1971) and human (Paraskevas et al, 1971a; Wilson and Nossal, 1971.) The contradictory findings are believed by some authors (Mond et al, 1972; Unanue et al, 1971; Katz and Benacerraf, 1972) to be due to the specificities of the antisera which may contain in some cases other antibodies. However, since "T" cells have been shown to respond to antigens by mitosis (Davies et al, 1966) and to bind antigens (Greaves and Hogg, 1971;

Möller and Greaves, 1971; Basten et al, 1971), the possibility that receptors of Ig nature are present on "T" cell surfaces can not be excluded. The lack of detection of such a receptor on "T" cells is believed also (Unanue et al, 1971; Katz and Benacerraf, 1972) to be due to:

a) Ig molecules are deeply buried in the plasma membrane of the lymphocyte where they are unlikely to be detected;

b) The "T" cell may bear Ig, but in a concentration below the level of detection by the present day techniques;

c) Only a small number of "T" cells bear surface immunoglobulin and are obscured from detection due to large numbers of Ig bearing cells; and

d) "T" cells may have Ig on their surfaces which is antigenically different from the currently known classes of immunoglobulin.

While the presence of Ig on the "T" cell surface is controversial, the evidence for the presence of Ig on the "B" cell surface is abundant. This evidence also indirectly supports the view of the lack of surface Ig from "T" cells.

Surface Ig on B lymphocytes has been explored using the following approaches:

(i) The lowest concentrations of lymphocytes with surface immunoglobulin were observed in organs where the thymic-derived cells are thought to be predominant (Raff and Wortis, 1970; Bankhurst and Warner, 1971).

(ii) It has been shown in experimental animals that conditions known to deplete "T" cells will increase the number of immuno-

globulin carrying cells and conditions known to deplete "B" cells will diminish immunoglobulin carrying cells. Thus lymphocytes from thymectomized irradiated and bone marrow reconstituted mice showed a high proportion of immunoglobulin carrying cells (Unanue et al, 1971; Raff, 1970a) and lymphocytes from agammaglobulinemic chickens produced by bursectomy at hatching showed complete absence of immunoglobulin carrying cells (Kincade et al, 1971; Rabellino and Grey, 1971).

(iii) In human immune deficiency diseases such as Bruton's type of agammaglobulinemia absence of immunoglobulin carrying cells was observed in the peripheral blood (Kincade et al, 1971; Naor et al, 1969; Fröland et al, 1971). Virtually all cells in the peripheral blood of patients with "B" cell type chronic lymphatic leukemia (CLL) were shown to bear surface immunoglobulin (Wilson and Nossal, 1971; Pernis et al, 1971).

(4) <u>The Number of Ig Classes on the Surface of the Lymphocyte</u>

One cell one specific antibody may be considered a central dogma of the clonal selection theory (Burnet, 1959) and much evidence indicates this to be true (Mäkelä, 1967; Peterson and Ingraham, 1969; Gershon et al, 1968a). Of equal importance is the question related to the number of Ig classes or allotypes produced by a single cell. It is widely held that one cell produces one Ig class (Mellors and Korngold, 1963; Bernier and Cebra, 1965; Burtin and Buff, 1965; Cebra et al, 1966; Nordin et al, 1970), one type of light chain (Bernier

and Cebra, 1965; Pernis and Chiappino, 1964) and one allotype among those controlled by allelic genes (Pernis et al, 1965). Cells examined in the above experiments are more or less well differentiated cells, blasts or plasma cells. The restriction although true for antibody producing cells may not apply to the precursor cells. Recently even among cells at different stages of differentiation, contradictory views to the above results have been expressed in which a small number of antibody or Ig secreting cells are producing more than one class of Ig (Nussenzweig et al, 1968; Nossal et al, 1971; Greaves, 1971a; Takahashi et al, 1968; Costea et al, 1967), more than one allotype (Greaves, 1971b) and even more than one specificity (Liacopoulos et al, 1971; Attardi et al, 1964; Hiramoto and Hamlin, 1965). It is not known at which stage after stimulation phenotypic restriction will take place. It has been shown by Greaves (1971a) who employed inhibition of rosettes that between days 15 and 30 after antigenic stimulation the antigen binding cells appeared to express only a single class of Ig on their surface. The antigen binding cells examined before that time period were expressing more than one class of Ig or allotype.

It is well known that normal resting lymphocytes carry Ig on their surface and can be easily detected by various techniques. These surface Ig molecules are lost when lymphocytes differentiate to plasma cells (Pernis et al, 1970; Paraskevas et al, 1971b; McConnell, 1971; Zucker-

Franklin,1972) which,however,are actively secreting large amounts of Ig. It is likely that in cells actively synthesizing Ig,phenotypic restriction is invariably expressed. It is not clear,however,whether in the resting lymphocytes phenotypic restriction is expressed.

Although class (Rabellino et al,1971) and allotypic (Pernis et al,1970; Davie et al,1971b) restriction and even the exclusive presence of IgM class (Uhr and Vitetta,1973) on the surface of lymphocytes has been described,several investigators have recently shown that in the resting lymphocyte population, phenotypic expression is less restricted. More than one class of Ig (Lawton et al,1972;Kincade et al,1970;Manning and Jutila, 1972),more than one type of light chain (Heller et al,1971) and even more than one allotype (Sell et al,1970;Greaves,1971b) can be expressed simultaneously on the surface of a lymphocyte. In other words,resting multipotent lymphocytes do exist with respect to the classes,type of light chains and allotypes of surface Ig.

V. SURFACE ANTIGENIC MARKERS FOR DISTINGUISHING T AND B CELLS

Since the lymphoid organs contain a heterogeneous population of lymphocytes the evaluation of the role of T and B cells in various immunological phenomena was made possible with the discovery of individual antigenic markers present on these cells.

T and B cells evolved from a common stem cell which resides in the bone marrow in adult animals (Ford,1966). The differentiation of T lymphocytes takes place in two steps:

first: the differentiation of stem cells to thymocytes within the thymus (Moore and Owen, 1967a; Owen and Ritter, 1969) and second: further differentiation of thymocytes to peripheral T lymphocytes. Both steps have been found to involve quantitative changes of surface alloantigen which can be followed by alloantisera (Takahashi et al, 1970b; Owen and Raff, 1970). These antigens are called differentiation antigens by Takahashi et al (1970b, 1971b). The differentiation of B cells may take place in one step and involves the direct seeding of bone marrow cells to the peripheral lymphoid organs (Ford, 1966; Moore and Owen, 1967b). The maturation of B cells may be influenced by the local microenvironment (Moore and Owen, 1967b).

1. Surface Markers on T Lymphocytes

Reif and Allen (1964) first described the existence of a θ alloantigen on thymocytes using dye exclusion cytotoxicity tests. By absorption studies they found this antigen to be present in brain and in some lymphocytes of the lymph nodes and spleen. They later demonstrated that antigen was controlled by a single locus with two alleles: θ-AKR found in AKR, RF and a few substrains and θ-C3H found in most other inbred strains of mice (Reif and Allen, 1966). Anti-θ antisera can be prepared by injecting thymocytes of AKR into C3H mice or vice versa. Monospecific anti-θ serum has been raised in congenic mice differing only at the θ locus (Aoki et al, 1969).

Boyse and Old's group have described several other antigens including alloantigens TL (thymus leukemia) (Boyse and Old, 1969) and Ly (Boyse et al, 1968) and a heteroantigen MSLA (mouse specific lymphocyte antigen) (Shigeno et al, 1968) present on the surface of thymocytes. The TL antigens (in TL+ strain) are restricted to thymocytes and are not expressed on the surface of the peripheral T cell (Aoki et al, 1969; Takahashi et al, 1970b). These antigens are lost during differentiation when the thymus cells emigrate from thymus to peripheral lymphoid organs (Aoki et al, 1969; Raff, 1971). Partial loss in strength of θ, Ly and MSLA antigens has also been described during differentiation from the stage of virgin thymus cells to thymus-derived cells, but enough is retained on the thymus-derived cell surface to be detected (Raff, 1971; Takahashi et al, 1970b, 1971b).

The detection of θ, Ly and MSLA antigens in the peripheral lymphoid tissue suggested that these antigens might serve as useful markers for T lymphocytes, a suggestion which has been substantiated by the following evidence:

(i) All thymocytes possess these antigens, as verified in many studies (Reif and Allen, 1964; Raff, 1970a; Raff and Owen, 1971).

(ii) Cells of thymus origin possess these antigens whereas cell populations of marrow origin do not. Thus lymphatic leukemia of thymus origin possess θ and Ly antigens (Takahashi et al, 1971b) whereas myeloma cells (Takahashi et al, 1971b),

plasma cells (Aoki et al 1969), plaque forming cells (Greaves and Moller, 1971; Takahashi et al, 1971b; Cerottini et al, 1970 and Schlesinger, 1970) and bone marrow cells (Raff and Owen, 1971), do not.

(iii) In mice treated with ALS (antilymphocytic serum) (Schlesinger and Yron, 1969; Raff, 1969) or in neonatally thymectomized mice (Schlesinger and Yron, 1970; Raff and Wortis, 1970) or in mice with congenitally hypoplastic thymus (nude mice) (Raff and Wortis, 1970) there is a greatly reduced number of θ or Ly antigen bearing cells in the peripheral lymphoid tissues.

Recently, heterologous antiserum against thymocytes has been raised in guinea pigs (Stevach et al, 1972) and was made specific for T cells by absorption with guinea pig leukemic cells of bone marrow origin. The antiserum has been shown to interfere with T cell functions without affecting those of B cells, thus behaving somewhat like anti-θ serum in the mouse system.

Specific heterologous antiserum to chicken T lymphocytes has also been reported (Potworowski, 1972), but the usefulness of this antiserum is less clear.

2. Surface markers on B lymphocytes

Several antigenic markers on B lymphocytes have been recently described, especially in the mouse system.

(i) Surface Ig

One of the most widely studied B cell antigenic

markers is surface Ig which exists on the surface of lymphocytes in all species under investigation as previously discussed (Section III). Surface Ig is generally considered as a B cell property. In the mouse system Ig carrying and θ carrying lymphocytes belong to two distinct separate populations of cells (Raff, 1970a; Rabellino et al, 1971; Takahashi et al, 1971a, 1971b; Unanue et al, 1971). In other words, B cells are distinct from T cells when surface Ig is used as a marker. In the guinea pig system, it has also been used with some degree of success (Stevach et al, 1972). The same conclusion has also been reached in chicken (Kincade et al, 1971; Rabellino and Grey, 1971; Potoworoski 1972), rabbit (Pernis et al, 1970) and human systems (Wilson and Nossal, 1971; Froland et al, 1970) where as yet no distinct T cell marker has been identified.

It should be emphasized, however, that surface Ig is restricted to B lymphocytes and not for the antigen-induced progeny of B lymphocytes such as plasma cells which has lost surface Ig during differentiation (Pernis et al, 1970; Paraskevas et al, 1971b; McConnell,1971; Zucker-Franklin and Berney, 1972).

(ii) Mouse B lymphocyte antigen (MBLA)

A specific antiserum against a mouse B lymphocyte antigen (MBLA) has been raised in rabbits by Raff et al (1971). It was produced by immunizing rabbits with lymph node lymphocytes from mice which had been thymectomized, lethally

irradiated and reconstituted with syngeneic fetal liver cells. It was made specific by absorption with mouse liver, erythrocytes and thymocytes until it was not cytotoxic to thymocytes. The antiserum has been successfully used to show that bone marrow and bone marrow-derived B cells were distinct from θ carrying T cells in the mouse (Raff et al, 1971; Raff, 1971).

(iii) Fc receptors on B lymphocytes

Recently, a receptor for the Fc fragment of antibody has been described on mouse B lymphocytes (Paraskevas et al, 1971b, 1972a; Miller et al, 1971; Basten et al, 1972). The Fc receptor on B lymphocytes could be detected only when the intact 7S antibody was complexed with antigen or when Fc isolated by papain digestion was used. 5S antibody-antigen complexes were inactive (Paraskevas et al, 1971b; 1972a, 1972b; Miller et al, 1971). The Fc receptor was detected by the ability of antibody-antigen complexes to bind to lymphocyte surface and to interfere with the detection of surface Ig on B lymphocytes (Paraskevas et al, 1971b, 1972a) or by direct binding of radioactive antibody-antigen complexes on the lymphocyte surface using autoradiography (Basten et al, 1972). Using this unique receptor, Basten et al (1972) have successfully separated B cells from θ carrying T cells in normal and immune spleens of mice.

(iv) Complement receptor

Besides the Fc receptor on B cells, Lay and Nussenzweig (1968) and Bianco et al (1970) have reported that

there is a distinct population of lymphocytes which could be differentiated by the presence on the plasma membrane of a receptor for modified C3 component of complement when the complement was in antibody-antigen-complement complexes (Ab-Ag-C). The receptor was detected by the fixation of the complex to the lymphocyte surface. When the antigen was SRBC, fixation on lymphocyte surface could be visualized because it led to the formation of clusters or rosettes. The receptor could also be detected by autoradiography when one of the components of the complex was radioactively labelled. It has been shown by Bianco and Nussenzweig (1971) that Ig and C3 receptors co-existed on the surface of the same cell which were distinct from θ carrying T cells in the mouse, implying that C3 receptor carrying cells are of bone marrow origin. Using the property of rosette formation through the C3 receptor, Nussenzweig et al (1971) have been successful in separating mouse B and T cells.

MATERIALS AND METHODS

I. MICE

BALB/c, C3H and AKR mice were obtained from Jackson Laboratories, Bar Harbor, Maine.

II. ANTIGENS

1. Mouse myeloma proteins

Myeloma proteins of IgG (IgG2a), IgF (IgG1), IgH (IgG2b), IgA and IgM classes were isolated from ascitic fluids of plasmacytoma bearing BALB/c mice by diethylaminoethyl (DEAE) anion exchange columns equilibrated in 0.005M phosphate buffer pH 7.5. The myeloma protein was prepared by stepwise elution with increasing buffer molarity as recommended by Potter (1967). The peaks or fractions from the columns were concentrated and examined by immunoelectrophoresis against a polyvalent rabbit anti-mouse Ig serum in order to identify the fractions containing the myeloma proteins. The myeloma proteins were further purified by gel filtration on Sephadex G-200 equilibrated in 0.15M borate buffered saline at pH 8.0. The purity of each myeloma protein was finally examined by immunoelectrophoresis and Ouchterlony techniques using the polyvalent rabbit anti-mouse Ig antiserum. IgG (7SIgG2a), IgF (7SIgG1) and IgH (7SIgG2b) showed only one precipitin line by both techniques. IgA and IgM, however, contained trace amounts of albumin as shown by immunoelectrophoresis.

A Bence Jones protein (BJ) of K type was isolated from urine of plasmacytoma bearing mice according to methods given by Potter (1967). The urine was dialysed against

distilled water at 4°C for 72 hours, centrifuged at 10,000 rpm for 10 minutes and concentrated using ultrafiltration. The sample was then dialysed and equilibrated in 0.05M Tris acetate buffer pH 5.5. The light chain protein was isolated from the first peak of a DEAE cellulose column equilibrated in 0.05M Tris-acetate buffer pH 5.5. Further purification was carried out on a Sephadex G-200 column.

2. Horse spleen ferritin

Ferritin (Fe) 2x crystalline, cadmium free was purchased from Nutritional Biochemicals, Cleveland, Ohio.

3. Chicken egg albumin

Chicken egg albumin (EA) was purchased from Pentex, Kankakee, Illinois.

4. Polymerized flagellin

The method of preparation of polymerized flagellin from Salmonella adelaide, strain SW1338 was that of Ada et al (1964). We are grateful to Dr. W. D. Armstrong of the University of Alberta for providing the bacterial culture and for demonstrating the isolation of the flagellar organisms.

(i) Growth of organisms

The bacteria of S. adelaide were grown in trays (25x20 cm) containing a layer (0.5 cm) of medium consisting of heart infusion broth (Difco) in 0.9% agar. The culture trays were incubated at 37°C for 24 hours. At the end of the incubation, saline containing 1/10,000 merthiolate (W/V) was added to each tray, the organisms were scraped off the surface of

the agar and the fluid containing the bacteria was harvested.

(ii) Isolation of flagella

Flagella were isolated from the organisms by homogenization (Waring blender) and subsequent centrifugation (6,000 rpm for 20 minutes). The organisms were re-homogenized again in saline containing merthiolate, centrifuged and supernates were filtered through a funnel packed with glass wool to remove agar. Centrifugation at high speed (26,000 rpm for 45 minutes) resulted in a pellet containing the flagella. The pellet was re-homogenized and centrifuged at low speed (6,000 rpm for 30 minutes.) The supernate was re-subjected to the above procedures and the flagella were isolated in pure form (pellet) by centrifugation at 26,000 rpm for 45 minutes. This material was suspended in 0.01M phosphate buffer pH 7.5 and stored at-26° C.

(iii) Production of polymerized flagellin (POL)

To a flagella suspension, 1N HCl was added to a final concentration of 1/20 in volume. This caused the immediate decrease in opacity of the flagella suspension, due to the formation of monomeric flagellin. The monomers were separated from the acid insoluble flagella by centrifugation at 35,000 rpm in a Spinco SW 39 rotor. The supernatant was saved and neutralized with 1N NaOH. Polymerized flagellin was obtained by addition of saturated ammonium sulfate to the flagellin solution to a concentration of 15%. Polymerization was allowed to proceed overnight at room temperature. The

polymerized flagellin was isolated as sediment by centrifugation at 26,000 rpm and resuspended in distilled water. The final preparation was dialyzed against distilled water for two days to remove ammonium sulfate and then it was sterilized by passing through millipore filters and stored at -26° C. The protein content of polymerized flagellin can be measured in a U.V. spectrophotometer at 215 mu in monomeric form by dissolving in acid.

III. RABBIT IMMUNIZATION AND COLLECTION OF ANTISERA

Groups of two or three rabbits were injected intramuscularly with the following antigens: IgG, IgF myeloma proteins, egg albumin or horse spleen ferritin. The proteins were given emulsified in complete Freund's adjuvant (Difco, Detroit) and each animal was injected once a week for five weeks with one ml containing 1 mg of protein. One week after the last injection, the rabbits were test bled through the vein of the ear or by heart puncture. The antibody activity of each antiserum was checked by immunoelectrophoresis. Once the rabbits showed the proper antibody activities, they were bled by heart puncture once a week for four or five times. Following a rest period, a booster injection of 1 mg protein was given. Thirty to fourty ml of blood was collected and the serum was stored at -26° C.

IV. SEPHADEX G-100 AND G-200 GEL FILTRATION

The Sephadex G-100 and G-200 columns were packed according to the instructions given by Pharmacia Ltd. (Montreal, Canada). The complete swelling of the Sephadex gels was carried out at room temperature for three days in the desired buffer. The gels were added gradually to an excess of buffer with mixing to facilitate dispersion of the beads in the swelling medium. Decanting and stirring were carried out intermittently during swelling. Before packing, the air bubbles trapped in the gel-buffer mixture (1:1 in volume) were removed by a vacuum pump. A Sephadex laboratory column was mounted vertically on a stand and 15-20 cm of buffer was poured into it. Any air bubbles trapped in the polyethylene disc were removed by forcing the buffer back and forth through the disc by a syringe attached to the outlet tubing. The gel-buffer mixture was dispersed evenly and poured down the column gradually until it reached the top of the column. The outlet was kept at the same level with the top of the gel slurry. After about half and hour, when approximately 10 cm of bed was settled, the outlet was lowered, so that the pressure was equal to 1 cm. As the packed bed rose, the outlet was lowered to maintain an optimum pressure equal to one tenth of the packed bed length. To prevent boundaries, the excess buffer was removed and another portion of gel slurry was added before the previous portion had settled completely. Care was taken not to disturb the packed bed during addition of the gel slurry.

After the column was packed, the upper surface of the bed was protected by inserting a sample applicator. The column was then allowed to equilibrate for 24 hours at 10-15 cm pressure at a rate of about 20 ml/hour. Before starting an experiment, about 5 mg blue dextran dissolved in 3 ml buffer were filtered through the column in order to check the homogeneity of the packing and determine the void volume.

In the experiments, the flow of the column was kept at a rate of 5-10 ml/hour, under a pressure head of 2-3 cm.

V. ION-EXCHANGE CHROMATOGRAPHY BY DEAE-CELLULOSE

The DEAE-cellulose ion exchange column was packed according to the method of King (1968). The dry DEAE-cellulose ion-exchanger (Carl Schleicher and Schuell Co., Keene, N.H. .89 meq/gm dry weight) was washed by suspending it in .5N NaOH+ .5N NaCl with stirring. After settling for 30 minutes, the cloudy supernatant was decanted. The cellulose was then resuspended in IN NaCl. The process of stirring and decanting was continued for once or twice and the cellulose was filtered by suction on Buchner funnel through filter paper. The moist cake was suspended in IN HCl, immediately filtered by suction, and washed with distilled water until the pH was neutral. The ion-exchanger was then equilibrated to the desired pH and molarity by suspending and washing with the starting buffer. The cellulose-buffer mixture was prepared in thick slurry form which was first dispersed in a waring blender and trapped air bubbles were removed by a vacuum pump. The uniform suspension

was poured into the column. The cellulose was allowed to settle to 4 or 5 cm length at the bottom of the column with the outlet closed. Excess buffer was removed with the outlet open and more slurry was added until the column was packed. A 3-5 cm column of the buffer above the bed was left behind to avoid any disturbance of the packed bed with next addition of cellulose suspension. The whole process of packing was carried out under the atmospheric pressure. The upper surface of the bed was protected by inserting a piece of filter paper or a sample applicator. The DEAE-cellulose ion-exchange column was run under a pressure generally greater than atmospheric pressure at a rate of 30 to 40 ml per hour.

VI. IMMUNOELECTROPHORESIS

The method was that of Scheidegger (1955). The glass slides (25 x 76 mm) were first coated with 2 ml .5% melted agar (Difco Noble) in distilled water and dried at 80 C for 4 hours. In the immunoelectrophoretic experiments, 3 ml 2% melted agar in .025M barbital buffer pH 8 were layered on the coated slides. The agar gel was allowed to solidify for a few minutes, and the required wells and troughs were cut. The materials to be tested were placed in the wells and the slides were then placed in electrophoretic apparatus and ran at 14 ma approximately 70 to 75 volts for three and a half hours. The slides were then removed from the apparatus, the troughs were filled with proper antisera and placed in a

moist chamber at room temperature overnight to allow the development of the precipitation lines. The slides were washed in .9% saline for 24 hours, desalted in distilled water for 8 hours, and then dried overnight by placing filter paper on the slides. The dried slides were stained with amido black (1 gm/1000 ml sodium acetate buffer) for 10 minutes, decolorized in acetic acid (acetic acid: methanol: water = 150: 750: 750 in volume) for another 10 minutes and then air dried.

VII. OUCHTERLONY GEL DIFFUSION

Coated slides as described in immunoelectrophoresis were used with 3 ml 1.5% melted agar in .15M saline. The gel was allowed to solidify for 5 to 10 minutes in a moist environment. Required patterns of antigen-antibody precipitation reaction were arranged by filling the wells with antigen or antibody solution. The slide was placed in a moist chamber at room temperature overnight for the precipitation lines to develop. It was then washed and stained as described for immunoelectrophoretic slides.

VIII. IMMUNOABSORBENTS

1. Bis-diazotized benzidine method (BDB)

The BDB reagent was prepared according to the method given in the Handbook of Experimental Immunology by Herbert (1967) and stored in 2 ml volumes at -26° C. All the mouse immunoglobulins were aggregated according to the

method of Bernier and Cebra (1965). To 10 mg of myeloma protein in 5 ml buffer (0.1M phosphate buffer pH 6.8) 5 ml of 1/15 dilution of BDB reagent in the same buffer were added and incubated at room temperature for 5 hours. The aggregates formed were washed 3 times with the same buffer before use.

2. Ethyl chloroformate method

Ethyl chloroformate was purchased from K and K Laboratories Inc., Plainview, N.Y. Chicken egg albumin was aggregated by this reagent using the method of Avrameas and Ternynck (1967). To 100 mg of EA in 4 ml of 0.1M acetate buffer pH 4.5, 0.2 to 0.3 ml ethyl chloroformate was added dropwise over a period of one minute with efficient stirring for 15 minutes. Further incubation was carried out for one hour with occassional stirring. The aggregates were then washed successively before use with large excess of phosphate buffered saline, 0.1% sodium carbonate followed by glycine-HCl buffer until the optical density of the eluates was 0 at 280 mu. The aggregates were then maintained at neutral pH by washing in phosphate buffered saline.

IX. PREPARATION OF ANTIBODIES BY IMMUNOABSORBENTS

1. Anti-mouse Ig and anti-egg albumin antibodies

One hundred mg of aggregated mouse IgG, IgF or egg albumin were added to 10 ml of rabbit anti-IgG, anti-IgF or anti-EA respectively. The samples were stirred in the cold overnight. The aggregates were then separated from the serum

by centrifugation at 10,000 rpm, washed three times in cold 0.15M saline. The antibodies were eluted in 3 ml 0.1M glycine-HCl buffer pH 2.5 for one hour. The supernatant containing the antibodies was recovered after centrifugation and immediately neutralized.

The antibodies recovered contained 80-90% specifically precipitable protein as determined by a quantitative precipitin method. They reacted with the respective antigens and formed only one line by immunoelectrophoresis in the IgG region when examined against a polyvalent goat anti-rabbit serum.

2. Anti-ferritin antibody

Anti-ferritin antibody was isolated following the method suggested by Hämmerling et al (1968). To 10 ml of rabbit anti-ferritin serum sufficient ferritin was added to produce maximal precipitation. This was determined by a quantitative precipitin test before use. The specific precipitates were isolated by centrifugation at 4° C at 10,000 rpm, dissolved in 0.1M glycine-HCl buffer pH 2.5 and left at 4°C for one hour. Subsequently the solution was centrifuged in a Spinco centrifuge for 60 minutes at 35,000 rpm using a SW 39 rotor. Ferritin being heavier sedimented to the bottom of the tube. The supernatant was collected and neutralized immediately. The protein which was recovered precipitated with ferritin in an Ouchterlony plate and formed only one line in the IgG region with goat anti-rabbit serum on immuno-electrophoresis. The sample was found to contain 80-85%

specifically precipitable protein.

X. SPECIFIC PURIFICATION OF ANTI-IgG AND ANTI-IgF ANTIBODIES

The anti-IgG and anti-IgF antibodies eluted from the immunoabsorbents cross-reacted with other classes of mouse immunoglobulins. They were made highly specific for the heavy chain of each class by exhaustive absorption, thus anti-IgG preparation was absorbed with aggregated IgF and BJ myeloma protein and the anti-IgF antibody with IgG and BJ aggregates. Each preparation after absorption was examined by Ouchterlony technique and gave one line with the corresponding antigen but no line against the other mouse Ig's or BJ proteins. The specificity of each antibody preparation was also examined by passive hemagglutination, the anti-IgG antibody did not react with IgF or BJ coated SRBC and the anti-IgF antibody did not react with IgG or BJ coated SRBC, but both showed strong reactions when SRBC were coated with the homologous antigen.

XI. PREPARATION OF 5S HYBRID ANTIBODIES

1. Pepsin Digestion of antibodies

The antibody preparations were digested by pepsin (Mann Research Laboratories, New York) according to the methods of Nisonoff et al (1960) and Utsumi and Karush (1965) to obtain $F(ab)_2$ fragments. The antibody at a concentration of 1-2% was digested with pepsin for 4-5 hours at 37°C at a protein-enzyme ratio of 100:1 by weight, in 0.1M sodium

acetate buffer pH 4. The digestion was stopped by neutralization with 1N NaOH to pH 8, and the sample was applied on a Sephadex G-100 column (23 x 970 mm) equilibrated with 0.15M borate buffered saline pH 8 to separate 5S $F(ab')_2$ fragments from non-digested 7S globulins.

2. Hybridization of $F(ab')_2$ fragments of antibodies of two specificities.

Hybrid antibody molecules of two specificities were prepared according to the method of Nisonoff and Rivers (1961). The $F(ab')_2$ fragments of two antibodies, for example anti-IgG and anti-ferritin or anti-IgF and anti-EA were mixed in equal amounts in 1-2% concentration and dialysed against one litre of 0.1M sodium acetate buffer pH 5 overnight at 4° C. The mixture was reduced with 2-aminoethanethiol hydrochloride (2-mercapto-ethylamine/HCl) (Matheson Coleman and Bell, Norwood, Ohio) at a final concentration of 0.015M under nitrogen at 37°C for 60 minutes. The reducing agent was then removed by passage through a column (10 x 200 mm) of cation exchange resin AG50WX4 (mesh 100-200, Bio-Rad Laboratories, Richmond, Calif.) packed in 0.1M sodium acetate buffer pH 5. The protein recovered from the column was immediately neutralized with 1N NaOH and re-oxidized with gentle stirring at room temperature in an atmosphere of oxygen for two hours. The re-oxidized material was applied on a Sephadex G-100 column which separated the $F(ab')_2$ recombinants from the univalent fragments. The $F(ab')_2$ fragments were concentrated by ultra-filtration to 5 to 10 mg/ml and stored at 4° C.

3. Hybrid antibody purification

The 5S re-oxidized material obtained from the Sephadex G-100 column contained at least three recombinants. In anti-IgG and anti-Fe recombination, for example, the three possible bivalent recombinants are:

i. anti-IgG-anti-Fe hybrid recombinants,

ii. anti-IgG-anti-IgG bivalent recombinants and

iii. anti-Fe-anti-Fe bivalent recombinants.

In order to isolate the hybrid recombinants, a two step absorption-elution procedure was carried out. The $F(ab')_2$ material was first absorbed with BDB-aggregated ferritin. This absorption leaves behind the bivalent anti-IgG recombinants. In the second step, the anti-IgG-anti-Fe hybrid molecules were obtained by absorption with BDB-aggregated IgG myeloma protein. About 2 mg of hybrid antibody were isolated out of 10 mg of 5S re-oxidized material, a recovery of 20%.

The following hybrid antibodies were prepared:

i. anti-mouse Ig-anti-Fe (αMIG-αFe)

ii. anti-mouse IgG-anti-Fe ($\alpha\gamma$-αFe) and

iii. anti-mouse IgF-anti-EA ($\alpha\phi$-αEA)

XII. SPECIFICITY OF HYBRID ANTIBODIES

The hybrid antibody αMIg-αFe could detect other mouse Ig and the BJ light chains. On Ouchterlony plate, it formed a line against a mixture of mouse IgG and ferritin or IgF and ferritin, but no line when IgG or IgF or ferritin

were present alone. Similarly, in passive hemagglutination tests, it reacted with a mixture of IgG coated and ferritin coated SRBC or a mixture of IgF coated and ferritin SRBC to a $\log_2$ titre of 15. No agglutination was observed with IgG or IgF or ferritin coated SRBC used alone.

The specificity of $\alpha\gamma$-αFe and $\alpha\phi$-αEA hybrids was also examined by passive hemagglutination technique even though the bivalent 7S antibodies used for their preparation were highly specific. Results are shown in Table 1. As can be seen the hybrid antibody $\alpha\gamma$-αFe did not react with either IgG coated or Fe coated SRBC alone, or with mixture of IgF coated and Fe coated SRBC, but agglutinated strongly when IgG coated and Fe coated SRBC were mixed together. Similarly the $\alpha\phi$-αEA hybrid strongly agglutinated a mixture of IgF coated and EA coated cells, but not a mixture of IgG coated and EA coated cells, or any of the cell populations present alone. These results indicate that the hybrid antibodies $\alpha\gamma$-αFe and $\alpha\phi$-αEA are highly specific for one class of mouse Ig.

TABLE 1

THE SPECIFICITY OF $\alpha\phi$-αEA AND $\alpha\gamma$-αFe HYBRID ANTIBODIES, AS CHECKED BY PASSIVE HEMAGGLUTINATION.

Hybrid antibody	SRBC coated with	Titer log 2
$\alpha\phi$-αEA (150 μg/ml)	IgF	0
$\alpha\phi$-αEA (150 μg/ml)	EA	0
$\alpha\phi$-αEA (150 μg/ml)	IgF + EA [a]	6
$\alpha\phi$-αEA (150 μg/ml)	IgG + EA [a]	0
$\alpha\gamma$-αFe (300 μg/ml)	IgG	0
$\alpha\gamma$-αFe (300 μg/ml)	Fe	0
$\alpha\gamma$-αFe (300 μg/ml)	IgG + Fe [a]	15
$\alpha\gamma$-αFe (300 μg/ml)	IgF + Fe [a]	0

[a] Mixture of two SRBC populations each coated with one protein.

XIII. INDICATOR SYSTEMS FOR RICA

Three different cell types, sheep red blood cells (SRBC), chicken red blood cells (CRBC) and cells of Bacillus cereus were used in this work. They were coated with different proteins according to the following method.

1. Formalinization

Formalinized red blood cells or bacteria were used in all the experiments. The method of formalinization was that of Wede (1962). The cells in Alsever's solution were first washed 3 to 4 times with 0.15M saline and one volume of 8% red blood cells or 1% of bacteria was incubated with an equal volume of 3% formaldehyde pH 7 (adjusted by 0.1N NaOH). The cells were incubated at 37°C for 18-24 hours and then washed 4 times with distilled water. They were suspended in distilled water as a 10% stock suspension in the case of red blood cells and 1.6×10^{10} cells/ml in the case of B. cereus.

2. Coating of SRBC and CRBC

The method used for coating of SRBC and CRBC was essentially the same as described by Boyden (1951). Two ml of a 2% formalinized RBC suspension were washed 3 times in 0.15M phosphate buffered saline pH 6.4, suspended finally in the same buffer containing 0.0025% tannic acid and incubated at 37°C for half an hour. The samples were washed twice and resuspended in two ml of buffer containing the proper concentration of protein and incubated at 37°C for one hour. It was found that 0.2 mg ferritin/ml and 0.3 mg egg albumin/ml were suitable for RICA. The coated cells were washed 3 times and finally suspended in 1.5 ml of buffer

containing 0.25% human albumin(HSA). This resulted in a suspension of approximately 2.5% cells.

3. Coating of Bacillus cereus

One ml of formalinized B. cereus suspension containing 1.6×10^{10} cells was tanned with the same method used for the RBC. Various amounts of either ferritin or egg albumin were tested to coat a certain number of bacteria. It was found that 1.5 mg of ferritin or 1 mg of egg albumin per 1.6×10^{10} bacteria in one ml was suitable for the rosette formation.

XIV. PASSIVE HEMAGGLUTINATION TEST

The method is that described by Herbert(1967) with some modifications. The test is carried out in capillary tubes which hold a series of doubling dilutions of the antiserum to be examined in 0.01 ml volume of 0.15M phosphate buffered saline pH 6.4. To each capillary tube 0.01 ml of a 2.5% SRBC suspension coated with antigen is added. The agglutination reaction is observed with a magnifying glass after half an hour incubation. A positive reaction is indicated by the aggregation of SRBC by the antibody to form a rough surfaced column in the capillary tube as compared to a negative reaction which is indicated by a smooth defined column of SRBC.

XV. CELL SUSPENSIONS

Cell suspensions from mouse spleen and thymus were prepared by gently teasing the cells into Hanks'solution. They were then passed through a stainless steel cloth to

remove tissue debris and washed three times with Hanks' solution before use. Bone marrow cells were prepared by flushing the cells out of the thigh bone and subsequent washing. Cells in each preparation were counted in a hemocytometer, and approximately 3 million cells/ml were used in each test.

XVI. REVERSE IMMUNE CYTOADHERENCE (RICA)

1. Single RICA

The technique of RICA has been described in detail previously (Paraskevas et al 1971a). Basically it involves the interaction of a hybrid 5S antibody with the cell surface associated Ig by its anti-Ig site, while the other site reacts with protein coated indicator cells (SRBC, CRBC or Bacteria) to form a rosette. We called this version the single RICA technique. In each test, 0.05 mg hybrid antibody and 0.08 ml of a 2.5% protein coated indicator cells were added to a suspension of mouse lymphocytes (3 x 10^6 cells/ml) and the mixture was incubated at 4° C overnight. The rosette forming cells were counted by introducing a drop of cell suspension into the chamber of a Bellco slide (Bellco Glass Inc., Vineland, N.J.) Each time a total of a 1,000 nucleated cells were counted and the number of rosette forming cells was also recorded. The term rosette forming cells (RFC) by RICA and the Ig-carrying cells are used here interchangeable.

2. Mixed RICA

A modification of RICA was devised in which two hybrid antibodies were used, each with a different specificity for Ig and two corresponding different indicator cells. This is referred to as the mixed RICA. In this study two hybrid antibodies were used with anti-Ig specificities against two major mouse Ig classes. One with an anti-IgG specific site ($a\gamma$) carried an anti-ferritin site (aFe) (hybrid$a\gamma$-aFe), the other with an anti-IgF specific site ($a\phi$) carried an anti-egg albumin site (aEA) (hybrid$a\phi$-aEA).

In addition to SRBC as indicator cells, CRBC and B. cereus were used. For mixed rosette formation, either CRBC or B. cereus was used with SRBC. If a cell carries both IgG and IgF globulins it should bind both hybrid antibodies, resulting on the attachment of both indicator cells around the lymphocytes (mixed rosette). If a cell carries only IgG or only IgF globulin, it should form a single rosette. The mixed RICA was performed in two different ways. In the first, the two hybrid antibodies were added simultaneously to the spleen cell suspension and after incubation, both types of indicator cells each coated with a different protein were added. We called this form of RICA the simultaneous mixed RICA. In the second method, one hybrid was incubated first with spleen cells followed by the proper indicator cells. After 6-8 hours at 4° C the second hybrid and indicator cells were added to the suspension after gentle resuspension of the

tube contents. This time interval was found to be the minimum time required for maximum rosette formation. We called this form of RICA the sequential mixed RICA. In this test, for example, the $\alpha\phi$-αEA hybrid was added first with EA coated SRBC and rosette formation was allowed to take place for 6-8 hours. This hybrid will be able to react without interference by the second hybrid, with all cells carrying IgF globulin. The contents of the tube are re-suspended 6-8 hours later and the $\alpha\gamma$-α Fe hybrid and Fe coated bacterial cells were added. The rosettes were counted after overnight incubation at 4° C and characterized as SRBC or bacterial or mixed rosettes. Since the first hybrid does not react with cells carrying only IgG globulin (γ cells), these cells will be left to react with second hybrid. The second hybrid will also react with variable number of cells carrying both IgG and IgF globulins. The addition of $\alpha\phi$-αEA followed by $\alpha\gamma$-αFe was called sequence 'a' and was used to determine the number of γ cells.

In another aliquot of the same spleen cell suspension, the sequence of the addition of the hybrid was reversed ie: the $\alpha\gamma$-αFe was added first, followed by $\alpha\phi$-αEA. This was sequence 'b' and was used to determine the cells carrying only IgF globulin (ϕ cells).

Finally, the cells carrying both IgG and IgF globulins ($\gamma\phi$ cells) were calculated by subtracting from the number of IgF cells (single RICA with $\alpha\phi$ - α EA hybrid) the

number of ϕ cells as determined by mixed RICA by sequence (b).

A mixed rosette was counted as such when at least a total of 4 cell elements, two from each indicator cells, were attached to the lymphocytes. The vast majority of RFC have usually more than 4 indicator cells. Controls were always set up without hybrid antibody.

XVII. PAPAIN DIGESTION OF RABBIT IMMUNOGLOBULINS

The purified rabbit anti-mouse IgG and anti-mouse IgF antibodies were digested with crystalline mercuripapain (Worthington Biochemical Corp.) as described by Porter (1959). The Ig was digested with papain in an enzyme : substrate ratio of 1:100 at 37 C for 4 hours in 0,1M sodium phosphate buffer pH 7.0 with 0.01M cysteine and 2mM EDTA. The digest was applied on a Sephadex G-100 column. The 3.5S peak obtained was concentrated against 0.001M phosphate buffer pH 7,0 at 4 C. to separate Fab from Fc fragments which crystallize under these circumstances. The Fab fragments were further purified by elution on a column of Carboxymethyl-cellulose (CM) equilibrated with acetate buffer of 0.01M pH 5.5. Most of the Fab fragments was eluted at this molarity. Small amounts of Fab were subsequently eluted with gradient elution. The first Fab peak was used in all the experiments.

XVIII. CYTOTOXICITY TESTS

1. Antisera

i. Anti-θ antiserum: The AKR anti-C3Hθ antiserum was

prepared in AKR mice against C3H thymocytes according to the method of Reif and Allen (1964). C3H thymocytes (10^7) were injected intraperitoneally into AKR mice at weekly intervals for 6 weeks. The serum was collected by heart puncture 10 days after the last injection. Decomplementation of the antiserum at 56° C for 30 minutes was followed by absorption with packed red blood cells from C3H and BALB/c mice (1/20 in volume) at 4° C for 30 minutes. The antiserum was distributed into small aliquots and stored at -26° C.

ii. Anti-mouse Ig antiserum (anti-MIg)

The antiserum was the same as that used for preparation of hybrid antibody αMIg-αFe. This antiserum reacts with IgG and IgF globulins as well as with light chains. It was decomplemented at 56° C for 30 minutes and absorbed with packed BALB/c RBC (1/20 in volume) at 4° C for 30 minutes to remove non-specific cytotoxic activity. The antiserum was stored in small aliquots at -26° C.

2. Complement

Lyophilized guinea pig serum obtained from The Department of Health, Ottawa was used as complement source after reconstitution with distilled water. The reconstituted guinea pig serum was absorbed with BALB/c spleen cells (20×10^6 cells per ml) at 4° C for 30 minutes to remove non-specific cytotoxic substances. It was then distributed into small aliquots and stored at -26° C.

3. Test system

The method used was that of Takahashi et al (1970b). Using Hanks' solution as diluent, tubes were set up containing (i) 0.1 ml of a serial dilution of antiserum. (ii) 0.1 ml of non-diluted guinea pig serum and (iii) 0.2 ml of cells ($2\text{-}3 \times 10^6$ cells). They were incubated at 37 C for 45 minutes. The viable cells were counted in a hemocytometer by adding 0.1 ml of cell suspension to 0.1 ml of freshly prepared 1% dye trypan blue.

Each test included controls in which the cells were incubated with either guinea pig serum or antiserum alone (1/4 dilution).

The anti- antiserum had a cytotoxic titre of 1/256 for C3H and BALB/c thymocytes. It was not cytotoxic to thymocytes when used alone and not cytotoxic even in the presence of complement to bone marrow cells.

The rabbit anti-MIg antiserum was titrated on BALB/c spleen cells which normally contained approximately 30-35% immunoglobulin carrying cells as determined by RICA (Paraskevas et al, 1971a). The antiserum had a slight prozone effect (up to 1/16 dilution) and a cytotoxicity titre of 1/1024. It was not cytotoxic to thymocytes.

The per cent (%) cytotoxicity was calculated as follows:

$$\text{Cytotoxicity} = \left(1 - \frac{\text{Viable cells in antiserum and complement}}{\text{Viable cells in antiserum alone}} \right) \times 100$$

Per cent θ carrying cells and Ig carrying cells in the normal as well as immune mouse spleens were determined by cytotoxicity tests as described above. In each test, 1/4 dilution of the anti-θ serum and 1/20 of the anti-MIg serum were used for 5×10^6 cells in one ml volume.

XIX. IMMUNIZATION OF MICE

Polymerized flagellin (POL) was used as the antigen. Twenty-five micrograms (μg) of antigen mixed in Freund's complete adjuvant (FCA) were injected intraperitoneally (IP) into groups of mice. Another group of mice received the antigen without FCA. At different time intervals after immunization, the spleen cell suspensions were prepared and examined with various techniques.

XX. IMMUNE SERA FROM MICE

Sera were collected from mice by heart puncture at different time intervals after IP injection of 25μg of POL in FCA. These sera samples were named according to the time they were collected after immunization. For example, "6 hour serum" was the serum collected 6 hours after immunization; "24 hour serum" was the serum collected 24 hours after immunization and so on. These serum samples were always collected and used fresh. When cells from spleen, thymus or bone marrow were treated with immune serum, it was always used in a concentration of 20% for 10^7 cells in 1.0 ml volume. Incubation of the mixture was carried out at 37°C for

different time intervals as will be described below.

XXI. IRRADIATION AND RECONSTITUTION OF MICE WITH THYMUS AND BONE MARROW CELLS

BALB/c mice were irradiated at zero hour with 640 rads of total body irradiation administered with an Eldorado A^{60} Cobalt therapy machine (Atomic Energy of Canada Ltd.). Groups of irradiated mice were injected with thymus (7.5×10^7) and bone marrow cells (7×10^7) intravenously (IV) from the tail veins at 48 hours followed by antigenic challenge (POL in FCA) at 72 hours. The cells harvested from the spleen 6 hours after the antigenic stimulation (at 78 hours) were examined by RICA and the total number of cells harvested from each spleen were also recorded. As controls, irradiated animals without any injection, or injected with antigen only (at 72 hours) or with cells only (at 48 hours) were used. In all control animals, spleen cells were examined by RICA at 78 hours and the total number of cells from each spleen was recorded.

XXII. SHORT TERM MOUSE SPLEEN CELL CULTURE FOR RICA TESTS

The technique for mouse spleen cell culture was that of Mangi and Mardiney Jr. (1970). Sterilized procedures were used throughout the whole culturing period.

Mouse spleen cell suspensions were prepared in medium RPMI 1630 containing 15% mule serum (inactivated at 56°C for 30 minutes) and washed in the same medium twice. Per cent viability was determined in the spleen cell suspension

by trypan blue dye exclusion tests. Samples containing over 90% viable cells were used for the experiments. For cultures spleen cells treated with anti-θ serum and complement were always used.

To 5-6x10^6 viable cells, 0.15 ml immune serum was added and made up to a final volume of 1.0 ml, the sample was incubated at 37° C in an incubator supplied with 5% CO_2 and humidity. In each experiment, four such samples were prepared; one was incubated with immune serum for one hour, washed and used for RICA test. The second sample was incubated for 4 hours, washed and examined by RICA test. The third sample was washed at 4 hours and continued culturing for 3 additional hours, (a total of 7 hours). The sample was then washed and used for RICA. The fourth sample was incubated straight for 7 hours, washed and examined by RICA test. In controls, NMS was used in the place of the immune serum and the cells were examined as described above.

XXIII. TREATMENT OF 6 HOUR SERUM

1. Dialysis

Two ml 6 hour serum was dialysed in 1,000 ml Hanks' solution at 4° C overnight. The dialysed sample was collected and passed through millipore filter (0.45 pore size). Normal mouse serum equally treated was used for controls.

2. Precipitation with specific rabbit anti-mouse IgG antibody

The specific anti-mouse IgG antibody was the same as that used for preparation of specific hybrid $a\gamma$-aFe. To

2 ml of 6 hour serum, 0.1 mg of anti-IgG antibody was added. The sample was first incubated at 37° C for half an hour and then at 4° C overnight. The process of precipitation was repeated several times until only a small amount of visible precipitate came down after overnight incubation with the last addition of antibody, thus no excess antigen-antibody complexes were formed or no free antibody was left behind. The sample was then sterilized by passing through a millipore filter.

Any increase in volume due to addition of antibody was corrected to the original volume when it was used.

3. Precipitation and dialysation

One ml 6 hour serum precipitated with rabbit anti-IgG antibody was dialysed at 4° C in Hanks' solution overnight and sterilized with millipore filter.

XXIV. TREATMENT OF 7 DAY SERUM

1. Dialysis

Two ml 7 day serum were dialysed at 4° C in Hanks' solution as described for 6 hour serum. The sample was kept in sterilized condition by passing through the millipore filter.

The variously treated 6 hour and 7 day sera were used in short term culture system with the enriched spleen B cells as described in short term culture section. The sera were added to a final concentration of 15% for 5 x 10^6 cells/ml.

XXV. TREATMENT OF ENRICHED SPLEEN B CELLS WITH CONCANAVALIN A (ConA)

Spleen cells prepared in tissue culture medium RPMI 1630 were treated with anti-θ serum in the presence of complement to eliminate T cells. The sample was then washed and exposed to ConA at a concentration of 100 μg ConA/20x10^6 cells for half an hour at 37° C. The cells were washed free of excess of ConA and used in the culture system.

XXVI. STATISTICAL ANALYSES

All the statistical analyses were performed by a two sample Student t test using an Olivetti programma 101 electronic desk computer.

EXPERIMENTAL RESULTS

SECTION A: THE NUMBER OF B AND T CELLS IN THE NORMAL MOUSE SLEEN

I. Ig CARRYING CELLS IN THE NORMAL MOUSE SPLEEN

1. Single RICA

(i) The total number of Ig carrying cells in the mouse spleen

The number of rosette forming cells (RFC) per 1000 spleen cells using the αMIg-αFe hybrid antibody and Fe coated SRBC has been found to be 338. This is the average number from over 50 determinations in BALB/c mice 8-12 weeks of age. The number of RFC (Ig carrying cells) using the two highly specific hybrid antibodies was also determined using the SRBC as indicator cells. The average number from over 20 experiments is given in Table 2.

It will be noted that there are more cells detected as carrying IgF globulin than IgG globulin. The sum of the cells detected by both hybrid antibodies is much greater than the number found with the hybrid αMIg-αFe. This indicates that some of the cells must carry both immunoglobulins. It is also noted that the number of RFC detected by the αMIg-αFe hybrid is higher than that detected by either of the other two. The difference may be due to cells carrying other Ig or perhaps only L chains. Alternately the H chains of some of the surface associated Ig in some cells may not be detectable.

(ii) Specificity of rosette formation

Fab fragments were prepared from the two highly specific antibodies, anti-mouse IgG and anti-mouse IgF. Spleen cells (10^6/ml) were first treated with 0.7 mg of Fab fragment,

TABLE 2

NUMBER OF ROSETTE FORMING CELLS IN MOUSE SPLEEN.

Hybrid antibody	Rosettes per 1000 spleen cells±S.D.
αMIg-αFe [1]	338±21
$\alpha\gamma$-αFe [1]	190±10
$\alpha\phi$-αEA [2]	308±13
None [3]	6-8

1. Indicator cells = Fe-coated SRBC
2. Indicator cells = EA-coated SRBC
3. The control in each group of experiments was the same SRBC added to spleen cells without addition of hybrid.

and the number of rosettes was determined using the highly specific hybrid antibodies in separate tests. The results are shown in Table 3. Each Fab fragment completely inhibits rosette formation by the hybrid with the same anti-Ig specificity. However, Fab of anti-mouse IgG results in significant blocking of the rosette formation by the $a\Phi$-aEA hybrid antibody. The same holds true for Fab of anti-mouse IgF which reduces the number of rosettes formed by $a\gamma$-aFe. This indicates again that some cells must carry both classes of surface Ig so that Fab fragment of one specificity blocks the rosette formation by the hybrid antibody of the other specificity.

(iii) RICA using various indicator cells

Various indicator cells were used in RICA tests in order to examine whether they could be used interchangeably. Indicator cells were examined with each hybrid alone to assure that they yielded the same number of RFC as the SRBC in the standard technique. Table 4 shows the results. All three indicator cells gave the same number of RFC per 1000 spleen cells with each hybrid.

2. Mixed RICA

(i) Simultaneous Method

Spleen cells were incubated with both specific hybrid antibodies added simultaneously. Following incubation, properly coated SRBC and CRBC were added simultaneously. The same spleen cells were examined by the single RICA

TABLE 3

INHIBITION OF RICA BY Fab FRAGMENTS

Hybrid	Rosettes per 1000 spleen cells treated with[1]		
	Nothing	aγ	aφ
aγ-aFe	179	24	95
aφ-aEA	285	198	12
None	4 - 6	6	5

1. = Fab from rabbit anti-mouse IgG globulin.

= Fab from rabbit anti-mouse IgF globulin.

TABLE 4

ROSETTE FORMATION BY VARIOUS INDICATOR CELLS

Hybrid	Rosettes per 1000 cells					
	SRBC[1]		CRBC[1]		BACT[1]	
	Fe	EA	Fe	EA	Fe	EA
αMIg-αFe	328	-	326	-	343	-
αγ-αFe	197	-	178	-	189	-
αφ-αEA		308		289		314
None	4-6	4-8	5		14-17	15

1. Each type of cell was coated with either ferritin (Fe) or egg albumin (EA).

technique in which only one hybrid and one indicator cell were used. The results are shown in Table 5-A.

In experiment 1, in the single RICA the number of rosettes for each hybrid represents the number of RFC for that hybrid. For the general hybrid αMIg-αFe the rosette count indicates the total number of Ig carrying cells (Ig cells), that for αγ-αFe the total number carrying IgG globulin (IgG cells) and for αφ-αEA the total number carrying IgF globulin (IgF cells). In the mixed RICA the rosettes were classified according to the type of red blood cells surrounding the lymphocyte. Rosettes with SRBC only indicated lymphocytes carrying only IgG globulin (γcells) while lymphocytes carrying only IgF globulin would form rosettes with only CRBC (φ cells). A mixed rosette indicated that the lymphocyte carried both Ig classes. It will be noted that a number of mixed rosettes were formed thus directly demonstrating the presence of both Ig on the surface of the lymphocyte. Such a mixed rosette is shown in Fig. 1. It was found, however, that the total number of RFC with the mixed rosette technique was much below the number obtained with the αMIg-αFe of the single RICA. Furthermore, the total number of lymphocytes carrying IgG globulin as detected by the mixed RICA (Column γcells + mixed) was much smaller than that detected by hybrid αγ-αFe used alone in the single RICA (IgG cells in section of single RICA, Table 5-A). The same was true for lymphocytes carrying IgF globulin (compare

TABLE 5

MIXED REVERSE CYTOADHERENCE - SIMULTANEOUS METHOD

A. SRBC and CRBC

Method	Single RICA[1]			Mixed RICA[2]				
Hybrid	aMIG-aFe	aγ-aFe	aφ-aEA	aγ-aFe + aφ-aEA			Total	
Lymphocytes[3]	Ig cells	IgG cells	IgF cells	γ-cells	Mixed	φ-cells	γcells +mixed	φcells +mixed
Exp.1	315	175	298	30	45	65	75	110
Exp.2	336	171	295	18	84	80	102	164

B. SRBC and B. Cereus[4]

Exp.1	372	168	352	90	70	161	170	231
Exp.2	342	198	331	80	111	159	191	270

1. Properly coated SRBC were used in the single method as indicator cells both in section A and B, according to the specificity of the hybrid antibody. Controls (without hybrid antibody) are similar to those of Table 4.

2. Both indicator cells were added simultaneously. In Exp. 1 Fe-SRBC and EA-CRBC and Exp. 2 EA-SRBC and Fe-CRBC. As controls both indicator cells were mixed with spleen cells but without hybrid and gave a background of 4 - 8 RFC.

3. Ig-cells = cells carrying immunoglobulin
   IgG-cells = cells carrying IgG globulin
   IgF-cells- cells carrying IgF globulin
   γ-cells = cells carrying only IgG globulin
   φ-cells = cells carrying only IgF globulin

4. In both experiments in the mixed method we used EA-SRBC and Fe-Bact. cells. The controls in the mixed RICA gave a background of 10 - 15 RFC, of which most were bacterial rosettes.

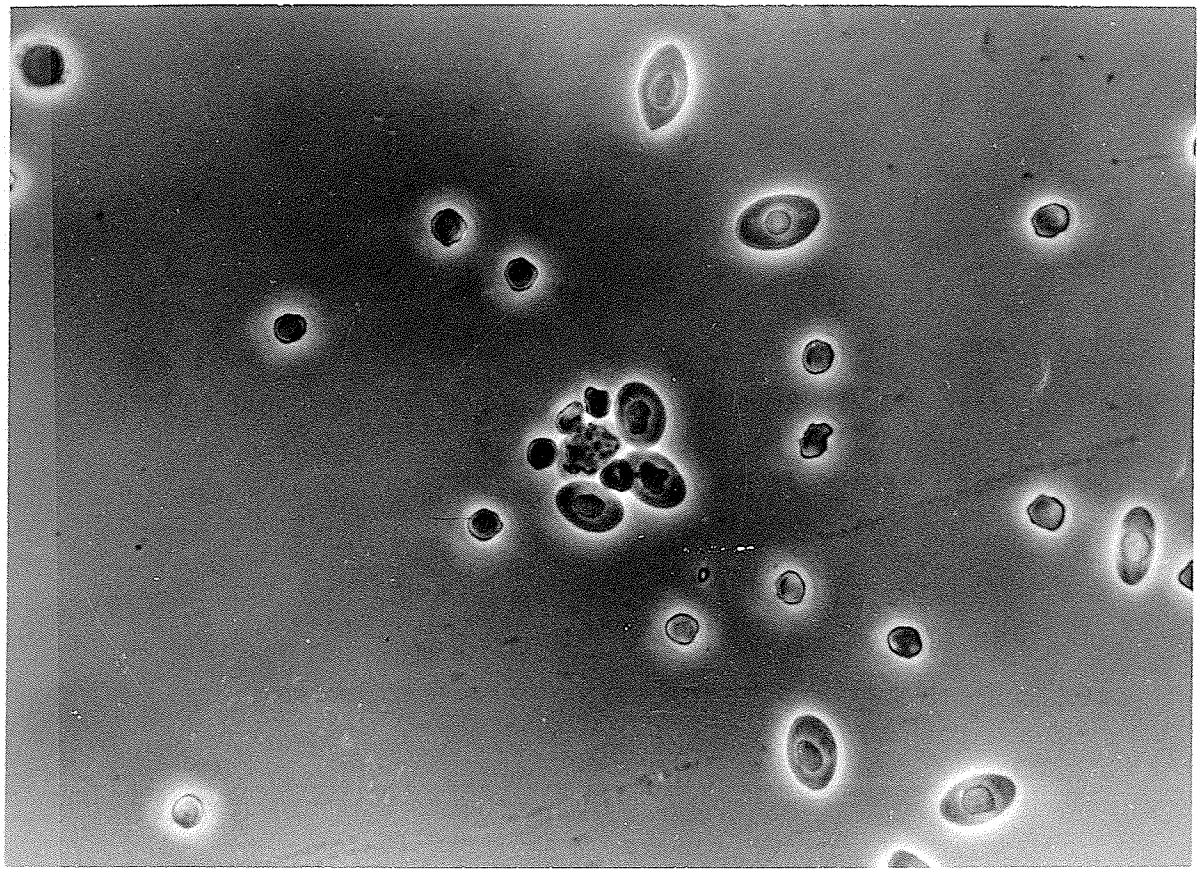


FIGURE 1: Mixed rosettes formed around a mouse spleen lymphocyte by EA coated CRBC and Fe coated SRBC.
Hybrid antibodies = $a\gamma$-aFe and $a\phi$-aEA.

column Φ cells + mixed with that of IgF cells in the section of single RICA, Table 5-A). This discrepancy was also found when we used the same two indicator cells but reversed the protein coat (Exp. 2, Table 5-A). It was thought that the discrepancies were probably an artifact due to the size of the CRBC as related to the surface area of the lymphocyte. Thus one indicator cell could sterically interfere with another and prevent approximation and attachment to the lymphocyte. For these reasons we used a smaller cell and selected the B. cereus. EA coated SRBC and Fe coated B. cereus were used and the results are shown in Table 5-B. Again the rosettes were classified according to the type of indicator cell surrounding the lymphocyte and again it was found that many lymphocytes fixed both indicator cells. Such a mixed rosette is shown in Fig. 2. The total number of all three rosettes formed in the mixed method now closely approximated the number detected by **a**MIg-**a**Fe hybrid.

Although the first number is always somewhat smaller than the second, the difference is small and the sum of the bacterial plus mixed rosettes agrees within the limits of the method with the number detected by **a**γ-**a**Fe hybrid alone. However, the sum of the SRBC plus mixed rosettes is much lower than the number detected by the **a**Φ-**a**EA hybrid in the single technique. This might occur if the majority of the cells detected in the mixed method as carrying only IgG globulin actually carried both immunoglobulins and for

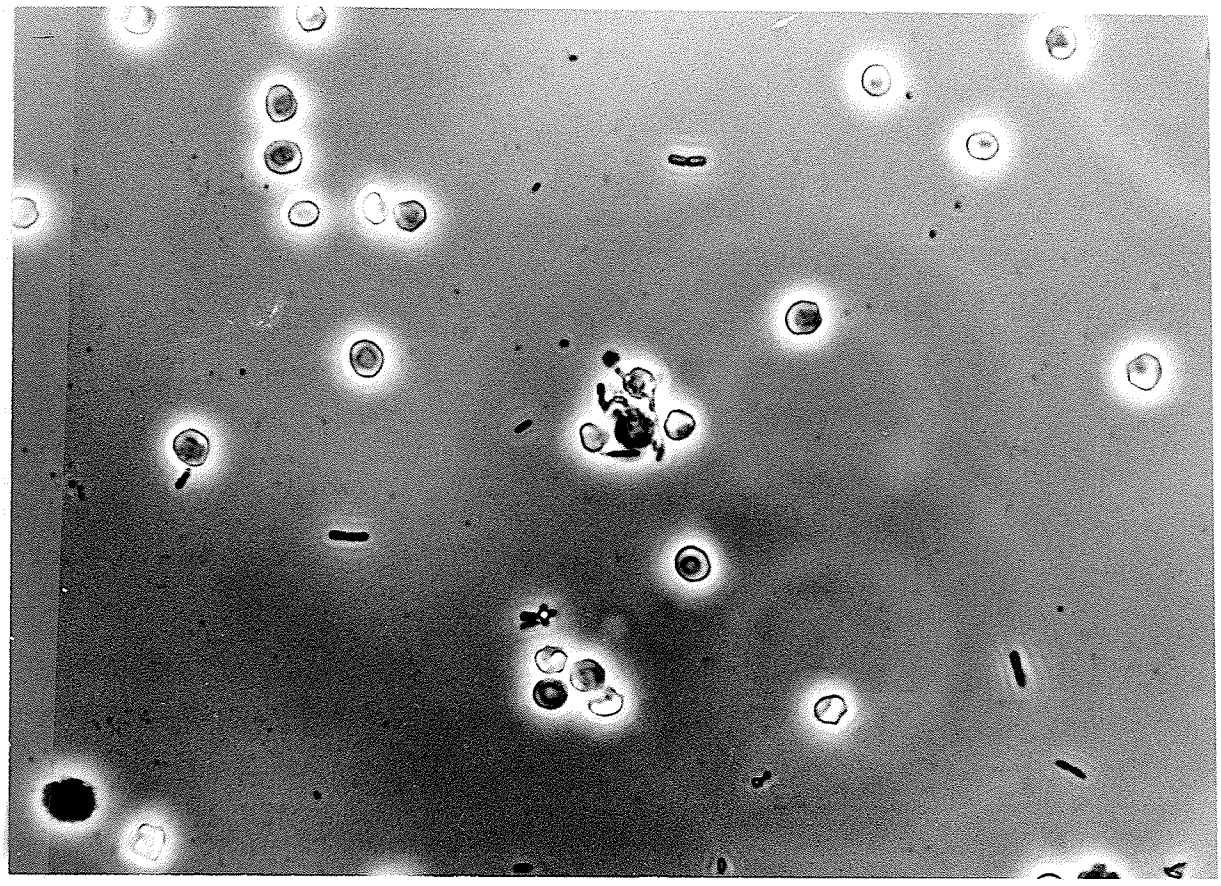


FIGURE 2: Mixed rosette formed around a mouse spleen lymphocyte by Fe coated bacteria and EA coated SRBC.
Hybrid antibodies = $\alpha\gamma$-αFe and $\alpha\phi$-αEA.

unknown reasons the bacterial cells interfered with the attachment of the SRBC. This forced us to further modify the method of mixed RICA to a sequential method.

(ii) Sequential Method

In this modification the spleen cells are first incubated with one specific hybrid antibody followed by the proper indicator cell and rosette formation allowed to take place for 6-8 hours at 4° C. At the end of this period the tube contents were gently resuspended and the second hybrid antibody and its indicator cells added. The rosettes were counted after overnight incubation. If the lymphocyte population consists of cells carrying IgG or IgF or both of them, the addition of the first hybrid $a\phi$-aEA with anti ϕ activity and its indicator cell should not form rosettes with the population bearing only IgG globulin. The subsequent addition of the hybrid with anti-γ activity (eg. $a\gamma$-aFe) and its indicator cell should reveal this second population. If the sequence of the addition of the hybrids and indicator cells is reversed the other population is determined. The exact number of cells carrying both receptors can be determined.

The results of ten experiments are shown in Table 6. In each experiment representing a spleen cell suspension from a different mouse, both single and mixed (sequential) RICA technique were used. In the single method, all three hybrid antibodies were used again separately and each count represents the number of RFC for that hybrid. The notation of the cell types is the same as that used in the simultaneous method in

TABLE 6

MIXED REVERSE CYTOADHERENCE - SEQUENTIAL METHOD

Method	Single RICA			Mixed RICA[1]					
Hybrid[4]	αMIg-αFe	αγ-αFe	αφ-αEA	a[2]	αφ-αEA		αγ-αFe		
				b[3]	αγ-αFe		αφ-αEA		
RF lymphocytes[5]	Ig cells	IgG cells	IgF cells		γ-cells	Mixed	φ-cells	γCells +mixed	φCells +mixed
Exp.1	333	206	321	a	10	96	213		309
				b	69	132	112	201	
2	330	197	307	a	17	137	144		281
				b	69	126	108	195	
3	311	169	297	a	10	96	190		286
				b	94	98	106	192	
4	309	186	297	a	19	135	138		273
				b	109	81	91	190	
5	298	179	278	a	17	111	164		275
				b	132	76	85	208	
6	321	198	306	a	23	133	153		286
				b	144	75	83	219	
7	352	195	287	a	13	107	184		291
				b	144	83	79	227	
8	321	195	306	a	20	116	170		286
				b	108	76	124	184	
9	320	198	294	a	16	123	159		282
				b	90	91	104	181	
10	294	183	281	a	10	108	158		266
				b	78	98	99	176	
Average	319± 15.5	190± 10.5	297± 12.5	a	15	-	-		
				b	±4.4	-	99 ±13.7	197 ±15.7	283 ±11
11	312	192	287	a	15	135	140		275
				b	92	111	101	203	

1. In all experiments of the mixed RICA we used EA coated SRBC and Fe coated bacteria except in exp. 11 where the SRBC were coated with Fe and bacteria with EA.

2. a= the sequence of the addition of the hybrids is αφ-αEA followed by αγ-αFe.

3. b= the sequence is reversed, αγ-αFe followed by αφ-αEA.

4. Controls (one or both indicator cells) mixed with the spleen cell suspension are the same as in Table 4 and 5.

5. The designations Ig cells etc. are the same as in Table 5.

Table 5. In the mixed RICA the two specific hybrids were used in two sequences. In (a) of mixed RICA section of Table 6 the **aΦ-a**EA hybrid was added, followed by the proper indicator system (EA-SRBC). After 6 hours at 4° C the hybrid **aγ-a**Fe was added with the Fe coated bacterial cells. This sequence should determine the lymphocytes carrying only IgG sites as they have been excluded by the hybrid **aΦ-a**EA which reacts with both Φ cells and γΦ cells. This number is shown in Table 6 in column γ cells of sequence (a), in the mixed rosette section. The range of this number is 10 to 23 cells, with an average of 15 cells per 1000 spleen cells. Since in control tests (no hybrid) the background of bacterial rosettes is 12-15, there are an insignificant number of cells carrying only IgG globulin. In order to exclude the possibility that the lack of γ cells may represent an artifact due to the peculiarity of the bacteria we had reversed the indicator systems for the hybrids. In experiment 11 of Table 6, we have used EA bacteria and Fe-SRBC. The results are identical with the other ten experiments.

When the sequence is reversed, the number of lymphocytes carrying only IgF globulin is determined and the average of the ten experiments is 99 lymphocytes per 1000 spleen cells (Table 6, sequence b, Φ cells).

Since there are virtually no cells carrying only IgG globulin, the total number of cells carrying IgG and IgF globulins is represented by the cells detected by **aΦ-a**EA hybrid.

The average of this number is 297 (Table 6, single method). If we subtract the average number of cells carrying only IgF globulin (99 in Table 6) we find that 198 cells carry both IgG and IgF globulins on their surface. This is remarkably similar to the number of cells detected by $a\gamma$-aFe hybrid. (190, Table 6).

From the sequential method the sum of γcells plus mixed cells in sequence (b) should yield the total number of IgG carrying cells. If one compares the numbers in column "γ + mixed" with that of the "IgG cells" by the single method (Table 6) they are seen to be identical within the range of the method. Similarly the sum of "ϕcells" plus "mixed cells" in sequence (a) should give the total number of cells carrying IgF globulin. The numbers in column "ϕ + mixed" and that of IgF cells of the single method section are considered remarkably similar again within the range of the method (Table 6)

The numbers in Table 6 for IgG carrying cells and IgF carrying cells, whether calculated from the single method experiments or by the mixed method are again comparable to the numbers given in Table 2. The total number of all three types of rosettes formed by the mixed method is usually lower than that detected by the general hybrid aMIg-aFe.

3. Cytotoxicity test with rabbit anti-MIg serum

The total number of Ig carrying cells in the mouse spleen detected as rosette forming cells by RICA using

general hybrid (**a**MIg-**a**Fe) was about 330 RFC/1000 spleen cells. The number of Ig carrying cells was determined also by a cytotoxicity test as described in Materials and Methods. The results from ten experiments are shown in Table 7. The average of all the Ig carrying cells in the mouse spleen as detected by cytotoxicity tests was 32% (column 2) which was quite comparable to the level of RFC as detected by RICA (Table 7, column 3)

The total number of Ig carrying cells or B cells in the normal mouse spleen is about 330/1000 spleen cells as detected by both RICA and cytotoxicity tests.

II. THE NUMBER OF θ CARRYING T CELLS IN THE NORMAL MOUSE SPLEEN

1. Cytotoxicity test with anti-θ serum

The θ carrying cells in the mouse spleen can be detected by anti-θ serum cytotoxicity test in the presence of guinea pig complement. The cytotoxicity tests were performed as described in Materials and Methods. Normal mouse spleen cell suspensions were incubated with anti-θ serum and complement for 45 minutes at 37° C. The viable cells in the control (anti-θ serum alone) and the test (anti-θ serum + C) was determined using the trypan blue exclusion method. The results of ten experiments performed on ten separate mice are shown in Table 7 (column 1). The average number of cells killed by anti-θ serum was about 27.3% or 270 per 1000 spleen cells.

TABLE 7

DETERMINATION OF Ig AND CARRYING CELLS IN NORMAL MOUSE SPLEENS.

	CYTOTOXICITY 1. % θ cells	2. % Ig cells	RFC PER 1000 SPLEEN CELLS BY RICA 3. Nontreated cells	4. αθ' Obs[2]	Exp[2]	5. Cells treated with αθ + C' Obs	Exp	6. αMIg' Obs	Exp	7. αMIg + αθ' Obs	Exp
1.	27	33	322	562	592	462	441	36	0	301	270
2.	27	32	308	546	578	472	422	34	0	301	270
3.	23	29	292	525	522	431	380	24	0	265	230
4.	29	35	327	613	617	509	474	34	0	312	290
5.	33	36	318	656	648	509	474	34	0	330	330
6.	26	31	306	537	566	431	413	32	0	331	260
7.	27	33	321	565	591	465	440	42	0	307	270
8.	27	32	318	568	588	468	435	30	0	282	270
9.	27	30	289	524	559	403	395	38	0	314	270
10.	27	29	275	522	545	401	376	26	0	-	270
Ave.	27.3 ±2.3	32.0 ±2.2	308 ±16	561 ±40	580 ±34	455 ±36	423 ±32	34 ±5	0	305 ±20	273 ±24

[1] αθ = anti-θ serum
αθ + C = anti-θ serum + complement
αMIg = rabbit anti-mouse Ig serum
αMIg + αθ = rabbit anti-mouse Ig serum followed by anti-θ serum

[2] Obs = observed value
Exp = expected value. For calculation see text Section a. II 2.

2. The θ carrying cells by RICA following treatment with anti-θ serum

Since the anti-θ antibody is a mouse Ig, it can be detected by the hybrid antibody on the surface of the θ carrying cells. If the θ carrying cells have no Ig on their surface, it would be possible to determine their number after treatment with anti-θ serum using RICA technique.

Normal spleen cells were separated into aliquots of $7x10^6$ cells and were treated as follows:

i. anti-θ serum

ii. anti-θ serum + C

iii. anti-MIg serum

The cells were incubated at 37°C for 45 minutes and the viable cells were counted by trypan blue exclusion tests.

The first and second aliquots were washed three times with cold Hanks' solution and used for RICA. The third sample after washing was further subdivided into two parts. One was used for RICA and the other was incubated with anti-θ serum without complement for 30 minutes at 37°C. It was then washed and used for RICA.

If the θ carrying and Ig carrying cells are populations distinct from each other, the total number of RFC/1000 spleen cells detected after anti-θ treatment alone should equal the sum of θ-carrying and Ig carrying cells as detected by cytotoxicity and RICA respectively. The results of ten experiments in Table 7 (column 4), indicate that the

observed values of RFC/1000 spleen cells after treatment with anti-θ are approximately the same as the expected value. The latter is obtained simply by adding the number of θ carrying cells determined by cytotoxicity to the number of RFC of the same spleen cell suspension as determined by RICA. Based on the same reasoning, the elimination of θ carrying cells from the spleen cell suspension should cause a relative increase of Ig carrying cells. The results of ten experiments are shown in Table 7, (column 5). The expected value in that column was calculated according to the formula:

$$\frac{\text{Number of RFC/1000 spleen cells by RICA}}{\text{1000-number of } \theta \text{ carrying cells by cytotoxicity}} \times 1000 = \text{Expected Value}$$

For example, in experiment 1, Table 7, the number of RFC is equal to 332/1000 spleen cells and the number of θ carrying cells as determined by cytotoxicity is 27% or 270/1000 spleen cells. Therefore, the expected value is:

$$\frac{322}{1000-270} \times 1000 = 441$$

This number is quite comparable to the observed value of 462. The observed values are always slightly higher than the expected ones as calculated above. This may be due to the fact that some θ carrying cells may not be eliminated after treatment with anti-θ serum and complement and thus are detected as RFC.

From these two sets of experiments, it can be

concluded that the θ carrying cells detected by RICA technique after treatment with anti-θ serum are equal to those detected by cytotoxicity tests. Furthermore, the θ carrying and the Ig carrying cells are distinct from each other.

The number of θ carrying cells in the mouse spleen was also determined after blocking the detection of normal rosette forming cells with anti-MIg serum. Such cells were subsequently treated with anti-θ serum without complement. As can be seen in Table 7 (column 6), treatment of spleen cells with anti-MIg serum inhibits rosette formation in the mouse spleen by 85-90% with only 34 RFC per 1000 spleen cells remaining. When these cells were subsequently treated with anti-θ serum the number of Ig carrying cells was found to be 304/1000 spleen cells (column 7). If one subtracts from this value 34 RFC we find that the number of θ carrying cells which were rendered Ig carrying after treatment with anti-θ serum is identical to that obtained by cytotoxicity tests.

The blocking of normal RFC by anti-MIg antisera without any interference in the detection of θ carrying cells also leads to the conclusion that Ig carrying cells are distinct from θ carrying cells. If there is any overlapping of the two populations, the percentage must be very small and beyond the limits of detection by these methods.

SECTION B: CELL SURFACE MARKER CHANGES OF B AND T CELLS AFTER ANTIGENIC STIMULATION.

I. CHANGES OF Ig CARRYING CELLS DURING PRIMARY RESPONSE

1. Changes of spleen cells in mice immunized with POL in FCA

(i) Single RICA

The results are shown in Fig. 3. The hybrid **a**MIg-**a**Fe which gives the total number of Ig carrying lymphocytes (Ig cells) shows that there is a statistically significant ($p<0.001$) increase of RFC at 6 hours, from a normal average of 319 to 371. It indicates the 'addition' to the pool of Ig carrying cells of new cells which were previously not detectable by RICA. The use of the specific hybrid antibodies indicates that this increase at 6 hours is contributed by cells which acquired IgG globulin. The **a**γ-**a**Fe hybrid (IgG cells) shows an increase of RFC to 238 from 190 before immunization ($P<0.001$). On the contrary the number of RFC by **a**ϕ-**a**EA hybrid (IgF cells) show a significant drop of 50 cells ($p<0.001$) which amounts to 16% of all IgF cells present before immunization. Following the initial increase, the Ig carrying cells decrease at 24 hours to 267 RFC which is below the pre-immunization level ($p<0.001$). The IgG cells return to normal levels while the IgF cells show a further drop. During the first two weeks all cell categories show a progressive decrease and all reach their lowest point at 15 days. At this time Ig, IgF and IgG cells decrease by 38%, 43%, and 46% respectively from the pre-immuniation levels. No statistically significant difference in total spleen cell counts are detected at any time during the primary response.

TABLE 16

TOTAL SPLEEN CELL COUNTS DURING PRIMARY IMMUNIZATION WITH POL IN FCA

TIME	CELL COUNT x 10^6 ±S.D.	SIGNIFICANCE *
0 hr.	120±13	
6 hr.	113±9	$p < 0.5$
24 hr.	115±10	$p < 0.7$
3 day	125±14	$p < 0.7$
7 day	140±23	$p < 0.3$
15 day	128±6	$p < 0.5$
24 day	117±11	$p < 0.7$
30 day	115±9	$p < 0.7$
40 day	113±8	$p < 0.5$

* The p value was determined by the Student t-test.

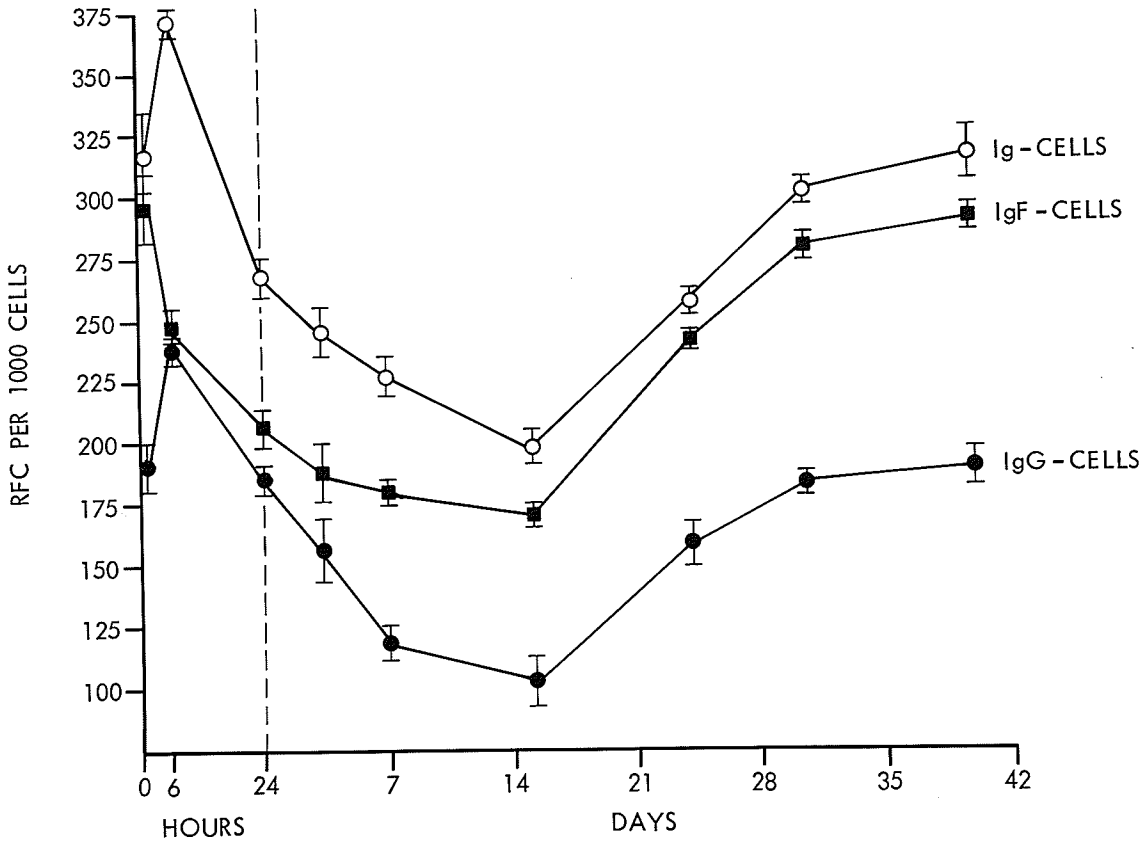


FIGURE 3: Changes of the Ig carrying cells during primary response as detected by single RICA.

Each point represents the average of 4 - 5 animals.

(ii). Sequential mixed RICA

This modification of RICA shows that the Ig carrying cells can be subdivided into cells carrying both IgG and IgF globulin ($\gamma\phi$ cells) and those carrying only IgF globulin (ϕ cells). In normal spleen the cells carrying only IgG globulin (γ cells) are not detectable or present in very small numbers (Section A I.2.ii). The results of the changes of RFC by mixed RICA are shown in Fig. 4. The striking finding is that at 6 hours there is an increase of γ cells, to 85 per 1000 cells ($p<0.001$). The $\gamma\phi$ cells decrease to 139 cells ($p<0.001$) a decrease which is similar to the drop of IgF cells detected by single RICA (Fig. 4) and the ϕ cells show no changes. Thus mixed RICA reveals that among the IgF cells (total number of cells carrying IgF globulin) only the $\gamma\phi$ cells but not the ϕ cells are affected. After this time interval the $\gamma\phi$ cell population shows a progressive and marked decrease with only 90 cells detectable at 15 days (a decrease of 55% from the pre-immunization level). The drop of $\gamma\phi$ cells by 55% is greater than the drop of IgF cells (by 43%) in the single RICA the difference being due to the ϕ cells which are not substantially changed. Thus the sum of the decrease of $\gamma\phi$ cells and ϕ cells (total 123 cells) is identical to the decrease of IgF cells (124 cells) at 15 days. No significant changes of the ϕ cells occur during this period while the γ cells return slowly towards normal levels. These results clearly demonstrate that the changes of the $\gamma\phi$ cells can account for the changes detected

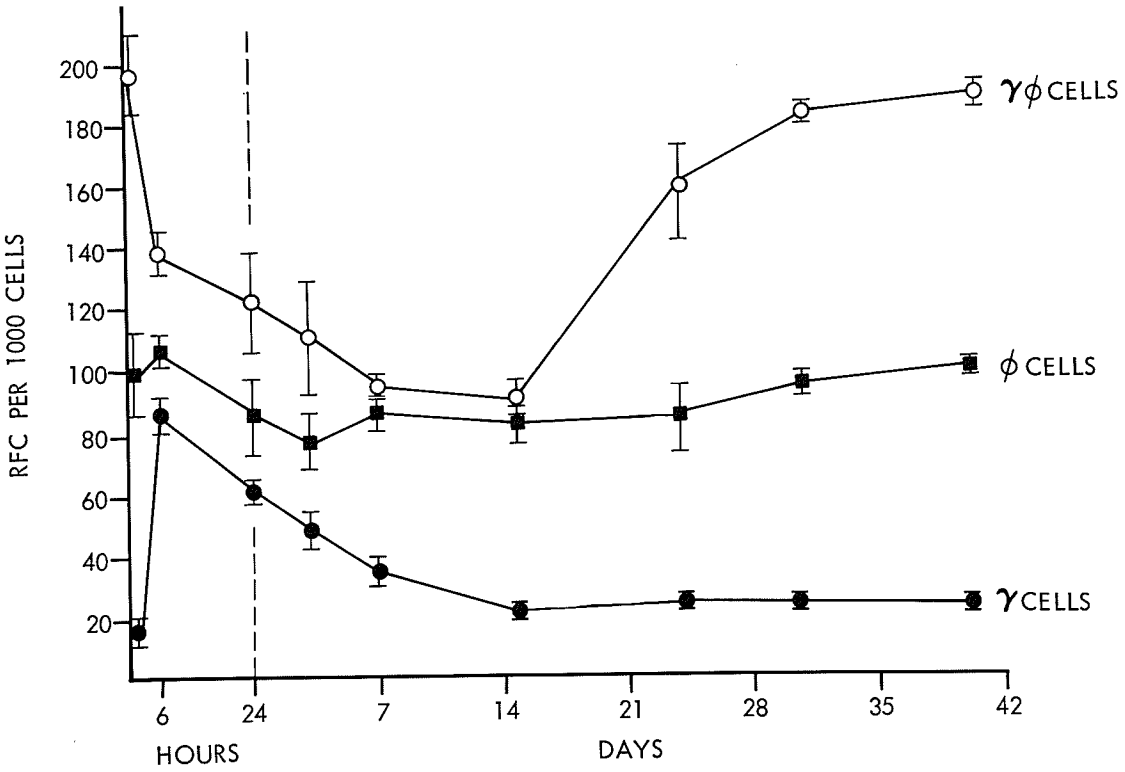


FIGURE 4 : Changes of Ig carrying cells during primary response as detected by mixed RICA.
Each point represents 4 - 5 animals.

by single RICA of the Ig carrying cells. The $\gamma\phi$ cells return to normal levels by the end of the sixth week. In conclusion the results from single and mixed RICA indicate that two large scale events take place in the spleen during primary immunization. First, 6 hours after stimulation, a lymphocyte which does not carry surface Ig (or carries it below levels of detection by RICA) acquires an IgG globulin resulting in a net increase of total Ig carrying lymphocytes. Second, on cells which carry surface Ig before immunization (B cells) such globulin becomes undetectable as early as 6 hours after stimulation. The number of B cells progressively diminishes in the first two weeks and the decrease involves more than 50% of these cells. From the various categories of cells which are detected by mixed RICA before immunization only the one previously defined as pluripotential is affected.

(iii) Cytotoxicity test with anti-MIg serum

The B cell changes induced in mouse spleen by antigen were followed by cytotoxicity tests with a rabbit anti-MIg antiserum. Spleen cell suspensions from immunized mice were prepared at different time intervals after immunization and were treated with anti-MIg serum in the presence of complement. The number of Ig carrying cells determined by cytotoxicity is the same as that detected by RICA using the hybrid antibody αMIg-αFe (Fig. 5). Six hours after antigenic stimulation, the number of Ig carrying cells detected by cytotoxicity is higher than the normal level by 8%, and these cells decreased at 24 hours below the normal level reaching the lowest point by 15 days.

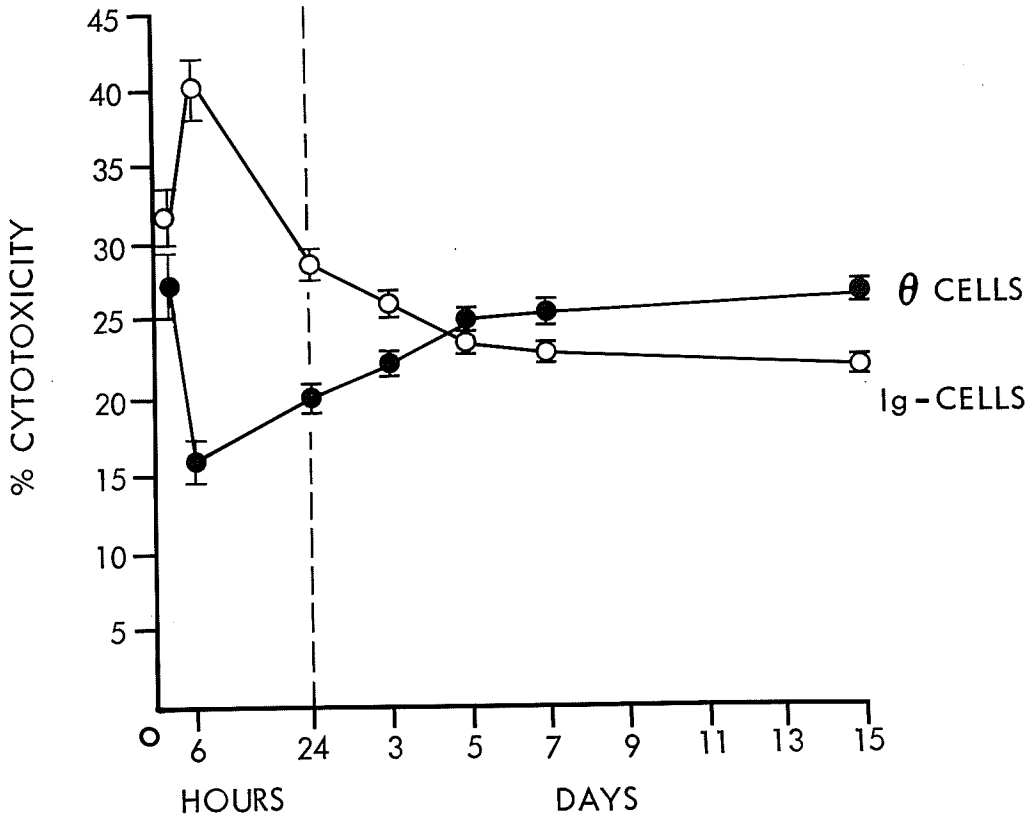


FIGURE 5: Changes of the θ carrying and Ig carrying cells during primary response.

2. Changes of Ig carrying cells in the spleen of mice immunized with POL without FCA

The results are shown in Table 8 and represent the average from duplicate experiments. POL without FCA induces the same type of changes of Ig carrying cells in the spleen as described above.

II. CHANGES OF θ CARRYING CELLS DURING PRIMARY RESPONSE

1. Cytotoxicity test with anti-θ serum

The number of θ carrying cells in the immunized mouse spleen was determined by cytotoxicity tests with anti-θ serum in the presence of complement. The average pre-immunization level of θ carrying T cells by this method is 27.3% (Table 7). Six hours after antigenic stimulation, there is a decrease of the number of T cells by 10% (Fig. 5). The number of T cells slowly returns to normal by the end of the fifth day. It is clear from Fig. 5 that at 6 hours the T and B cell changes are inversely related and this evidence may suggest that the T cells which become undetectable at 6 hours make up for the increase of Ig carrying cells.

2. The θ carrying cells by RICA after treatment with anti-θ serum during primary response

Spleen cell suspensions from mice were prepared at various time intervals after antigenic stimulation and used for RICA as follows:

i. non-treated cells

ii. after treatment with

a. anti-θ serum

TABLE 8

SEQUENTIAL MIXED RICA WITH SPLEEN CELLS FOLLOWING ANTIGENIC STIMULATION WITH POL

	Single RICA			Mixed RICA		
Time	a MIg-aFe Ig cells	aγ-aFe IgG cells	aφ-aEA IgF cells	γ cells	φ cells	γφ cells
6 hr.	373	229	248	80	101	147
24 hrs.	271	190	236	55	85	151
7 days	254	148	227	33	87	140
15 days	241	139	226	24	94	132
24 days	278	166	268	20	94	174
30 days	308	180	282	20	99	183
40 days	326	186	288	22	108	180
0 hr.	319	190	297	15	99	199

The numbers represent averages from 2 experiments

In mixed RICA the two hybrid sequences were used as described in Section A.I.2.ii.

The γ cells were determined by sequence (a) and the φ cells by sequence (b).

The γφ cells represent the difference of IgF cells - φ cells.

b. anti-MIg serum

c. anti-MIg followed by anti-θ serum.

The results are shown in Table 9. Column 1 shows the number of Ig carrying cells during the first two weeks of primary response. These results are similar to those reported in Section B, I.1. After treatment with anti-θ serum (without C) RICA detects the sum of B and T cells for reasons given above (Section A, II.2).

The number of T cells is determined when one subtracts the number of RFC of untreated spleen cells (column 1) from that of the same cells treated with anti-θ serum (column 2). By this approach the number of T cells given in column 3 is found to be quite comparable with that determined by cytotoxicity.

Finally, since B and T cells are not overlapping cell populations (Section A. II.2), we have attempted to determine the number of T cells available after blocking the rosette formation of B cells by the rabbit anti-MIg serum. After such blocking the number of RFC decreased to a base line level of about 30 RFC per 1000 spleen cells (column 4). After treatment of such cells with anti-θ serum, the number of T cells could be determined by RICA and it is shown in column 5. The number of T cells is again given (column 6) if one subtracts the number of RFC of anti-MIg treated spleen cells (column 4) from that of the same cells treated sequentially with anti-MIg and anti-θ sera (column 5). Both of these approaches of determining T cells indicate that the

TABLE 9

DETERMINATION OF θ CARRYING CELLS BY RICA ON ANTI-θ SERUM TREATED SPLEEN CELLS DURING PRIMARY RESPONSE

	RFC per 1000 Spleen Cells					
	1	2	3	4	5	6
Time	Cells Non-treated	Cells treated with αθ	θ carrying cells	αMIg	αMIg +αθ	θ carrying cells
0	308± 16	562± 40	254	34± 5	303± 20	269
6 hrs.	380± 10	539± 12	159	34± 2	212± 9	178
24 hrs.	270± 9.2	465± 2.5	195	32± 3	236± 66	204
3 days	248±9	462± 15	214	33±2	244±4	211
5 days	232±2.5	456±14	224	35±1	269±2.5	234
7 days	222±3	464±9	242	37±1	272±10	235
15 days	204±2	458±12	254	36±2	291±5	255

The abbreviations in this Table are the same as in Table 7.

The number of RFC represents the average of 3 - 5 experiments ± S.D.

For calculations of θ carrying cells, see text Section B.II.2

number of T cells decreases 6 hours after immunization and slowly returns to normal level by the end of the first week.

Thus the changes of θ carrying cells during primary response as determined by RICA after rendering them Ig carrying with anti-θ serum treatment are quite similar to those obtained by cytotoxicity test as shown in Fig. 5.

SECTION C: UPTAKE OF CYTOPHILIC Ig BY T CELLS

I. THE EFFECT IN VITRO OF '6 HOUR SERUM' ON CELLS FROM VARIOUS ORGANS

1. Spleen cells

An increase of 70-80 rosettes per 1000 spleen cells comparable to that observed in vivo 6 hours after antigenic stimulation, can be reproduced in vitro by exposing normal spleen cells to 6 hour serum (Table 10 and Table 11, line 2.). Three hybrid antibodies were used in the single RICA tests (Table 10). The results were comparable to those obtained in the immunized 6 hour mouse spleens. Thus a non-RFC present in normal spleen takes up a cytophilic Ig and is transformed to RFC. NMS and NMS plus complement have no effect (Table 11, line 3 and 4). Serum collected 6 hours after injection of FCA alone was also inactive (Orr and Paraskevas, 1973). Anti-θ serum in the presence of guinea pig complement eliminates 25-27% of spleen cells (T cells). Normal spleen cells treated with anti-θ serum and complement and then examined by RICA showed a relative increase of RFC because of the elimination of the non-rosette forming T cells. When the observed value is corrected by the anti- cytotoxicity factor, the corrected value is still higher by 25-30 cells from the expected value (line 5, Table 11) probably because some T cells are not eliminated by the antiserum and since they now carry the anti-θ antibody (mouse Ig) they are detected as RFC. When normal spleen cells were first treated with anti-θ serum and then exposed to 6 hour serum, no increase of RFC was observed (line 6, Table 11). Thus the elimination of the

TABLE 10

EFFECT OF 6 HOUR SERUM ON NORMAL SPLEEN CELLS

Treatment	RFC/1000 Spleen Cells		
	Ig Cells	IgG Cells	IgF Cells
Normal	319±15.5 (10)	190±10.5 (10)	297±12.5 (10)
6 hour serum	371±6 (4)	247±5 (4)	239±2 (4)

The values represent the average ± S.D from the number of experiments given in parentheses.

TABLE 11

THE EFFECT OF '6 HOUR SERUM' ON SPLEEN CELLS TREATED IN VARIOUS WAYS.

Treatment	Exp.1	Exp.2	Exp.3	Exp.4	Exp.5
1. None	314	319	275	328	268
2. '6 hour serum'	381	387	347	404	341
3. NMS [a]	308	318	282		
4. NMS + C	312	321			
5. $\alpha\theta$ + C [b]	444(328) [c]	460(331)	409(298)	497(362)	398(298)
6. ($\alpha\theta$ + C) + '6 hour serum'	441(326)	459(330)	412(300)	502(366)	403(302)
7. ($\alpha\theta$ + C) + NMS	447	458			
8. ($\alpha\theta$ + C) + (NMS + C)	451	459			
9. αMIg [b]	34	36	29	37	27
10. αMIg + '6 hour serum'	107	112	93	115	95
11. αMIg + NMS	37	38	33		
12. αMIg + ($\alpha\theta$ + C)	64(47)	76(54)	74(54)	86(62)	
13. αMIg + ($\alpha\theta$ + C) + '6 hour serum'	67(49)	83(59)	78(56)	97(70)	
Cytotoxicity by anti-θ serum (%)	26	28	27	27	25

[a] NMS = Normal mouse serum

[b] $\alpha\theta$ + C and αMIg, same abbreviations as in Table 7.

[c] The number in parentheses represents the rosette counts corrected for the elimination of T-cells. The factor used is based on the % cytotoxicity by anti-θ serum for each experiment.

T cell population had eliminated the cell upon which the 6 hour serum is acting to transform it to a RFC. Exposure of the anti-θ treated spleen cells to NMS and NMS plus complement had no effect (lines 7,8).

Treatment of spleen cells with rabbit antimouse immunoglobulin almost completely inhibited rosette formation (line 9, Table 11). Such cells exposed to 6 hour serum showed an increase of 64 to 78 RFC comparable to untreated cells (line 10, Table 11). Again NMS has no effect on spleen cells treated by anti-immunoglobulin. When the anti-immunoglobulin treated cells are subsequently treated with anti-θ serum and then exposed to the 6 hour serum the increase was abolished (line 13). As above when the observed values in lines 12 and 13 are corrected by the cytotoxicity factor the corrected value is higher by 25 cells as compared to the counts in line 9, most likely for the same reasons discussed above.

2. Thymus cells

The results are shown in Table 12. RICA detects no Ig on mouse thymocytes as we have reported previously (Paraskevas et al, 1971a). When thymocytes are first exposed to 6 hour serum we are able to detect 50 RFC per 1000 cells. Normal mouse serum has no effect on thymocytes when examined by RICA. Thus approximately 5% of the mouse thymocytes can take up an Ig component from 6 hour serum and be transformed to a RFC.

TABLE 12

THE EFFECT OF '6 HOUR SERUM' ON THYMUS AND BONE MARROW CELLS

Cells	Exp. 1	Exp. 2	Exp. 3	Average
Thymus	6	8	4	6 ± 1
Thymus + NMS	4	6	6	$5.3 \pm .9$
Thymus + 6 hour serum	49	52	50	50.3 ± 1.2
BM	98	138	113	116 ± 16
BM + NMS	99	136	118	117 ± 15
BM + 6 hour serum	111	133	111	118 ± 10

Abbreviations used: NMS = normal mouse serum

BM = bone marrow

3. Bone marrow cells

Bone marrow cells show no increase of RFC after exposure to the 6 hour serum (Table 12). Thus the B cell makes no contribution to the increase of RFC observed at 6 hours after antigenic stimulation. Controls with normal mouse serum show no changes in RFC.

II. RICA WITH SPLEEN CELLS FROM IRRADIATED AND IRRADIATED - RECONSTITUTED MICE

The total spleen cell count and the number of RFC in mice given sublethal dose of irradiation is shown in Table 13. When the mice are injected with antigen in FCA no change is observed in the total cell count and RFC count 6 hours later, (lines 1, 2).

Irradiated mice received thymocytes (7.5×10^7) intravenously (IV) and approximately 10% of the injected thymocytes homed to the spleen producing a doubling of total cell count. Since they are non-RFC, they resulted in a relative decrease of RFC counts to almost half of the value observed in the control animals (line 3). When the mice are injected IP with antigen in FCA and spleen cells were examined 6 hours later the RFC are increased by 43 rosettes (line 4). This increase is comparable to that of thymus cells treated in vitro with 6 hour serum. Thus the thymus cell which homed to the spleen contributed to the 6 hour increase of RFC.

Bone marrow cells (7×10^7) were given IV to irradiated mice as outlined in the methods. The bone marrow

TABLE 13

RICA WITH SPLEEN CELLS FROM IRRADIATED RECONSTITUTED MICE CHALLENGED WITH ANTIGEN

Mice injected with[a]	RFC/1000 Spleen cells					Total spleen cell counts x 10^6				
	1	2	3	4	Av.	1	2	3	4	Av.
Nothing	32	54	39	41	41.5±7.9	4.4	8.1	6.6	5.3	6.1±1.3
Ag[b]	58	40	42	35	43.7±8.6	4.5	3.2	6.4	7.9	5.5±1.7
Thymus cells	17	18	26	-	20.3±4	12.0	14.0	10.0	-	12±1.6
Thymus cells+Ag	67	65	60	63	63.7±2	11.2	13.6	8.3	7.9	10.2±2.3
BM cells	102	111	84	-	99±11	8.4	9.6	9.6	-	9.2±0.5
BM cells + AG	106	97	102	-	101±3	9.2	8.4	7.5	-	8.4±0.69

a = All mice were given 640 rads. See Materials and Methods for details.

b = Ag = POL + FCA

lymphocytes which homed to the spleen almost doubled the total cell count and at the same time more than doubled the RFC count (line 5). Antigen in FCA was injected intraperitoneally (IP), and spleen cells were examined 6 hours after antigenic stimulation by RICA. No increase of RFC is detected (line 6).

SECTION D: LOSS OF SURFACE Ig FROM B CELLS

I. THE EFFECT OF MOUSE IMMUNE SERA ON Ig CARRYING CELLS IN VITRO

The results are shown in Table 14. When cells were treated for 45 minutes with 6 hour serum two different changes were detected. The αMIg-αFe hybrid antibody detected an overall increase of the Ig carrying cells from a base line of 304 RFC per 1000 cells to 387. Such an increase has been detected in vivo (Section B) and was conclusively shown to be due to the uptake of a cytophilic IgG (probably a complex of a Ig with antigen) by T cells (Section C). At the same time cells carrying IgF (IgF cells) show a decrease which amounts to 15% of IgF cells present in the normal mouse spleen. The mechanism of reduction of the IgF cells which has also been detected in vivo (Section B) is not apparent at present. Since the serum contains a cytophilic IgG globulin, which may represent an Ig-antigen complex (Orr and Paraskevas, 1973), it is possible that such an Ig taken up by the IgF cells may sterically hinder the detection of surface of IgF by the $\alpha\phi$-αEA hybrid antibody. Experiments designed to elucidate the mechanisms of the action of 6 hour serum are presented below. In contrast to the 6 hour serum all the other immune sera induced only a decrease of the number of RFC shown by both hybrid antibodies. These in vitro induced changes are comparable again to those observed in the spleen cells in vivo at the same time intervals (Section B) with the exception of the 15 day serum. The most marked decrease of Ig cells

TABLE 14

TREATMENT OF NORMAL SPLEEN CELLS WITH IMMUNE SERA

Treatment	RFC/1000 Spleen Cells	
	Ig Cells	IgF Cells
None	304 ± 5	287 ± 4
6 hour serum	387 ± 1	238 ± 1
24 hour serum	266 ± 2	226 ± 5
7 day serum	219 ± 8	208 ± 5
15 day serum	243 ± 3	232 ± 4

Each number represents the average of 3 - 5 experiments $\pm$S.D.

(38% of the preimmunization level) in vivo during primary response was detected 15 days after antigenic stimulation.

Since no changes in the viability of the cells were detected after the incubation with the immune sera, the decrease of the Ig carrying cells is considered to be due to loss of Ig from the cell surface. One should note that the cytophilic Ig of the 6 hour serum, which induces the increase of Ig cells is not detectable in the other immune sera. This finding agrees with the lack of the detection of the Ig-antigen complexes 24 hours after immunization, although such a complex is present at 6 hours (Orr and Paraskevas, 1973).

These results reported here emphasize the fact that loss of surface Ig induced by soluble factors, may be an important mechanism which operates in vivo and gives rise to marked decreases of Ig carrying cells detected during the first two weeks.

II. SHORT TERM CULTURES WITH ANTI-θ SERUM TREATED SPLEEN CELLS

1. Six hour serum

i. Culture at 37° C

In order to investigate the effect of 6 hour serum on cells carrying detectable surface Ig, we have used anti-θ serum treatment to eliminate the θ carrying cells. Such a treatment, it was shown previously, abolished the increase of Ig carrying cells above the preimmunization level (Section C).

Anti-θ treated spleen cells give a proportionally higher number of Ig carrying cells/1000 cells due to the elimination of 27% of T cells which do not carry surface Ig as detected by RICA. Such cells were cultured under sterile conditions as described in the Materials and Methods for 7 hours in the presence of 6 hour serum or NMS.

The cells were examined at 1, 4 and 7 hours for viability and by RICA. The number of viable cells decreased at the end of the culture period by 10 - 15% (Table 15) while no appreciable loss was detected earlier. No difference in the viability were detected between cells cultured in NMS and those cultured in immune sera. The cells were examined by RICA using two hybrid antibodies. The results are shown in Fig. 6. After one hour of culture the number of Ig cells (αMIg-αFe) did not change as compared with the control. However, the number of IgF cells decreased by 80 RFC/1000 cells. At 4 hours, both hybrid antibodies detect a comparable decrease below the control value, which is about the same after 7 hours of culture. The decrease is statistically significant ($p<0.001$), and since there is no significant loss of viable cells the decrease may indicate loss of surface Ig.

To support this interpretation we have washed one of the culture tubes at 4 hours and incubated it for 3 additional hours in fresh medium without 6 hour serum. As shown in Fig. 6 (interrupted lines) the RFC count returned

TABLE 15

VIABLE CELL COUNTS AFTER CULTURE OF SPLEEN CELLS WITH VARIOUS IMMUNE SERA.

Time of culture		-Immune sera- 6 hour	24 hour	7 day	15 day
0		5.75×10^6	5.5×10^6	5.5×10^6	5.75×10^6
1 hour	NMS	5.15×10^6	5.0×10^6	5.36×10^6	5.75×10^6
	Serum	5.5×10^6	5.0×10^6	5.43×10^6	5.95×10^6
4 hour	NMS	5.05×10^6	4.7×10^6	5.1×10^6	5.75×10^6
	Serum	5.35×10^6	4.8×10^6	5.06×10^6	5.15×10^6
7 hour	NMS	4.6×10^6	4.05×10^6	4.96×10^6	5.25×10^6
	Serum	4.15×10^6	4.2×10^6	4.93×10^6	5.35×10^6
7 hour	Washed *	4.45×10^6	4.05×10^6	4.6×10^6	4.85×10^6

* This culture was washed after 4 hours of incubation with immune serum and then it was resuspended in one ml of fresh medium and cultured for another 3 hours.

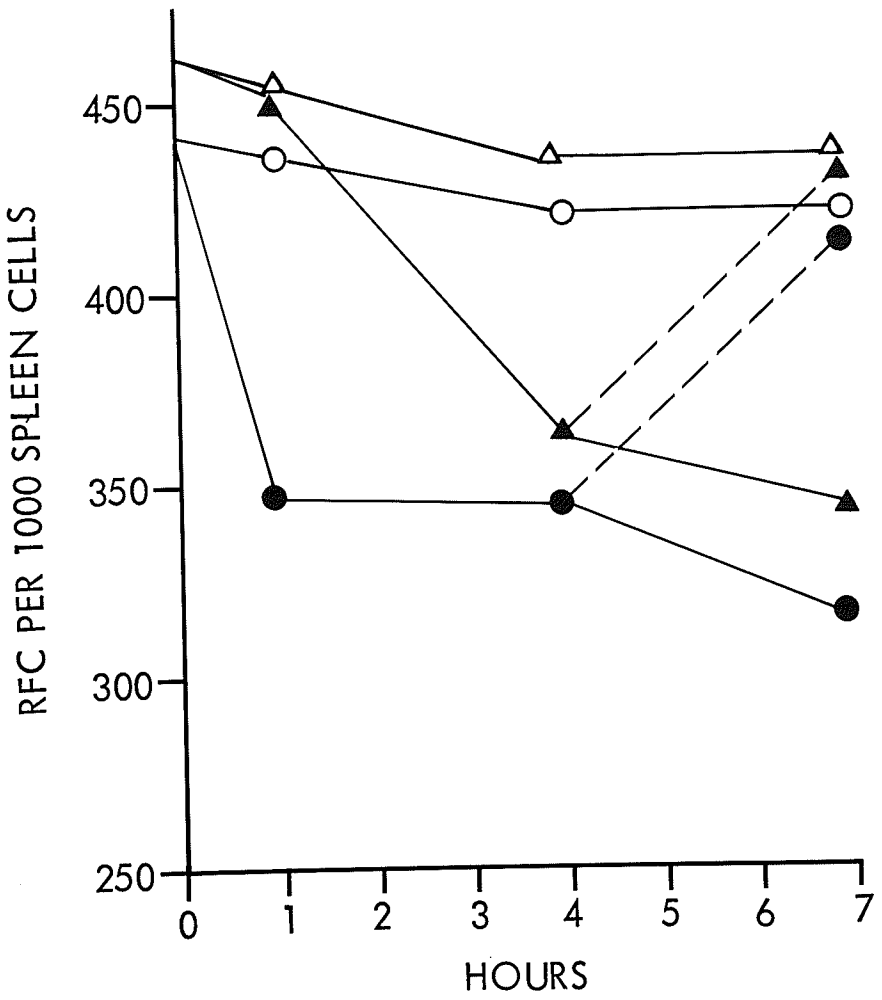


FIGURE 6: Short term culture of Ig carrying cells with 6 hour serum at 37°C.

to normal with both hybrid antibodies. It is likely that during this interval surface Ig was regenerated since studies have shown that its half life is 45 minutes (Lerner et al, 1972).

In order to understand further the phenomena observed, the following experiments were performed.

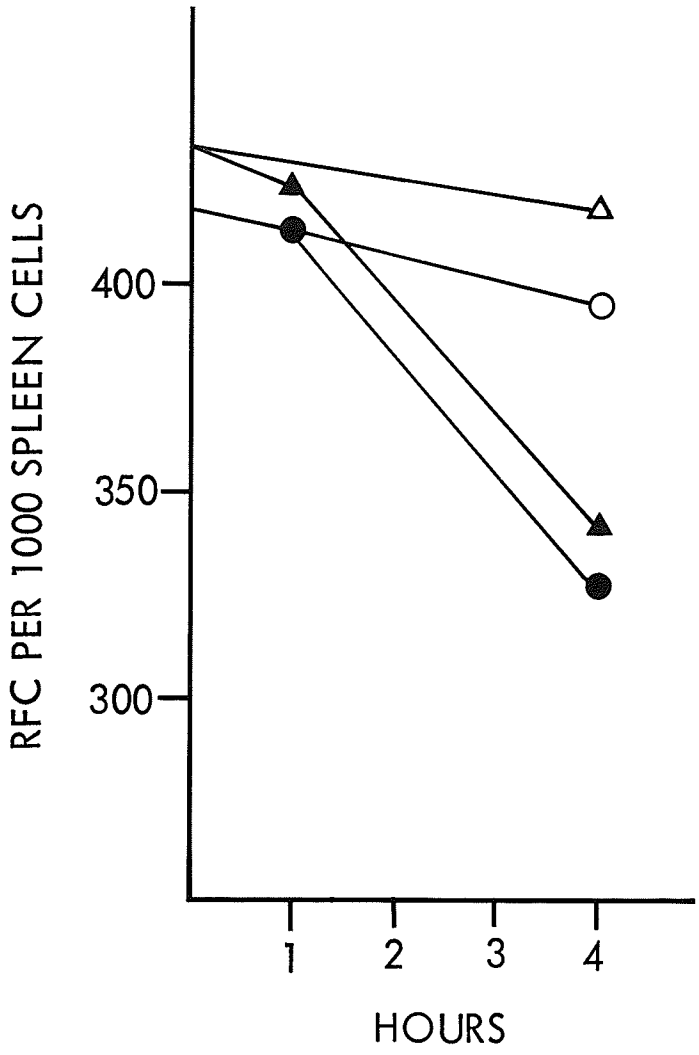
ii. Precipitation of IgG from 6 hour serum

As we have indicated earlier, previous studies have shown that the cytophilic Ig in the 6 hour serum belongs to the IgG class (Section B). A highly purified and specific rabbit anti-mouse IgG antibody was used to precipitate the IgG globulin from 6 hour serum as indicated in the Materials and Methods.

Anti-θ serum treated spleen cells were cultured as above in the presence of the serum from which IgG was precipitated. No decrease in the IgF cells was detected at 1 hour (Fig. 7). However, at 4 hours, the number of RFC by both hybrid antibodies was decreased to the same extent as with the non-precipitated serum. These results indicate first, that the decrease of IgF cells at 1 hour is due to the uptake by Ig carrying cells of a cytophilic IgG and second, that the loss of surface Ig at 4 hours is not due to the uptake of this globulin but to a different factor.

iii. Dialysis of 6 hour serum

Anti-θ serum treated spleen cells were cultured



	Ig CELLS	IgF CELLS
CONTROL NMS	△	○
IMMUNE SERUM	▲	●

FIGURE 7: Short term culture of Ig carrying cells with specific anti-mouse IgG precipitated 6 hour serum at 37° C. NMS was equally treated.

in the presence of 6 hour serum which was dialysed overnight. Similar cultures were done in the presence of dialysed NMS. The results are shown in Fig. 8.

A decrease of IgF cells was detected at 1 hour and remained the same at 4 hours. However, no decrease of the Ig cells was present at 4 hours. This finding indicates that dialysis removed from the serum a factor which caused the mobilization of surface Ig between 1 to 4 hours of culture. Furthermore, the cytophilic IgG which was still taken up from the dialysed 6 hour serum remained apparently on the cells during the entire 4 hour culture period and this globulin is not the cause of the loss of surface Ig.

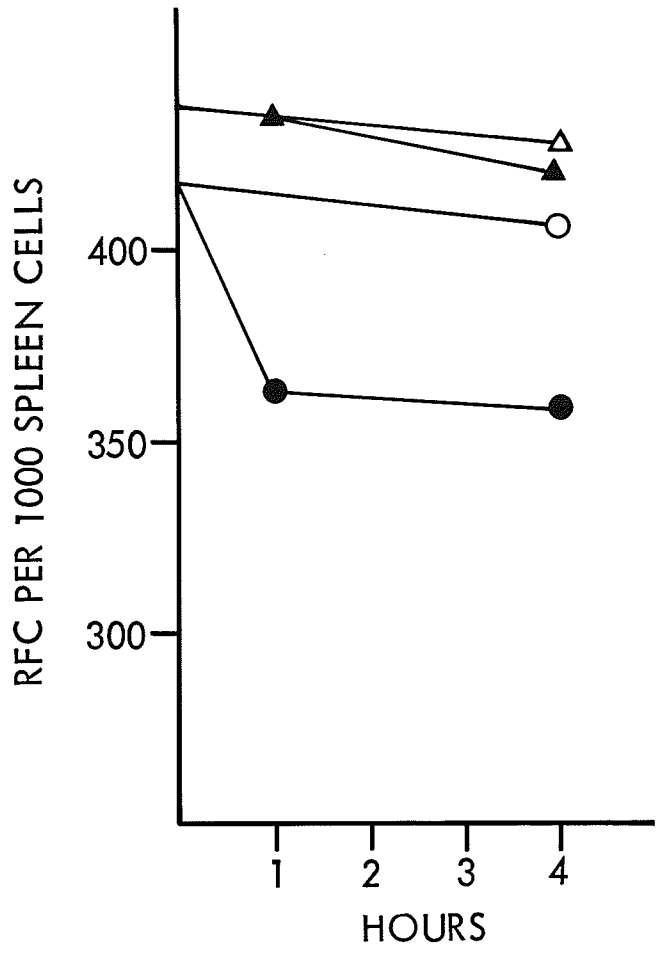
iv. Anti-IgG precipitated 6 hour serum followed by dialysis

Six hour serum was precipitated with rabbit anti-mouse IgG antibody and then dialysed as described above. When this serum was used in cultures, no decrease of IgF cells was observed at 1 or 4 hours. Such treatment resulted in a 6 hour serum which was in culture identical to NMS (Fig.9).

v. Culture at 4° C

Mobilization of surface Ig by heterologous anti-Ig has been shown to depend on pinocytosis and it could be prevented by inhibitors of pinocytosis or low temperatures (Taylor et al, 1971; Yahara and Edelman, 1972; Loor et al, 1972).

Spleen cells were cultured for 7 hours at 4° C in the presence of 6 hour serum. As shown in Fig. 10, the



	Ig CELLS	IgF CELLS
CONTROL NMS	△	○
IMMUNE SERUM	▲	●

FIGURE 8: Short term culture of Ig carrying cells with dialysed 6 hour serum at 37°C. NMS was equally treated.

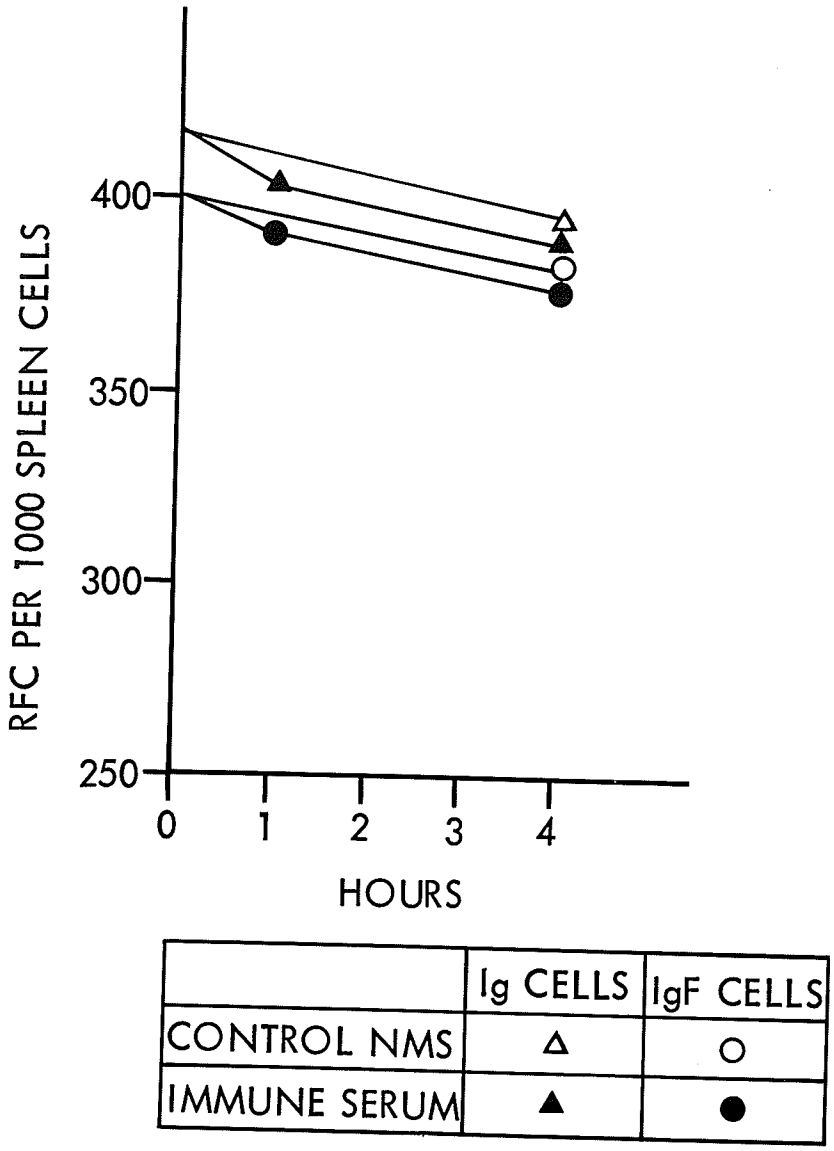


FIGURE 9: Short term culture of Ig carrying cells with anti-mouse IgG precipitated and then dialysed 6 hour serum at 37°C. NMS was equally treated.

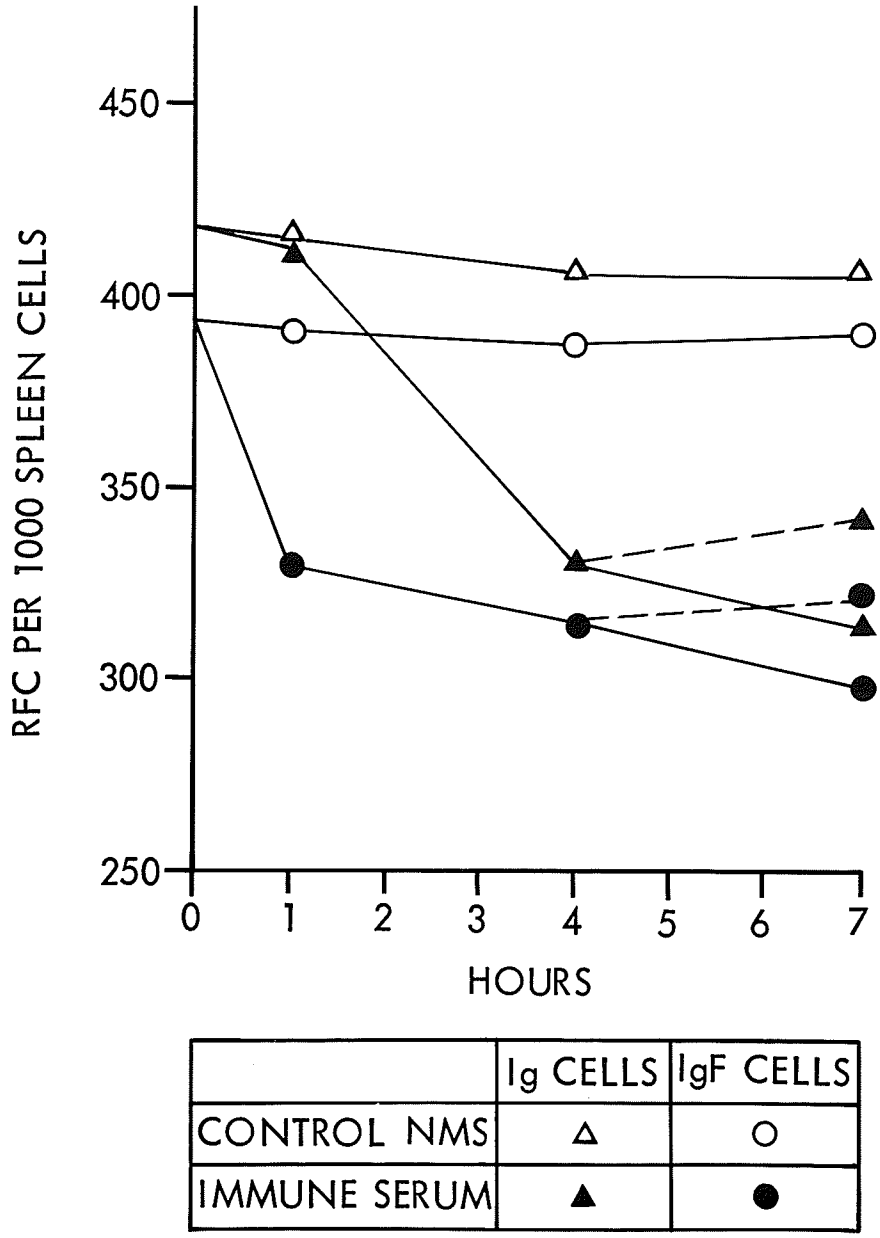


FIGURE 10: Short term culture of Ig carrying cells with 6 hour serum at 4°C.

changes induced as detected by both hybrid antibodies are identical with those induced in cultures at 37° C. Thus both the uptake of the cytophilic Ig at 1 hour and the mobilization of surface Ig by the dialysable factor at 4 hours are temperature independent and require no metabolic or membrane activities.

It appears that the mobilization of surface Ig described here does not depend on pinocytosis. It should also be noted that when the culture was washed at 4 hours and further incubation for additional 3 hours at 4° C in the absence of 6 hour serum, the RFC count did not rise to the normal level. Thus at 4° C regeneration of surface Ig did not take place, since it has been shown that this phenomenon depends on active metabolism (Lerner et al, 1972).

Sodium azide has also been known to inhibit the mobilization of surface Ig as inhibitor of pinocytosis (Taylor et al, 1971). Some experiments using azide were performed in our system, but unfortunately yet for unknown reasons azide interferes with rosette formation.

It is felt, however, that the 4° C culture experiments provide strong evidence against pinocytosis as a mechanism of mobilization of surface Ig.

2. 24 hour serum

Spleen cells treated with anti-θ serum and complement were cultured in the presence of 24 hour serum. No significant decrease of viability took place during the

culture period (Table 15). As shown in Fig. 11, the RFC by both hybrid antibodies are decreased at 1 hour. This contrasts with the result obtained with the 6 hour serum. It indicates that no cytophilic Ig is present 24 hours after immunization which agrees with previously reported findings that no Ig-antigen complexes were also present at this time (Orr and Paraskevas, 1973). Another striking difference between the 24 and 6 hour sera is that loss of surface Ig by the former is induced after only 1 hour incubation while in the 6 hour serum after removal of the cytophilic IgG by precipitation, the dialysable factor requires 4 hours for inducing the loss of surface Ig (Fig. 7). Whether the factors in the two sera are different or whether the difference is simply quantitative is not completely clear at present.

Some experiments performed to answer this question by using larger amounts of filtrate from 6 hour serum suggest that the last possibility may be true.

At 4 and 7 hours the number of RFC diminishes further more with both hybrid antibodies. When the culture is washed at 4 hours and incubated for 3 more hours in fresh medium the RFC counts returned to normal level, which again indicates regeneration of surface Ig.

3. Seven day serum

i. Culture at 37°C

The sera collected at 7 days after immunization

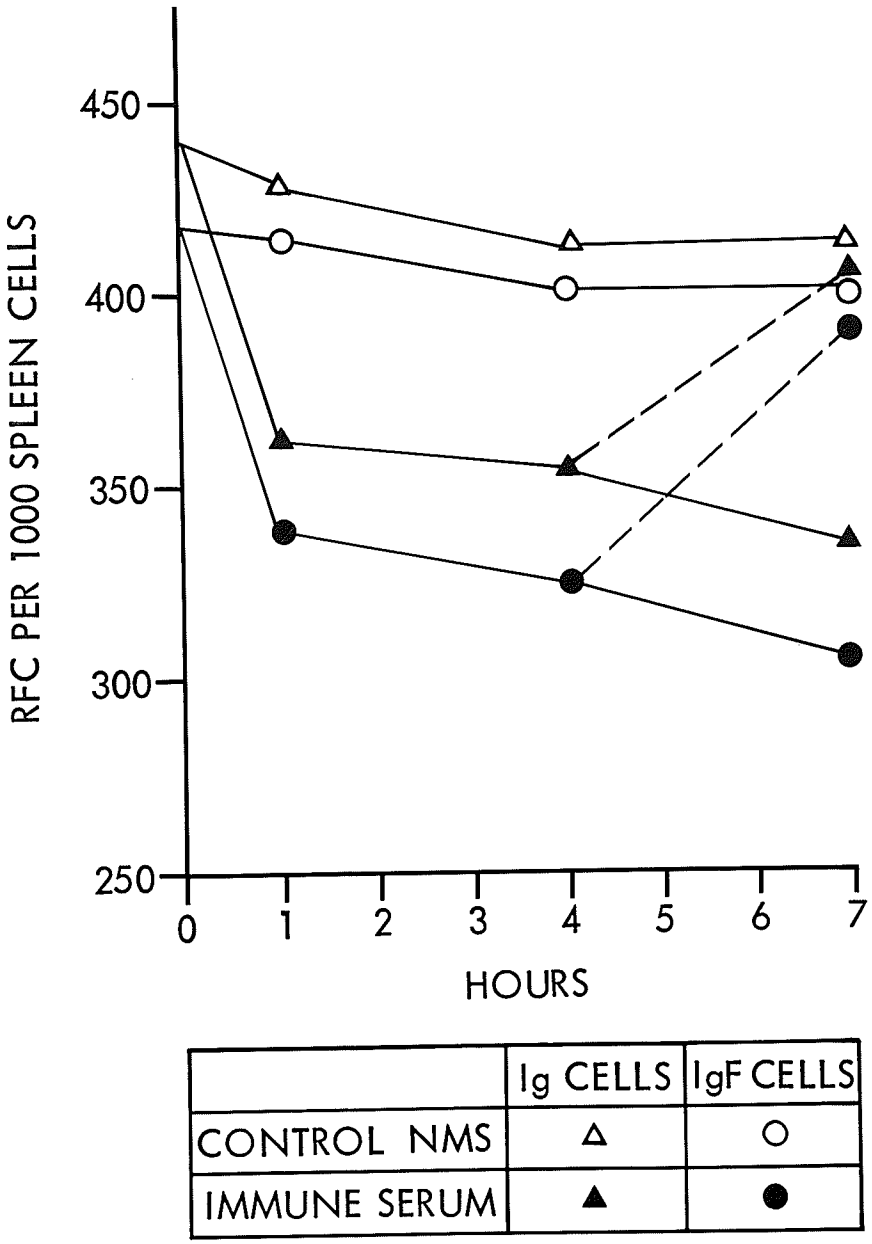


FIGURE 11: Short term culture of Ig carrying cells with 24 hour serum at 37°C.

was examined in the same culture system. As shown in Fig. 12, the changes are similar to those induced by 24 hour serum. Again no cytophilic Ig was detected and the serum contained a factor which mobilized surface Ig within the first hour of culture. The surface Ig re-generated again when the serum was removed by washing at 4 hours.

ii. Culture at 4° C

When the cells were cultured at 4° C, the 7 day serum induced identical changes as at 37° C (Fig. 13). However, as in the case of 6 hour serum the RFC did not return to normal after the removal of the serum from the culture, indicating again that regeneration of surface Ig did not take place at 4° C.

iii. Dialysis of 7 day serum

When dialysed 7 day serum was used in the culture, no changes were observed of RFC during the whole culture period (Fig. 14). Thus the factor present in the 7 day serum which induced loss of surface Ig is dialysable as in the 6 hour serum.

4. Fifteen day serum

The results are shown in Fig. 15 and are identical with those of 24 hour and 7 day sera.

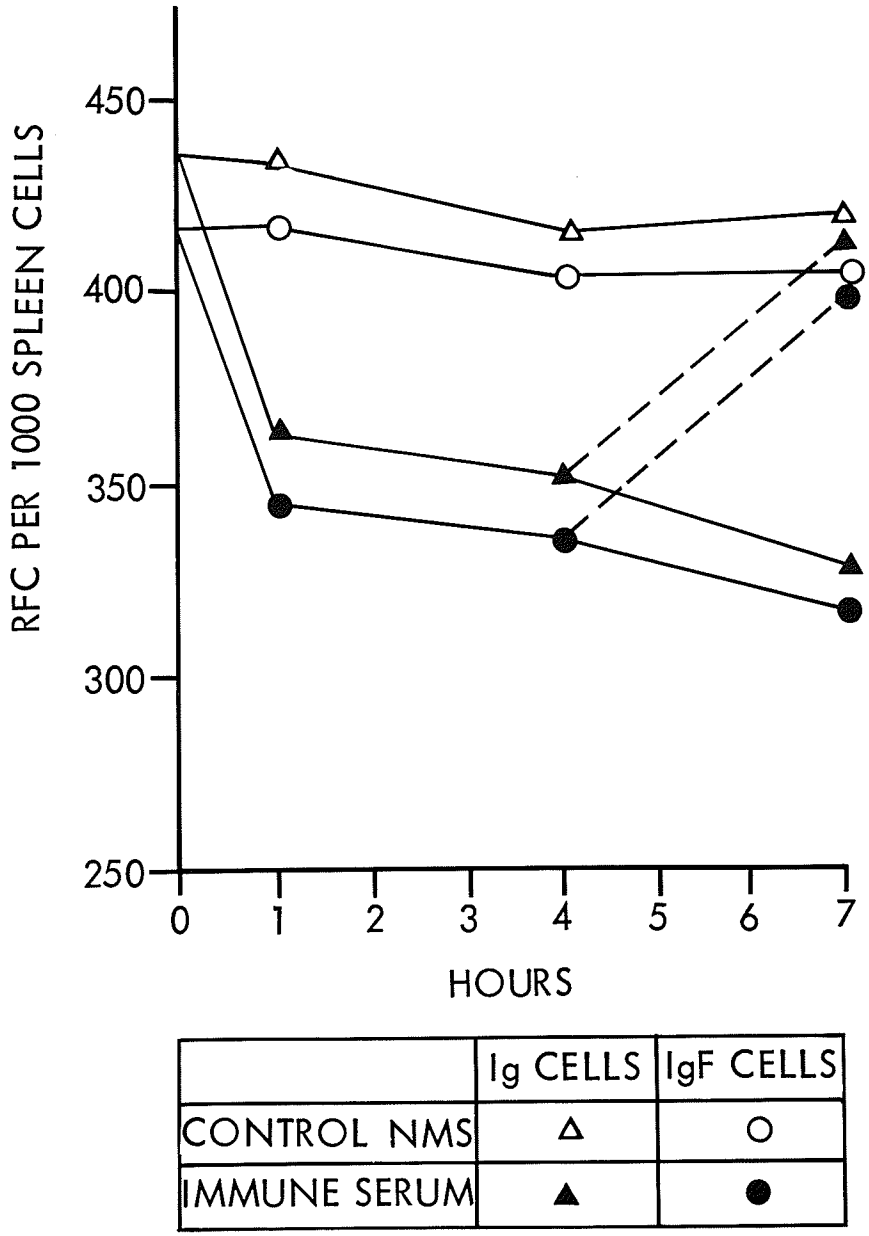


FIGURE 12: Short term culture of Ig carrying cells with 7 day serum at 37° C.

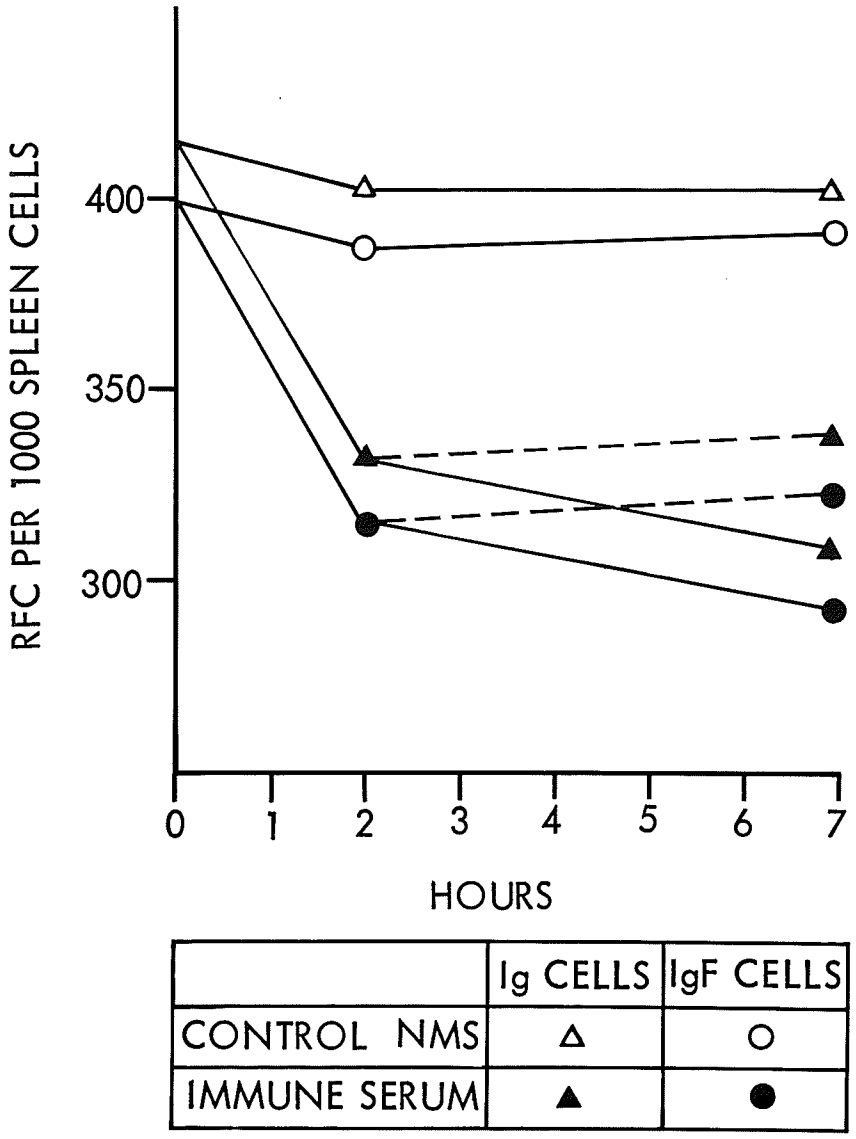
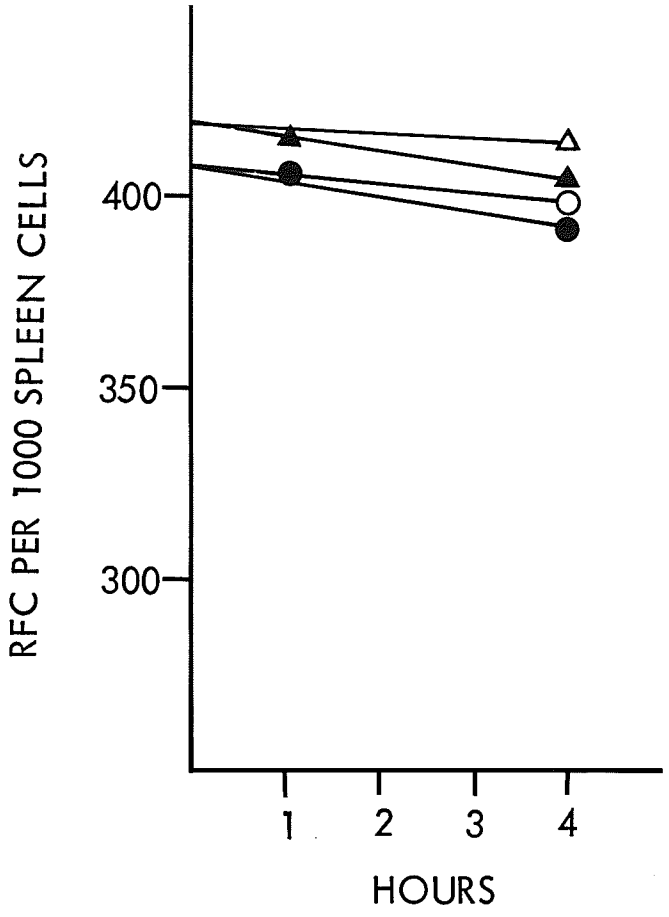


FIGURE 13: Short term culture of Ig carrying cells with 7 day serum at 4°C.



	Ig CELLS	IgF CELLS
CONTROL NMS	△	○
IMMUNE SERUM	▲	●

FIGURE 14: Short term culture of Ig carrying cells with dialysed 7 day serum at 37°C. NMS was equally treated.

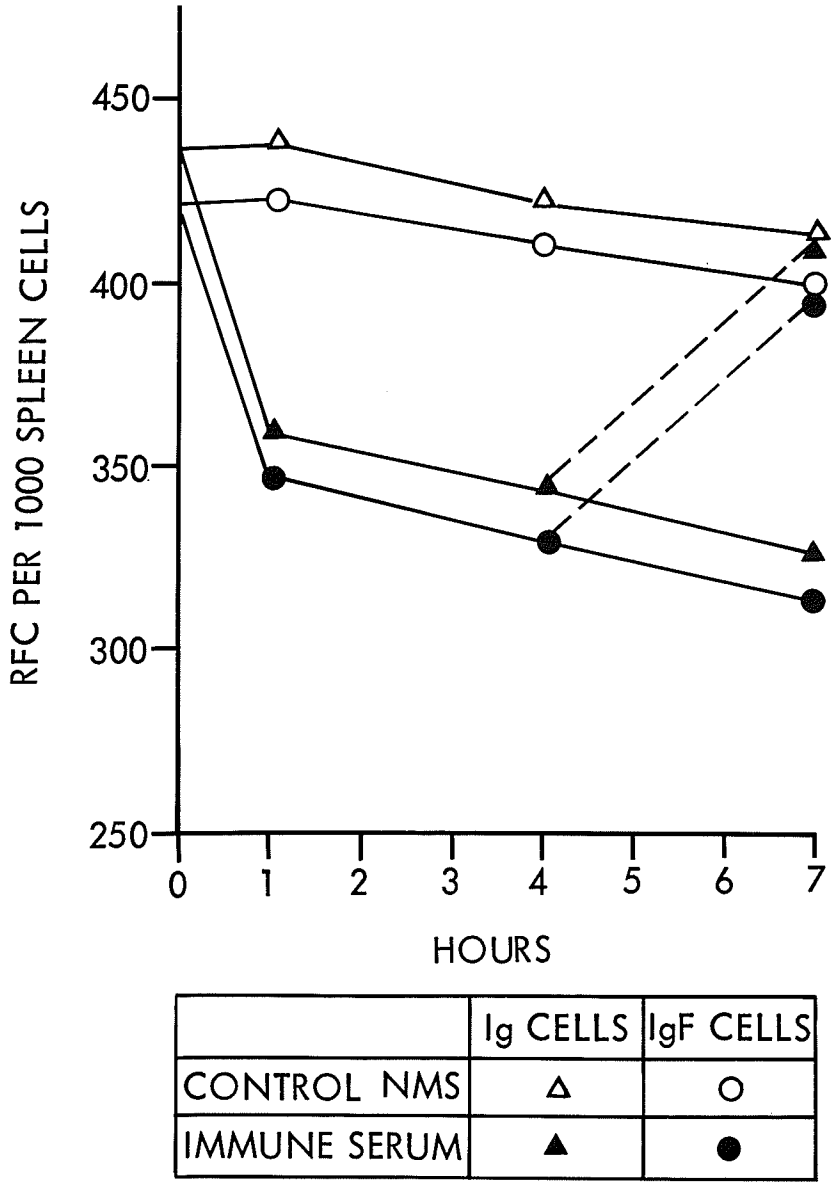


FIGURE 15: Short term culture of Ig carrying cells with 15 day serum at 37°C.

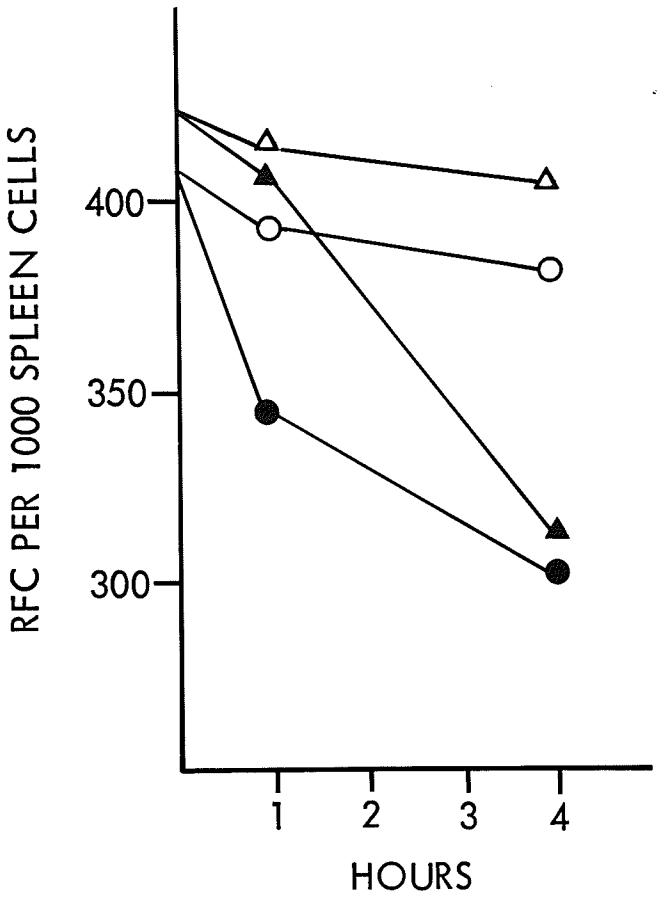
III. THE EFFECT OF CONCANAVALIN A ON THE MOBILIZATION OF SURFACE Ig BY IMMUNE SERA

1. Six hour serum

Spleen cell suspensions after they were exposed to anti-θ serum and complement, were treated with ConA in a concentration of 100 ug/ml for 20 x 10^6 cells for 30 minutes at 37°C. The cells were washed and used in culture in the presence of 6 hour serum or NMS. The effect of 6 hour serum on ConA treated cells was the same as on non-treated cells (Fig. 16). There was a decrease of IgF cells at 1 hour and of all Ig cells at 4 hours. These results show that the attachment of the cytophilic Ig and loss of surface Ig are not prevented by prior treatment of the cells with ConA. ConA has been shown to block the mobility of surface Ig induced by heterologous anti-Ig sera (Yahara and Edelman, 1972). Thus mobilization of surface Ig by the 6 hour serum may present some differences as compared to that induced by heterologous anti-Ig, it is temperature independent and is not prevented by prior treatment with ConA. Further work is required.

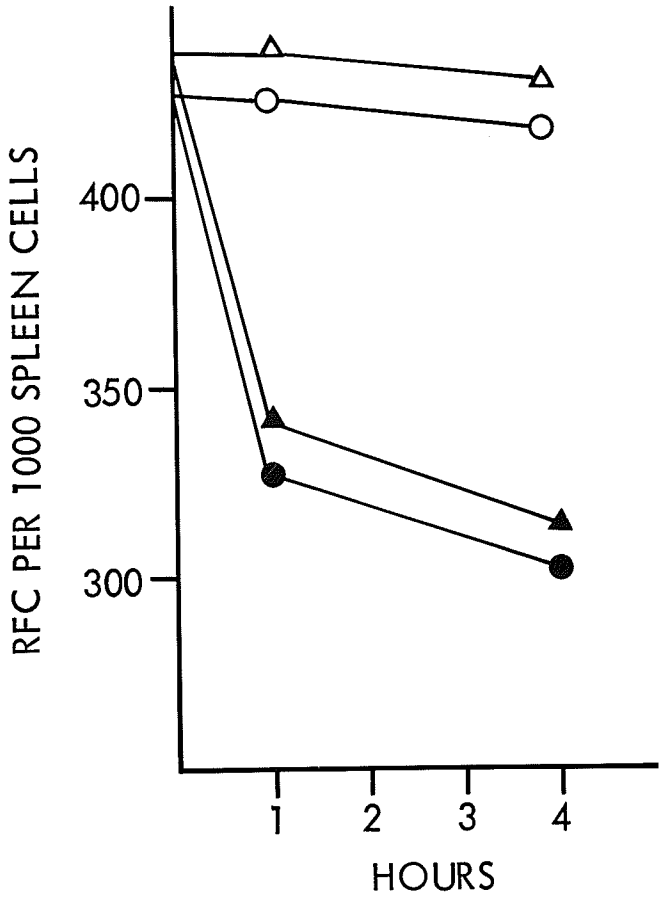
2. Seven day serum

This serum was examined in cultures after treatment of cells with ConA. As shown in Fig. 17, ConA treated cells did not show any difference from the untreated cells in their response to the factor in the 7 day serum.



	Ig CELLS	IgF CELLS
CONTROL NMS	△	○
IMMUNE SERUM	▲	●

FIGURE 16: Short term culture of Concanavalin A treated Ig carrying cells with 6 hour serum at 37°C.



	Ig CELLS	IgF CELLS
CONTROL NMS	△	○
IMMUNE SERUM	▲	●

FIGURE 17: Short term culture of Concanavalin A treated Ig carrying cells with 7 day serum at 37°C.

DISCUSSION

Two specific cell types - bone marrow derived B cells and thymus derived T cells are involved in immune responses to antigens. The B cells are mainly responsible for humoral antibody formation and the T cells for cellular immunity. For the thymus dependent antigens, however, both cell types are required to interact synergistically to bring about the humoral antibody response. It is widely accepted that antigen reacts with surface associated Ig on lymphocytes for the initiation of the immune response. Ig molecules are found in abundance on B lymphocytes and can be easily identified by various techniques. Whether T cells bear such molecules is still debatable and if they do they are not detectable in large numbers by present day techniques. T cells, however, can be identified by the presence of surface θ alloantigens. Most of the studies of the events following antigenic stimulation are concerned with antibody production, the morphological changes of the immune response or the DNA synthesis of the various cell types involved. There is no information regarding in general the changes of cells carrying surface Ig and θ antigen following interaction with antigen.

RICA has been successfully used to detect surface associated Ig on lymphocytes. This technique does not detect soluble Ig in the process of secretion as in normal (Paraskevas et al, 1971b) or neoplastic plasma cells (Paraskevas et al, 1970). Anti-θ antiserum on the other hand

has been used to identify T cells in the mouse system by cytotoxicity tests in the presence of complement. The changes of T and B cell populations during the primary response to polymerized flagellin antigen with or without complete Freund's adjuvant have been followed using the surface Ig and θ antigen as markers for identification of the respective cells.

With the RICA technique it was shown that the largest percentage of 'resting' lymphocytes in the mouse spleen carry both major Ig classes on their surface, the IgG (7SIgG2a) and IgF (7SIgG1). The total number of cells carrying surface associated Ig (about 330/1000 spleen cells) is slightly higher than the total number of cells carrying the IgG and/or IgF globulins (about 300/1000 spleen cells.) These extra cells may also carry other Ig classes or possibly L chain fragments. This study also revealed a peculiar distribution of cells carrying IgG and IgF globulins. Of 300 such cells, approximately 200 carry both immunoglobulins on the same cell. The other 100 carry only IgF. These 100 cells may also carry any of the three other mouse Ig classes (IgM, IgA, IgH). Cells carrying only IgG are not detected and either are present in very small numbers or carry IgG in amounts beyond the limit of detection by this method.

A fundamental question of immunology is related to the number of specific antibodies or the number of Ig

classes produced by one cell. One cell - one specific antibody may be considered a central dogma of the clonal selection theory (Burnet, 1959) and much evidence indicates this to be true (Mäkelä, 1967; Peterson and Ingraham, 1969; Gershon et al, 1968a). Although contrary views have been expressed that one cell produces more than one antibody specificity (Attardi et al, 1964; Hiramato and Hamlin, 1965; Liacopoulos et al, 1971). It is widely held that one cell produces one Ig class (Mellors and Korngold, 1963; Bernier and Cebra, 1965; Burtin and Buff, 1965; Cebra et al, 1966), one light chain type (Bernier and Cebra, 1965; Pernis and Chiappino, 1964) or one allotype among those controlled by allelic genes (Pernis et al, 1965). Some studies have claimed that a small number of cells produces more than one class of Ig (Nussenzweig et al, 1968; Takahashi et al, 1968; Costea et al, 1967; Greaves, 1971a) or more than one allotype (Greaves, 1971b). The cells examined in these studies were usually well differentiated cells, blasts or plasma cells. Phenotypic restriction may be true for antibody or Ig producing cells but may not apply to the precursors. Recent studies have indicated that 'resting' lymphocytes are pluripotent in terms of the number of Ig classes (Sell et al, 1970; Manning and Jutila, 1972b; Lawton et al, 1972; Kincade et al, 1970) or the type of light chains (Heller et al, 1971) they are able to produce and also less restricted than plasma cells in the expression of allotypic

specificity (Sell et al, 1970; Greaves, 1971b).

If surface associated Ig indicates the genetic potential of the lymphocyte to produce Ig, the direct and quantitative demonstration in this study, of cells carrying more than one class of Ig would imply that multipotent cells do exist in the resting lymphoid population in relatively large numbers. The lymphocytes shown to have on their surface only IgF among the two Ig examined, may still carry some of the other mouse Ig classes. Nevertheless, this subpopulation of lymphocytes is different from the other one which displays both IgG and IgF surface Ig. One may assume that subpopulation of more differentiated lymphocytes arise from one truly pluripotent, undifferentiated cell along various differentiation pathways, however, such evidence does not exist at the present time. It is possible of course, that antigen modifies the original uniform population. Such an example of extreme modification is the total lack of surface receptors on plasma cells (Paraskevas et al, 1971b) which arise after antigenic stimulation. If partial modification takes place by loss of one Ig then random loss should yield equal numbers of IgG and IgF cells. The absence of IgG cells speaks against partial modification of the original cell population.

One must consider that surface associated Ig is not always related to cell potential for Ig production. If the potential of Ig production for some cell is represented

on its surface by the display of surface associated Ig, the only cells where this relationship must be true are the cells carrying both IgG and IgF globulins, since no other cells exist to provide the cellular potential for IgG production. It is quite likely that IgF cell population may serve another immunological function unrelated to Ig production and belongs to a cell population not originally carrying Ig on its surface but has acquired such an Ig subsequently. However, further work is needed to explain the presence of IgF cells.

If 'resting' uncommitted lymphocytes are pluripotential in terms of Ig which they are able to synthesize, the important question is whether all receptors carried by one lymphocyte have the same specificity against an antigenic determinant. Since some evidence exists that various myeloma proteins share the same or similar V-regions (Wang et al, 1970), it is quite likely that the resting lymphocyte, although pluripotent in terms of Ig classes, it is restricted in terms of the number of specific antibodies it is able to synthesize.

The θ carrying cells in the resting spleen lymphocyte population are determined by cytotoxicity tests using AKR anti-C3Hθ serum in the presence of complement. The number was found to be 27.3%. This is in good agreement with other reports (Raff, 1971; Takahashi et al, 1970a).

Since Ig and θ antigens have been chosen to

follow the B and T cell changes in the primary response, it is important to know whether the Ig carrying and θ carrying cells as detected by RICA and anti-θ cytotoxicity are distinct cell populations. It was found that when spleen cells were treated with anti-θ serum in the absence of complement and then examined by RICA, the Ig carrying cells were equal to the sum of Ig carrying of untreated spleen cells detected by RICA and θ carrying cells detected by anti-θ cytotoxicity. Furthermore, when normal spleen cells were first treated with rabbit anti-MIg serum in order to block rosette formation by the B cells and then exposed to anti-θ serum in the absence of complement, the number of RFC detected by RICA was found equal to the number of θ carrying cells as detected by cytotoxicity (Table 7). These results demonstrate that the Ig and θ carrying cell populations are distinct from each other.

During the primary immune response, the same techniques are used to follow the changes of B and T cells in the spleen. Using single RICA, significant changes in the Ig carrying cells are observed after immunization. The changes are of two kinds. At 6 hours after antigenic stimulation, there is a significant increase of the total Ig carrying cells (Ig cells). This is followed by a progressive decrease of such cells in the first two weeks, which involved approximately 38-40% of the Ig carrying cells (Fig. 3). This phenomenon is not only observed with POL antigen but

is also true for other antigens such as SRBC, CRBC, KLH (keyhole limpet hemocyanin) and BSA (Paraskevas et al, 1972c). When the changes are followed with single RICA using specific hybrid antibodies, it was found that the increase at 6 hours of Ig cells could be accounted for by comparable increase of IgG cells. Mixed sequential RICA revealed that the increase of RFC was due to the appearance of γ cells, that is cells carrying only IgG globulin, which do not exist in normal spleen. Six hours after antigenic stimulation, these cells increased to 85/1000 spleen cells. While an overall increase of Ig cells takes place at 6 hours the IgF cells decreased as found by mixed sequential RICA and the decrease was contributed by the $\gamma\phi$ cells (Fig. 4) while the ϕ cells were not affected. (Fig. 4).

In the time interval between 24 hours and 15 days after immunization, the levels of Ig, IgG and IgF cells progressively decreased reaching the lowest point at 15 days (Fig. 3). The mixed sequential RICA revealed that the decrease of Ig carrying cells in this interval was contributed exclusively by a decrease of $\gamma\phi$ cells while ϕ cells were not changed. In the meantime, the γ cells returned to the normal base line level by the end of the 7th day.

We would first discuss the mechanisms of the cell changes at 6 hours. The increase of IgG carrying cells at 6 hours indicates that extra IgG carrying cells not previously

detected by RICA, are added to the normal Ig carrying cell pool following immunization. The changes may be argued to be due to proliferation or migration of Ig carrying cells. This is unlikely as the total spleen cell counts are not significantly different from that of unimmunized animals. DNA synthesis does not start until 24 to 32 hours after antigenic stimulation (Dutton and Mishell, 1967), and furthermore, the generation time is 5 to 8 hours (Rowley et al, 1968) or as long as 13 hours (Tannenberg and Malaviya, 1968). It is possible that the increase of Ig carrying cells in such a short period of time may be due to a preferential migration into the spleen of Ig carrying cells from other sources or a preferential migration out of the spleen of the non-Ig carrying cells. Although Zatz and Lance (1971) have described the trapping of lymphocytes in the spleen of mice , the cell type involved is not identified and it is not thought to occur until 24 hours after immunization. Perhaps the strongest evidence against proliferation or migration was the finding that similar changes can be reproduced in vitro by treating normal spleen cells with 6 hour serum (Table 10). The increase of Ig and IgG cells as well as the decrease of IgF cells was comparable to that observed in vivo. This indicates that some non-RFC in the spleen are transformed into RFC by taking up a 'cytophilic' IgG globulin and are thus detected as a RFC by the hybrid antibody. Since the T cells do not carry

surface Ig as detected by RICA, the possibility that this cell may be the non-RFC which contributed to the increase of RFC 6 hours after antigenic stimulation was investigated. In contrast to the increase of Ig carrying cells 6 hours after immunization, the θ carrying cells are decreased by 10% which is approximately of the same magnitude as the increase of Ig carrying cells. The number of θ carrying cells gradually returned to normal levels at the end of the 5th day (Fig. 5).

The inverse relationship of changes of B and T cells at 6 hours after immunization is taken as indirect evidence to suggest that some T cells may have been transformed into Ig carrying cells by the acquisition of a cytophilic IgG globulin on their surface. Upon such uptake of a cytophilic Ig by the T cells the θ antigen becomes undetectable by the anti-θ serum.

There are three other direct lines of evidence which clearly demonstrated that the cell which takes up the cytophilic Ig is a T cell (θ carrying). It has been shown that the changes of Ig carrying cells can be reproduced in vitro by treating normal spleen cells with 6 hour serum. It was found that elimination of θ carrying cells from normal spleen cells followed by the 6 hour serum treatment completely abolished the increase of RFC (Table 11). In contrast, treatment with anti-MIg serum has no effect on the ability of the spleen cells to show the increase after exposure to

the 6 hour serum (Table 11). When the spleen cells are first treated with anti-MIg serum followed by anti-θ serum treatment, exposure to 6 hour serum produces no increase. Since the anti-θ serum is not cytotoxic to cells from bone marrow but cytotoxic to all thymocytes, it is concluded that only T cells are affected by the treatment of spleen cells with this antiserum. These conclusions are furthermore supported by the experiments with thymus and bone marrow cells exposed to 6 hour serum (Table 12). Only thymus but not bone marrow contains a cell which can acquire the cytophilic IgG from the serum. About 5% of the thymus cells are thus detectable as carrying the proper receptor for acquiring the cytophilic IgG. Whether the other thymus cells lack this receptor or acquire the Ig in amounts not detectable by RICA is not known. Finally, other direct evidence comes from the experiments of irradiated and reconstituted mice (Table 13). Again, only the thymus cells homing to the spleen show the ability to produce an increase of Ig carrying cells after challenge with antigen. Spleen cells collected from mice injected with bone marrow cells lack this property.

From these lines of evidence, it is clear that 6 hours after immunization, a population of T cells can take up a cytophilic IgG globulin on their surface and be transformed into Ig carrying cells. Whether the θ antigens on a T cell which has acquired such a IgG globulin has lost

the ability to react with the anti-θ serum is only conjectural.

While an overall increase of Ig cells took place at 6 hours after antigenic stimulation, the use of specific hybrid antibodies showed that the total number of cells carrying IgF globulin (IgF cells) decreased. Sequential mixed RICA revealed that this was due to changes of the $\gamma\phi$ cells, while the ϕ cells showed no change. The mechanisms underlying the B cell changes are more difficult to interpret. It is possible that the decrease of $\gamma\phi$ cells at 6 hours may be due to loss of Ig from their surfaces. Since such a decrease of RFC is smaller than the increase contributed by the T cells the net result is an overall increase of Ig carrying cells at this time. Alternately, the same cytophilic IgG globulin which is taken up by the T cells may also be attached onto the surfaces of these cells and blocks the detection of the IgF globulin by specific hybrid $\alpha\phi$-αEA. Such B cells which have taken up the cytophilic IgG would still be detectable as Ig carrying cells by both αMIg-αFe and $\alpha\gamma$-αFe hybrid antibodies. Experiments were designed to test these hypotheses by exposing anti-θ treated spleen cells to 6 hour serum in vitro. Such a cell population was treated with 6 hour serum for different time intervals in a short term culture system and the changes of Ig carrying cells were followed using two hybrid antibodies: αMIg-αFe which detects all

mouse Ig classes and the specific hybrid antibody $\alpha\phi$-αEA which detects only IgF globulin.

If there is complete loss of surface Ig from the $\gamma\phi$ cell after treatment with the 6 hour serum, a decrease of RFC should be detected by both hybrid antibodies. On the other hand, if the decrease of IgF cells is due to a phenomenon of steric hindrance due to uptake of a cytophilic IgG globulin, a decrease in the number of RFC is expected only with the specific $\alpha\phi$-αEA hybrid antibody.

The results presented in Fig. 6 show that when anti-θ treated spleen cells are cultured with 6 hour serum for one hour, there is no decrease of Ig cells but a decrease of the IgF cells. When the cells were cultured for 4 hours with the 6 hour serum both Ig and IgF cells decreased by the same level. These data suggest that at the beginning a cytophilic Ig is taken up by the B cells and blocks the detection of surface IgF globulin possibly through steric hindrance. Four and 7 hours later, surface Ig is apparently lost from the cell surface since both Ig and IgF cells are decreased. If the cells are washed free of 6 hour serum at 4 hours, resuspended in fresh medium and cultured for another 3 hours, the number of both the Ig and IgF cells returned to normal levels which indicates that surface Ig is regenerated. The cells cultured in NMS show more or less a constant level of Ig and IgF cells throughout the whole culture period. A slight decrease at 4 and 7 hours

may be due to some cell death (Table 15).

Although the increase of IgG cells by single RICA and the γ cells by mixed RICA suggested that the cytophilic Ig taken up by these cells belongs to the IgG class, the nature of the cytophilic Ig taken up by the B cells was investigated by precipitating the IgG globulin in the 6 hour serum with an isolated highly specific rabbit anti-mouse IgG antibody. After such treatment, the 6 hour serum lost its ability to induce a decrease of IgF cells after one hour of culture (Fig. 7).

However, the number of RFC by both hybrid antibodies was decreased after 4 hours of culture to the same extent as with the non-precipitated serum. Thus, the decrease at 1 hour of IgF cells and the decrease of both Ig and IgF cells at 4 hours may be different phenomena.

To clarify further these mechanisms, the 6 hour serum was dialysed. The results (Fig. 8) from cultures of anti-θ serum treated spleen cells in the presence of dialysed 6 hour serum indicate that although the results after one hour are the same as with non-dialysed serum no decrease of Ig cells takes place at 4 hours and the difference between Ig and IgF cells present at 1 hour still exists till the end of the culture period at 7 hours. Thus, dialysis has removed a factor which induces a decrease of Ig cells at 4 hours. Dialysis and precipitation of IgG abolished all activities the 6 hour serum showed on Ig carrying cells (Fig. 9) It is

reasonable to conclude at this point that the serum of mice 6 hours after antigenic stimulation contains two different factors. One is a cytophilic IgG which probably, through steric hindrance blocks the detection of IgF by specific hybrid antibody $\alpha\Phi$-αEA. Since the cytophilic IgG is still detectable by αMIg-αFe hybrid antibody, no drop in the Ig cell count is detected and this gives an explanation for the discrepancy of the RFC counts by the two hybrids at 1 hour.

The second factor is dialysable and induces loss of surface Ig so that the RFC is low by both hybrid antibodies. Loss of surface Ig, rather than cell death, at 4 hours is the most likely interpretation of the data since when the 6 hour serum is removed by washing and the cells are cultured in fresh medium, the counts by both hybrid antibodies returned to normal level 3 hours later. Since the half life of surface Ig is about 45 minutes (Lerner et al, 1972) it is quite possible that the Ig has regenerated. This is even further supported by the experiments where the culture was carried out at 4° C (Fig. 10). The uptake of the cytophilic IgG was not inhibited at this temperature, neither the loss of surface Ig at 4 hours which suggested that both phenomena are temperature independent. However, no return to normal count was observed when the serum was removed after 4 hours of culture (Fig. 10 interrupted lines) since surface Ig regeneration depends on metabolic activity.

The results obtained with cultures at 4° C pointed out some important conclusions. The mobilization of surface Ig by the dialysable factor which is present in the 6 hour serum is in striking contrast to that induced by heterologous anti-Ig antisera since the last one is abolished at 4° C and also by inhibitors of pinocytosis (Taylor et al, 1971).

Sodium azide was used in some experiments in our system but for some obscure, as yet, reasons, this substance interfered with rosette formation in RICA, thus the effect of sodium azide on loss of surface Ig by the 6 hour serum could not be evaluated. We consider, however, the results obtained by the culture at 4° C to argue against pinocytosis as the mechanism of mobilization of surface Ig by the 6 hour serum.

The nature of the cytophilic IgG globulin which is detected in the 6 hour serum was characterized by passing the 6 hour serum through a Sephadex G-200 column. Only the 7S fraction containing radioactively labeled antigen could reproduce both the increase of RFC and the decrease of IgF cells induced by 6 hour serum (Orr and Paraskevas, 1973). This finding would suggest that the cytophilic IgG globulin may represent an antigen-antibody complex. Antigen complexed to IgG globulin was detected in rabbits 5 hours after stimulation by Yuan et al (1970). Early 7S antigen-antibody complexes with affinity to spleen cells have also been detected in immunized chickens (Ivanyi, 1970). The nature

of binding of this complex to the lymphocyte is unknown at present.

The mechanism of the decrease of the number of Ig carrying cells observed in vivo between 24 hours and 15 days following antigenic stimulation is undoubtedly highly complex. The same in vitro short term culture system was used in order to detect possible serum factors which may alter the surface Ig of B cells.

As shown in Table 14, the decrease of Ig carrying cell observed in vivo could be reproduced by serum collected at different time intervals after antigenic stimulation. Thus loss of surface Ig by these cells may be unimportant. contributing factor to the cell changes observed in vivo.

Normal spleen cells treated with anti-θ serum were cultured in the presence of 24 hour, 7 day and 15 day sera. Both the Ig and IgF cells were decreased by the same number after 1 hour or 7 hours of culture. The number of Ig and IgF cells affected were quite comparable to that observed in the spleen in vivo. No steric hindrance is detected with these sera at 1 hour as with the 6 hour serum which suggested that no cytophilic Ig is present (Figs. 11, 12 and 15). The surface Ig could be regenerated if the cells were washed free of immune serum and resuspended in the fresh medium. As with the 6 hour serum, dialysis of 7 day serum completely removed the ability of this serum to mobilize surface Ig (Fig. 14). Furthermore, 7 day serum was as effective at 4° C

as at 37° C, but regeneration of the surface Ig did not take place (Fig. 13).

Whether the factor in 6 hour serum is the same as that in the 7 day serum is not known. The difference in the time required to induce the loss of surface Ig by the two sera may represent a concentration phenomenon. Some experiments performed to answer this question tend to support this interpretation. Yahara and Edelman (1971) have shown that ConA prevents the mobilization of surface Ig by heterologous anti-Ig sera. We have also investigated the effect of ConA in our system and the results shown in Fig. 16 and Fig. 17 indicated that neither the uptake of cytophilic IgG nor the loss of surface Ig induced by the 6 hour or 7 day sera are blocked by ConA.

In this respect the mechanism of mobilization of surface Ig by the dialysable factor present in the immune sera, may be different from that induced by anti-Ig sera.

POL antigen injected without FCA induced changes similar to those described for the antigen given in FCA (Table 8). POL in FCA was used as antigen for all the studies because it has been shown by Paraskevas et al (1972c) that the presence of FCA is important for certain soluble antigens such as BSA, KLH, and fibrinogen. The 6 hour increase of RFC is produced only when these antigens are injected with FCA. For particulate antigens such as SRBC and CRBC, no FCA is required. In this respect, POL behaves

like a particulate antigen.

POL has been described as a thymus independent antigen (Armstrong et al, 1969), whereas SRBC is highly thymus dependent (Miller, 1961; Taylor, 1969) for eliciting the antibody response. The data presented here together with the observations made by Paraskevas et al, (1972c) seem to suggest that there is no difference between thymus dependent and independent antigens with respect to the increase of RFC 6 hours after immunization. The biological relevance of the phenomena described here is not clear at the present time.

We can summarize that the fundamental observations from our studies are:

1. Six hours after immunization
   - i. the uptake of a cytophilic IgG by T cells
   - ii. the uptake of a cytophilic IgG by B cells
2. During the first two weeks after immunization
   - i. large scale changes affecting B cells
   - ii. the presence in the sera of the animals during this time interval of soluble factors which induce in vitro mobilization of surface Ig on B cells.

It is possible that the changes of T and B cells induced by the cytophilic IgG at 6 hours are related to inductive phenomena underlying the initiation of the immune response. Since the cytophilic IgG most likely represents an Ig complexed with antigen, its uptake by both T and B

cells may provide the mechanism for the presentation of antigen to lymphocytes. Thus the uptake of the antigen in complex with Ig may give supporting evidence to Bretscher-Cohn suggestion that antigen is presented to the lymphocyte combined with a carrier antibody. In addition, these findings may provide a mechanism for concentration of the antigen.

An important question possibly related to the concentration of antigen is the observation that large numbers of T and B cells are affected. Antigen binding cells in unimmunized animal have been described by a number of authors (Naor and Sulitzneau, 1969; Humphrey and Keller, 1970; Ada et al, 1970; Dwyer and Mackay, 1970; Ada, 1970) for a number of antigens. The percentage, however, is small and never exceeds 0.5% of the total lymphoid cells. If this is the true number of cells sensitive to a given antigen, this percentage is at least 25 times lower than the number of cells taking up the cytophilic IgG (complex?) which is approximately 12% of the cells (120 B and T cells/ 1000 spleen cells). Even at the peak of primary antibody response to POL (5 to 6 days), Diener (1968) showed that approximately 0.1% spleen cells are producing antibody. At approximately the same time we have found here that 50 - 60 B cells per 1000 spleen cells are affected, or 5 to 6% which is 50 to 60 times larger than the cells producing specific antibody.

It has been demonstrated that the majority of newly synthesized Ig especially during the early periods after antigenic stimulation does not show antibody activity against the injected antigen and in addition, antibody and non-antibody Ig are synthesized by separate cells (Urbain-Vansenten, 1970). It has also been shown that a large number of cells in the spleen which proliferate do not develop into antibody producing cells (Harris and Pelc, 1970). Our findings may provide the interpretation for these two phenomena. Some cells involved in the early stages of the immune response may have been triggered non-specifically to a limited proliferative activity and the production of non-antibody Ig.

Since both T and B cells are taking up the cytophilic IgG (complex?) our observations may also form the basis of the cooperation of these two cell types. The decrease of the Ig carrying cells during the first two weeks in vivo was shown to affect mainly the $\gamma\phi$ cells. It is interesting that these cells, but not the ϕ cells (which are not affected) are likely to have the genetic potential for Ig (or antibody) synthesis. The decrease of Ig carrying cells was reproducible in vitro by exposing normal spleen cells to immune sera and was caused by the mobilization of surface Ig triggered by soluble and dialysable factors. Whether removal of surface Ig from B cells may underlie the mechanism of triggering of this cell for proliferation and

differentiation is entirely speculative.

More than seventy years ago Ehrlich suggested in his theory of antibody production that the interaction of the invading microorganisms with the 'side chains' on the cell surface results in the release of the side chains. Such a release triggers the cell for the production of more 'side chains' which constituted the circulating antibodies. The source of the soluble factors is unknown. Several studies (Doria et al, 1972; Gorczynski et al, 1972) have now demonstrated that soluble factors derived from T cells may replace these cells in the immune response to sheep erythrocytes. Whether the soluble factors from such studies are similar or identical to those we have described here remains an important challenging question.

It is to be noted that our observations raise more questions than they are able to answer. It is only hoped that the studies reported here may serve to trigger further experimentation in order to obtain answers for the several questions raised, which will undoubtedly help us to understand better the phenomena of the immune response.

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