THE UNIVERSITY OF MANITOBA

INDUCTION OF SOLUBLE FACTOR(S) BY FREUND'S COMPLETE ADJUVANT

by

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A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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TO MY WIFE MARIE

Man lives in a sea of microorganisms; the immune is his license to survive.

Robert Good

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RATIONALE

This section has been included in an attempt to rationalize the undertaking of this type of project as well as to clarify the format of this thesis.

Adjuvants are well known as substances which, when administered together with an antigen, increase the antibody response to that antigen. Such a property has been well documented. However, the mechanism by which this phenomenon operates is largely unknown.

This thesis is concerned with an in-depth study of this age-old problem. Thus we have attempted to investigate adjuvant action not in terms of increased antibody production; instead we have examined possible effects at the cellular level. That is, we have concentrated our efforts on examining possible adjuvant effects on the two main types of lymphoid cells - T and B cells - in an attempt to define a possible mode of action for Freund's complete adjuvant.

In our studies of the changes of immunoglobulin carrying cells during the primary immune response we observed a marked increase of such cells 6 hours after antigenic stimulation (Paraskevas et al, 1972c). This increase was produced by most of the soluble antigens when they were emulsified in FCA but not when they were injected in saline. Thus this initial observation has become the basis for this thesis and posed the following questions:

A. What is the mechanism which underlies this phenomenon?

B. How does FCA contribute to this phenomenon?

C. What is the active principle in FCA responsible for this phenomenon?

The questions posed above serve as main divisions in the body of this thesis in an attempt to describe the data in the most logical and easily understood sequence.

As will be noted, the introduction to this thesis is comprehensive in defining T and B cells as well as their characteristics and function. This is a deliberate attempt to acquaint the reader with these characteristics as they are used in this thesis for distinguishing T and B cells. Furthermore, the importance of the T cell as a regulatory and helper cell is described in an effort to further strengthen the results to be presented.

The last section of the introduction on adjuvants may appear quite short but the literature relevant to this thesis has been included. Since the end result of adjuvant action - increased antibody snythesis - is so well known by all Immunologists, only that data directly pertinent to this thesis has been described in any detail.

It is hoped that this section will avoid possible confusion which may otherwise arise over the presentation of the data.

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Ab	=	antibody
ABC	=	antigen binding cells
Ag	=	antigen
ALS	=	antilymphocyte serum
B cells	=	bone marrow derived lymphocytes
BCG	=	Bacillus Calmette-Guérin
BCGs	=	cell free supernatants obtained from lymphoid cells cultured in BCG
BDB	=	bis-diazotized benzidine
BGG	=	bovine gamma globulin
BSA	=	bovine serum albumin
C	=	complement
Con	=	control
Con A	=	concanavalin A
CRBC	=	chicken red blood cells
CRL	=	complement receptor lymphocyte
EA	=	egg albumin
EL	=	eluate
FCA	=	Freund's complete adjuvant
FCA-S	=	serum collected 6 hours after immunization with FCA
FCA-S(A)	=	serum collected 6 hours after immunization with FCA and absorbed with an insoluble aggregated antigen
Fe	=	ferritin

FIA	=	Freund's incomplete adjuvant
FIA-S	=	serum collected 6 hours after immunization with FIA
FIB	=	human fibrinogen
GvH	=	graft versus host
ΔH	=	heated at 56°C for 30 minutes
HRBC	=	horse red blood cells
6 HR-BSA	=	serum collected 6 hours after immunization with BSA in saline
6 HR-MS	=	serum collected 6 hours after immunization with antigen in FCA
24 HR-MS	=	serum collected 24 hours after immunization with antigen in FCA
Ig	=	immunoglobulin
IP	=	intraperitoneally
KLH	=	Keyhole limpet hemocyanin
LPS	=	lipopolysaccharide from E. coli 055:B5
MAAF	=	water soluble lipid free fraction from BCG
MLR	=	mixed lymphocyte reaction
MSH	=	maia squinado hemocyanin
NMS	=	normal mouse serum
NNP	=	4-hydroxy -3, 5-dinitrophenacetyl
NORs	=	cell free supernatants obtained from lymphoid cells cultured in Hank's solution
PFC	=	plaque forming cell
PHA	=	phytohemagglutinin
POL	=	polymerized flagellin
Poly A:U	=	polyadenylic and polyuridylic complexes

PPD	=	purified protein derivative of tuberculin
PVP	=	polyvinylpyrrolidone
RFC	=	rosette forming cell
RICA	=	reverse immune cytoadherence
RNase	=	ribonuclease
SIII	=	pneumococcal polysaccharide type III
SRBC	=	sheep red blood cells
TCA	=	trichloroacetic acid
T cells	=	thymus derived lymphocytes
TIGAL	=	synthetic polypeptide antigen consisting of polymeric side chains of DL alanine attached to a polylysine backbone. Tyrosine and glutamic acid residues are attached to the side chains
Θ	=	theta
$\alpha\Theta$	=	anti theta antiserum
αMIg	=	anti mouse immunoglobulin antiserum
αMIg - αFe	=	hybrid antibody which detects all classes of mouse Ig
$\alpha\gamma$ - αFe	=	hybrid antibody highly specific for the IgG class of mouse Ig
$\alpha\emptyset$ - αEA	=	hybrid antibody highly specific for the IgF class of mouse Ig

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ABSTRACT

The reverse immune cytoadherence technique (RICA) is one of many techniques designed to detect surface associated immunoglobulin on lymphocytes. This technique involves the use of a 5S hybrid antibody with one anti-mouse immunoglobulin site which could react with surface associated immunoglobulin. Through the other site, with a specificity to any protein, the hybrid antibody could react with protein-coated sheep red blood cells (SRBC), thus forming a rosette.

By RICA we have demonstrated that the immunoglobulin carrying or rosette forming cells (RFC) are significantly increased in the spleens of mice 6 hours after immunization. The results were reproduced in vitro by treating normal spleen cells with serum obtained 6 hours after injection of BSA in Freund's complete adjuvant but not when the antigen was injected alone or in incomplete Freund's adjuvant. Following gel filtration of the serum, antigen was detected in three forms: 1) native (4S), 2) bound to gamma globulin (>7S), and 3) fragmented (<4S). Normal spleen cells were treated with each fraction and examined by RICA. Increase of the RFC could only be induced by the 7S fraction containing a complex of IgG globulin and antigen. The binding of a cytophilic

IgG globulin on the lymphocyte surface was further supported with the use of highly specific hybrid antibodies.

Serum collected from mice 6 hours after injection of FCA, upon incubation in vitro with soluble antigens, induced an increase of the Ig carrying cells (RFC). Absorption of the same serum with aggregated or particulate antigen abrogated this activity which was recovered in the acid eluate. This property is most likely nonspecific since any antigen tested induced the increase and this property was lost to all antigens regardless of the antigen used for absorption. Fractionation of the serum on Sephadex G-200 showed that the activity resided in the 7S fraction. The 4S fraction also contained a factor which required the addition of both antigen and normal mouse serum to generate the increase. It is suggested that the interaction of this factor with normal mouse 7S Ig and antigen generates a cytophilic Ig which is taken up by 38-40% of the spleen T cells resulting in an increase of the RFC. About 15% of B cells were also affected. The cytophilic Ig belongs to the IgG(7SIgG2a) class.

Such a soluble factor (4S) could be induced in vitro in short term cultures of cells from thymus and spleen exposed to BCG or MAAF. As with the factor in vivo, this factor generated cytophilic Ig in normal mouse serum in the presence of antigen. Cultures of bone marrow cells and macrophages were inactive in this system.

The soluble factor induced in mice was demonstrated to be heat stable and trypsin sensitive. Upon acidification it dissociated to a size less than 10,000 molecular weight although the biological activity was retained. Circumstantial evidence suggested that the cytophilic Ig

induced by the 4S factor in the presence of antigen represented an Ig - antigen complex.

Possible modes of action of FCA are further discussed.

INTRODUCTION AND LITERATURE REVIEW

The initial side chain theory of antibody production described by Ehrlich (1900) ushered in a whole new era in Immunology. This was the first theoretical description of the existence of recognition units on the surface of lymphocytes. Very little experimental support emerged until Burnet (1959) postulated his clonal selection theory. He postulated the existence of antibody receptors of unique specificity on precursors of antibody secreting cells. The time was now ripe for experimentation and vigorous investigations into the cell types which recognize antigen as well as to the cellular manifestations of the immune response set the stage for the next decade of immunological research.

Recent investigations have led to the realization that antigen reactive precursor cells can differentiate via two pathways - the thymus dependent and bursa dependent pathways. The type of differentiation pathway selected determines functional as well as structural characteristics of these cells. Furthermore collaboration between these various cell types has been recognized and is presently the prime target for a majority of investigations in cellular immunology.

The data to be presented in this thesis is concerned with defining a possible mode of action of Freund's complete adjuvant. Classically, Freund's complete adjuvant has been used to enhance or prolong the production of antibody as well as to increase the ease with which delayed hypersensitivity is induced. Therefore the role of adjuvants in immunological phenomena is one of practical as well as theoretical importance.

The data to be presented will involve the reader with cell inter-

actions which occur after introduction of FCA. Therefore the literature review will be divided into a series of sections, each section defining certain intimate characteristics of T and B cells and the regulatory effects of one on the other. The results to be presented suggest that the T cell may have vast regulatory powers and the literature review will thus be inclined to defining other such examples of regulation, although literature pertaining to immunological phenomena other than the humoral immune response will also be cited.

I. SPECIFIC CELLS OF THE IMMUNE SYSTEM

Those cells which, although not actually engaged in an immune response, are nevertheless fully qualified to initiate such a response when appropriately stimulated by antigen, are termed immunologically competent cells (Medawar, 1963). In response to antigen, these cells either proliferate and differentiate to cells known as plasma cells which produce large amounts of specific antibody or respond by limited differentiation, resulting in sensitized cells which are responsible for cell-mediated immunity.

Thus immunocompetent lymphocytes can be distinguished according to their functional differences: 1) thymus or thymus derived cells - those small lymphocytes which are responsible for cell mediated reactions such as graft versus host reaction and homograft rejection and 2) bone marrow or bone marrow derived cells - those small lymphocytes which arise directly from the bone marrow and are the precursors of mature antibody producing plasma cells.

This section will be divided into two parts in an attempt to

evaluate the relevant roles of each of these two functionally distinct cell types in forming the matrix of the immune response.

i. Thymus and thymus derived cells

The thymus is a primary lymphoid organ which arises from the epithelium of the third and fourth pharynageal pouches (Venzke, 1952; Hammond, 1954). This gland is essentially composed of epithelial cells and large numbers of lymphocytes many of them in mitotic phase (Metcalf, 1964). Utilizing chromosomally marked cells and parabiosis experiments it was demonstrated that there is a continuous entry of new stem cells from extra-thymic sources with seeding of a small number of thymus cells to the peripheral organs (Ford, 1966; Weissman, 1967; Davies, 1969). However, it is still controversial whether the thymus actually produces these migrating cells or simply matures them under the influence of a thymic hormone (Trainin et al, 1969; Goldstein et al, 1970).

Miller's (1961) observation that neonatal thymectomy in mice prevents the development of cell mediated immunity and some antibody responses clearly demonstrated the prime importance of the thymus in the immune response. This became more clearly defined when Good and his colleagues (1962) demonstrated a correlation between thymic abnormalities and immunological deficiency disorders. Thus Bruton's type of agammaglobulinemia correlates well with lack of plasma cells and diminished humoral responses while cellular responses remain normal; Swiss agammaglobulinemia is characterized by a hypoplastic thymus while the Di George syndrome is consistent with the total absence of the thymus (Di George, 1955). In these latter disorders, cellular responses are impaired while antibody production is normal.

Although removal of the thymus has little effect on adult animals,

removal in the first few days of birth leads to depletion of cells in the periarteriolar areas of the spleen and paracortical areas of the lymph nodes (Miller, 1962). Such thymusless animals fail to reject grafts, to induce GvH reactions and to respond to some serum protein antigens but are not impaired in the antibody response to antigens such as haemocyanin and pneumococcal polysaccharides (Humphrey et al, 1964). Thus it appears that the immunological capacity of an animal depends on the thymus in the first few critical days of life. However the thymocytes themselves have been shown to be nonimmunocompetent as compared to lymph node or spleen cells (Vos et al, 1959; Billingham and Silvers, 1961; Thorbecke and Cohen, 1964). Consequently the thymus is regarded as a primary lymphoid organ not itself engaged in immune responses but required for correct development of the secondary lymphoid organs.

Those cells which fail to develop after neonatal thymectomy have been termed 'thymus dependent lymphocytes' and are dependent upon the thymus for their materialization either by production or maturation by a thymic influence. Such cells are responsible for those functions attributed to cell mediated immunity.

ii. Bone marrow and bone marrow derived cells

Reconstitution of lethally X-irradiated mice with bone marrow cells results in restoration of granulocyte, platelet, erythrocyte and lymphocyte production and is due to the donor inoculum (Ford et al, 1956). Repopulation of the spleen occurs in a focal manner with macroscopic nodules appearing on the surface of the spleen (Till and McCulloch, 1961). Each nodule is a single clone most likely arising from a single stem cell (Becker et al, 1963). Bone marrow cells repopulate both

primary and secondary lymphoid organs (Ford and Micklem, 1963). These experiments suggest that the bone marrow is the ultimate source of stem cells in the adult animal, with pluripotential precursors whose further differentiation probably depends on the prevailing local humoral environment (Wolf and Trentin, 1968).

Studies of the chicken lymphoid system have helped develop a clear cut separation between thymus-dependent cellular immunity and non-thymus or bursa dependent antibody production.

The bursa of Fabricius develops as an epithelial budding from the dorsal region of the cloaca (Ackerman and Knouff, 1964). After the fifteenth day of hatching lymphocytes appear and the organ develops into a typical lymphoid organ (Ackerman and Knouff, 1964).

Hormonal treatment of the embryo or surgical bursectomy immediately after hatching results in the complete loss of the bird's capacity to produce antibody against antigens without reducing its ability to reject tissue allografts (Glick et al, 1956). Although antibody producing cells are absent in the bursa (Dent and Good, 1965) these cells have the inherent capacity to transfer antibody production to irradiated birds (Gilmour et al, 1970) thus suggesting that this organ contains precursors of antibody forming cells. As with the thymus, the bursa in birds is considered to be a primary lymphoid organ responsible for providing the precursor cells which ultimately are responsible for the effects of humoral immunity.

Although no organ equivalent to the bursa has been defined in mammals, it is possible that the gut epithelium produces some factor that controls the differentiation of antibody-forming precursor cells (Cooper et al, 1966; Good and Finstad, 1971). It is commonly considered

that the bone marrow serves as the ultimate source of stem cells in the animal (Ford, 1966; Miller and Mitchell, 1969) and also as the bursa equivalent as previously discussed.

Thus two types of cells appear to be involved in the immune response; they arise from different primary lymphoid organs, and they are located in certain anatomical sites in the secondary organs. Such cells form the histological matrix of the immune response. Although no definite histological structures separate these cells in the secondary organs, they are separated on a functional basis. These cells will be referred throughout this thesis as T cells - thymus or thymus derived cells and B cells - bone marrow or bone marrow derived cells. Other characteristics distinguishing B and T cells will be discussed later.

II. CELL TO CELL INTERACTION IN HUMORAL IMMUNE RESPONSES

"Antibody production is a complex series of events beginning with the interaction of the antigen and ending with the production of specific antibody" (Claman and Chaperon, 1969). It is well established that the cells making antibodies belong exclusively to the lymphoid system. What is most crucial is whether the cell that responds to the antigen is the cell that makes the antibody. The preceding section described the existence of two cell types which are responsible for different manifestations of immunity. Division of these immunocompetent lymphocytes into T and B cells allows one to study immunological phenomena in terms of the participating cell types.

This section will demonstrate that these distinct cell lines not only perform different roles in the generation of different forms of

immunity but also interact synergistically for manifestation of immunity.

The initial suggestion for the requirement of T and B cell interaction in eliciting antibody responses arose from studies in neonatally thymectomized mice in which not only was cellular immunity affected but there was also depression of the antibody response to such antigens as sheep erythrocytes (SRBC) and foreign serum proteins (Miller, 1961; Miller, 1962). Moreover implantation of a thymus graft restored this defect to normal (Miller, 1961; Dalmasso et al, 1963; Taylor, 1963; Leuchars et al, 1965). Furthermore lethally irradiated mice reconstituted with syngeneic bone marrow recover immunocompetence only in the presence of the thymus (Miller et al, 1964). This was the first demonstration that a T cell or T cell component was required for full expression of immunocompetence.

Claman and his colleagues (1966) provided the first direct demonstration of cell to cell interaction in the humoral immune response to SRBC in mice. They observed that while transfer of marrow or thymus cells alone to lethally irradiated mice did not induce significant antibody production when challenged by antigen, transfer of a mixture of both cell types resulted in significant antibody production. Claman and Chaperon (1969) later demonstrated the radiosensitivity of the transferred cells.

In 1968, investigators in Australia published results which provided the first clear elucidation of specific cell interaction in the humoral immune response (Miller and Mitchell, 1968; Mitchell and Miller, 1968b; Nossal et al, 1968). They observed that transfer of syngeneic thymus or thoracic duct cells to neonatally thymectomized mice restored

the response to SRBC to normal levels (Miller and Mitchell, 1968). Employing either anti-H2 isoantisera (Miller and Mitchell, 1968) or the T6 chromosome marker (Nossal et al, 1968) these authors demonstrated that the hemolysin response of neonatally thymectomized mice given thymus or thoracic duct cells reflects antibody production by host cells.

The requirement for viable syngeneic thymus cells for synergism with marrow cells was demonstrated by Claman and his colleagues (1968, 1969). That is, sonicated, X-irradiated or minced thymus cells could not interact synergistically with marrow cells.

Cell to cell interaction between thymus and marrow cells has also been described in responses to bovine serum albumin (BSA) (Taylor, 1969) and human gamma globulin (HGG) (Chiller et al, 1970).

Cell interaction in vitro has been investigated utilizing tissue culture techniques (Mishell and Dutton, 1967; Marbrook, 1967). It has been recognized however that thymus and marrow cells must be cultured separately for a few days in the spleens of irradiated mice before transfer to the tissue culture system for efficient synergism to be achieved (Dutton et al, 1971a).

Thus the most important points arising from these studies are: 1) cell interactions between T and B cells do occur in vivo and in vitro and 2) B cells are the precursors of antibody forming cells.

The ability of T and B cells to interact synergistically in the immune response has opened the door to the application of these interactions to various immunological phenomena.

Although the carrier effect was first described in 1967 (Benacerraf et al, 1967; Rajewsky and Rottlander, 1967), cell to cell interaction

was not implicated until 1969 (Mitchison, 1969). Mitchison (1969) adoptively transferred spleen cells from animals immunized with a hapten carrier conjugate. He subsequently challenged these animals with the same conjugate or a conjugate consisting of the same hapten but an unrelated carrier. He observed that a response to the hapten was obtained only with the immunizing conjugate. However he could restore the response to the hapten-unrelated carrier conjugate by the addition of cells from another mouse primed to the heterologous carrier alone. This was the first demonstration that different cells were responding to the carrier and to the hapten; both responses being required for antihapten antibody to be elicited. This basic in vivo observation has been confirmed by many investigators (Mitchison et al, 1971; Hamaoka et al, 1971; Miller et al, 1971; Roelants and Askonas, 1971).

Raff (1970a) subsequently demonstrated that these carrier specific or helper cells are thymus derived cells as suggested by their sensitivity to anti-θ serum.

These studies clearly establish the operation of distinct recognition units for carrier and haptenic determinants corresponding to T and B cells respectively.

Synergism between T and B cells has also been described for immunological memory (Miller and Mitchell, 1967; Chan et al, 1970; Jacobson et al, 1970; Jehn and Karlin, 1971; Takahashi et al, 1971), antigenic competition (Gershon and Kondo, 1971a, 1971b) and tolerance (Taylor, 1968, 1969; Playfair, 1969; Chiller et al, 1970, 1971) and will be discussed in more detail in a later section. It is therefore evident that collaboration between thymus derived and bone marrow derived cells

is essential to many immune responses.

Thymus derived cells are stimulated by antigen to undergo mitosis (Leuchars et al, 1964; Davies et al, 1966; Koller et al, 1966; Miller et al, 1966) although they are incapable of secreting antibody (Kennedy et al, 1965; Claman et al, 1966; Davies et al, 1967; Mitchell and Miller, 1968a; Nossal et al, 1968). On the other hand, the bone marrow derived cell is the antibody secreting cell precursor but requires interaction with the thymus derived cell in order to perform its function (Miller and Mitchell, 1968; Mitchell and Miller, 1968a; Nossal et al, 1968; Miller and Mitchell, 1969; Davies, 1969; Claman and Chaperon, 1969).

It is conceivable therefore that thymus derived cells may subserve some non-specific ancillary function or may after specific interaction with antigen, elaborate a non-specific pharmacological factor which in some way facilitates the recruitment of antibody-forming cell precursors.

Before trying to solve this problem it is more important to define which cells recognize antigen and how this recognition is achieved. That is, it is necessary to define what structures on the surface of lymphoid cells are responsible for recognition of antigens and how this recognition is translated into the subsequent immune response.

III. SITE OF ANTIGEN SPECIFICITY

It is evident from the above discussions that the participation of two functionally distinct lymphoid populations is a prerequisite for induction of many humoral immune responses. This raises the question of the immunological specificity of these cell types. This section therefore is concerned with the site of antigen specificity and with

the recognition structures (receptors) responsible for this specificity.

i. Antigen binding cells

Preceding initiation of an immune response, antigen must be recognized by those cells (specific) which are responsible for triggering the chain of events which will eventually lead to humoral antibody synthesis. These precursor cells which presumably bind the antigen are called antigen binding cells (ABC). Such cells can be described as antigen sensitive or immunologically competent cells in the normal or primed cell populations but such properties may not necessarily apply in situations of immunological unresponsiveness.

Various techniques have been developed to observe antigen binding and localization of antigen binding receptors on the outer surface of lymphoid cells. The antigen can be rendered visible by tagging it with a radioactive isotope (Naor and Sulitzeanu, 1967; Byrt and Ada, 1969) or a fluorescent dye (Witten et al, 1963; Martins et al, 1965). Enzymes can also be used as antigens. These enzymes retain their activity after attachment and the subsequent enzymatic reaction identifies them (Sercarz et al, 1971).

Two main types of cytoadherence techniques have been employed to detect antigen binding cells - the bacterial adherence test (Makela and Nossal, 1961a, 1961b) and the rosette cell technique (Nota et al, 1964; Zaalberg, 1964). In this system, lymphoid cells bind particulate antigens to their surface and form rosettes.

The various techniques described above have provided the following observations:

a) Only a very small proportion of cells selectively bind the antigen (Naor and Sulitzeanu, 1967; Herzenberg et al, 1967; Byrt and

Ada, 1969; Sercarz et al, 1971).

b) Different cells bind different antigens (Sulitzeanu and Naor, 1969a).

c) The degree of antigen uptake depends on the antigen as well as the species of test animal (Byrt and Ada, 1969; Dwyer et al, 1971). That this may be a reflection in the absolute number of immunocompetent cells for that particular antigen has been suggested (Ada, 1970).

d) Binding of antigen to the cells is immunologically specific. That is, only 'cold' homologous antigen can inhibit binding (Baker et al, 1966; Herzenberg et al, 1967; Naor and Sulitzeanu, 1969a; Byrt and Ada, 1969; Howard et al, 1969).

e) Morphologically these cells are distinguished as lymphocyte-like cells (Pavlovsky et al, 1967; Storb et al, 1967a, 1967b, 1969; Byrt and Ada, 1969; Sulitzeanu and Naor, 1969; Biozzi et al, 1968; Duffus and Allan, 1969; Perrudet-Badoux and Frei, 1969; Chetter et al, 1969; Brain et al, 1970).

f) Antigen binding cells are observed in athymic mice (Dwyer et al, 1971) and in responders and nonresponders (Dunham et al, 1972).

g) Specificity of hapten protein conjugate antigen binding cells is largely directed towards the hapten (Davie and Paul, 1971a; Golan and Borel, 1972).

h) Antigen binding cells have been observed in species other than mice - humans (Witten et al, 1963; Martins et al, 1965; Dwyer and Mackay, 1970; Merler and Silbersmidt, 1972), rabbits (Herzenberg et al, 1967), chickens (Dwyer and Warner, 1971) and guinea pigs (Davie and Paul, 1971a).

i) Immunization leads to a selective increase of antigen binding cells to the immunizing antigen (Biozzi et al, 1968; Naor and Sulitzeanu, 1969a; Ada, 1970; Davie et al, 1971b; Dunham et al, 1972). This is correlated with an increase in the avidity of the cell receptor (Davie and Paul, 1972a).

j) Antigen receptors are present on antibody secreting cells in the early stages of the immune response but are subsequently lost (McConnell, 1971).

k) Conflicting reports exist as to whether tolerance induction leads to a selective depression of antigen binding cells (Naor and Sulitzeanu, 1969b; Humphrey and Keller, 1970) or a selective increase (Howard et al, 1969; Sjöberg and Möller, 1970; Sjöberg, 1971; Möller and Sjöberg, 1972).

l) No thymus derived antigen binding cells are observed in tolerant mice (Howard et al, 1969; Sjöberg, 1971; Howard, 1972; Argyris et al, 1972).

Thus various techniques have been described to detect antigen binding cells in normal, immune and tolerant animals. Although there is a difference in the sensitivities of these techniques, the same basic results have been observed as discussed above.

In conclusion, it appears that lymphocytes bind antigens by an immunologically specific process. The structures by which these cells recognize and bind antigen is the basis for the following section.

ii. Antigen receptors on T and B cells

In accordance with the clonal selection theory (Burnet, 1959), it was observed that only a small proportion of the total cell population is capable of binding any particular antigen. If one assumes that these

cells specifically bind antigen through antigen receptors, it becomes of vital importance to define the cell types binding the antigen.

The failure of most preparations of thymus cells to bind antigen in comparison to spleen cells (Byrt and Ada, 1969; Humphrey and Keller, 1970) suggests that it is the B cells which bind antigen. Furthermore, thymus independent antigens such as pneumococcal polysaccharide (SIII), endotoxin (LPS), polymerized flagellin (POL) and a synthetic polypeptide antigen (TIGAL) have few if any detectable thymus derived antigen binding cells (Raff, 1971).

I have previously mentioned that thymus derived cells respond mitotically to antigen and collaborate with B cells in many humoral immune responses. Since these cells must recognize antigen in order to respond it is only logical to assume that they also have antigen receptors.

Although little evidence suggests that T cells have receptors for antigen, there is some evidence which suggests that such receptors are present but at a density much lower than that of B cells.

Inhibition of spontaneous RFC formation by anti-θ antiserum suggests the presence of antigen receptors on virgin murine thymus derived cells (Greaves and Möller, 1970; Bach et al, 1971). These cells have been ascribed to the hydrocortisone resistant pool of thymocytes (Bach and Dardenne, 1972).

Thymus dependent antigens such as SRBC, MSH and BSA appear to have a proportion of ABC which are thymus derived as suggested by their sensitivity to anti-θ serum (Raff, 1971).

Antigen binding cells have been observed in fetal and mature thymus lymphocytes (Dwyer et al, 1972) and also in humans (Jondel et al,

1972; Fröland, 1972a).

Indeed a controversy exists as to whether thymus derived cells have antigen receptors. A receptor density difference between thymus derived and non-thymus derived cells coupled with differences in the sensitivities of the techniques employed may help explain the discrepancies cited above. There is, however, general agreement that B cells have structures on their surfaces called antigen receptors or antigen recognition structures.

iii. Functional activity of antigen binding lymphocytes

The evidence presented above clearly demonstrates that antigen is bound by lymphoid cells and that this reaction is immunologically specific. This raises the question concerning the functional activity of those cells which selectively bind antigen. This section will deal with defining such functional activity.

One method of studying the function of ABC is performed utilizing an antigen suicide technique (Ada and Byrt, 1969; Basten et al, 1971; Golen and Borel, 1972). Treatment of cells with very 'hot' radioactively labeled antigen in vitro at $4^{\circ}C$ selectively inactivates those lymphocytes to which the antigen is bound. Ada and Byrt (1969) and Unanue (1971a) have demonstrated that the subsequent adoptive immune response to that antigen is either reduced or abolished with no effect on the potential immune reactivity to other antigens. Both T and B cells have been demonstrated to dictate the specificity of the antibody response (Basten et al, 1971). Unanue (1971b) has found that inactivation of bone marrow ABC reduces the immune response, in contrast to that found by others (Basten et al, 1971).

Depletion of spontaneous RFC against an antigen specifically

abrogates the subsequent immune response to that antigen (Bach et al, 1970).

Columns of glass or plastic beads coated with antigen have also been used to deplete ABC. Specific elimination of immune cells by passage through antigen coated columns leading to a partial or complete abolition of specific immune reactivity of the filtered cell population allows one to determine the functional activity of these cells. Such an application of this technique has led to the demonstration that antibody-forming cells, memory cells (Wigzell and Andersson, 1969a, 1969b; Wigzell and Mäkelä, 1970), immunologically reactive cells in normal bone marrow (Singhal and Wigzell, 1969, 1970; Abdou and Richter, 1969) and potential antibody-forming cells (Wigzell and Mäkelä, 1970) express their capacity through the representation of antigen receptors on their surface.

These reports clearly establish that lymphocytes which selectively bind antigen are functional in either memory or humoral antibody production. Removal of these cells from an immunocompetent cell population by any one of a variety of ways selectively depletes this population from responding to that antigen.

iv. Mechanism of antigen binding to lymphocytes

Having established that lymphoid cells do selectively bind antigen and that this binding is involved with specific function, the question is raised on the mechanism of binding of antigen to the lymphocyte surface.

The clonal selection theory of Burnet (1959) advocates that antigenic specificity of lymphocytes develops by a selective process during the embryonic stage of development. During this time only those speci-

ficities not associated with self are allowed to develop in the lymphoid system. Recognition of antigens presumably takes place by receptors displayed on the lymphocyte surface. Jerne (1967) and Mitchison (1967) have logically concluded on the basis of the exquisite specificity of the immune system that only antibody recognizes antigen. Therefore, the receptor for antigen on the virgin lymphocyte should be immunoglobulin. If this is the case, then it should be possible to inhibit antigen binding by the use of anti-immunoglobulin reagents.

Lymphocytes which bind 4-hydroxy-3, 5-dinitrophenylacetyl (NNP) hapten or endotoxin from E.coli (LPS) are inhibited from binding the antigen by preincubation of the cells with anti-immunoglobulins (Möller and Sjöberg, 1972).

Inhibition of RFC formation against SRBC by anti-Ig antisera (Biozzi et al, 1967; Zaalberg et al, 1968; McConnell et al, 1969) suggests not only the immunoglobulin nature of the receptor but also that specific binding of antigen to the cell surface receptor Ig occurred. Anti-Fab and anti-Fc sera inhibit rosette formation to varying degrees (Biozzi et al, 1967) thus suggesting the presence of Fc and Fab fragments on the cell surface.

Addition of a rabbit anti-mouse Ig polyvalent antiserum to mouse lymphoid cells prior to radioactive antigen treatment significantly reduces the number of antigen binding cells to 10% of the control values (Byrt and Ada, 1969). Class specific anti-IgM antisera consistently reduces the number of antigen binding cells. Anti-light chain antisera have a similar effect as that of the polyvalent antisera (Ada et al, 1970). However, anti-Ig antisera are unable to inhibit antigen binding by bone marrow cells, thus suggesting the non-specific

binding by these cells (Byrt and Ada, 1969; Ada et al, 1970).

Inhibition of binding of immune cells on antigen coated columns occurs in the presence of anti-Igs (Walters and Wigzell, 1970). Walters and Wigzell (1970) and Wigzell (1970) have demonstrated that the surface bound receptor for antigen has the same heavy chain and probably light chain as will be present in the humoral antibody eventually produced by that cell.

Although this inhibition of antigen binding by anti-Igs could be the result of steric hindrance of the antigen binding site, it is likely that the anti-Ig and the antigen are competing for the same receptor. It is therefore likely that these immunoglobulin determinants on the lymphocyte membrane are the antigen recognition structures and are probably involved in the induction of humoral immune responses to that antigen.

v. Antigen receptors versus surface immunoglobulins

As previously discussed, anti-immunoglobulins have the ability to react with the lymphocyte and inhibit antigen binding. Antigen binding cells have also been shown to be specifically functional cells. Therefore, if the antigen receptor is immunoglobulin, not only would pretreatment of cells inhibit antigen binding but this should also result in the inhibition of a subsequent humoral response or cell mediated immunity to any antigen.

This section is designed to demonstrate that surface immunoglobulin and antigen receptors are indeed one and the same by examining the functional activities of cells which have been exposed to anti-immunoglobulins.

1. In vitro

The first demonstration of the presence of functional surface associated Igs was reported by Mitchison (1967). Treatment of cells immunized to a hapten-protein conjugate with either anti-Fab or anti-Fc sera drastically inhibited a secondary in vitro response. This result has been confirmed for a secondary response to keyhole limpet hemocyanin (KLH) (Vischer and Jaquet, 1972). Anti-Ig sera (Fuji and Jerne, 1969) and anti-K sera (Lesley and Dutton, 1970) but not anti-Fc sera (Hartmann et al, 1971) are able to inhibit the primary response of mice to SRBC. This is in contrast to results obtained by Sjöberg and Greaves (1971) in which only anti-IgM was capable of suppressing the primary and secondary responses to SRBC. This is, however, in agreement with the results of other investigators (Warner et al, 1970).

In vitro suppression of the secondary immune response in rabbits by anti-Igs (Daguillard and Richter, 1970), antiallotype sera (Mond et al, 1972) and anti-H chain sera (Kishimoto et al, 1971) serve to demonstrate the presence of functional immunoglobulin molecules on the surface of lymphoid cells.

Although reports exist on the failure of anti-Ig sera to inhibit such cell mediated reactions as graft versus host reactions (Sternberg, 1970; Ivanyi et al, 1970) other reports suggest that pretreatment of lymphoid cells with anti-Ig sera can diminish the GvH reaction when these cells are injected into appropriate hosts (Cole and Maki, 1971). Mason and Warner (1970) have reported that only anti-L chain sera suppresses GvH responses or the transfer of delayed hypersensitivity. On the other hand, others have found that only univalent fragments of anti-Igs are effective at suppressing these cell mediated reactions

(Riethmuller et al, 1971).

Pretreatment of human lymphocytes with the Fab monomer of rabbit antisera to human L chain determinants effectively suppresses the tuberculin response and mixed lymphocyte reaction (MLR) (Greaves et al, 1969; Greaves, 1970; Greaves et al, 1971; Tyan and Ness, 1972) without affecting transformation by PHA (Greaves et al, 1969).

There is general agreement that those cells responsible for in vitro humoral antibody synthesis display surface immunoglobulins and can be inhibited by anti-Igs. However, the results concerning in vitro cell mediated immunity are of a controversial nature.

More compelling evidence to suggest that the antigen receptor and surface immunoglobulin are the same is shown by the ability of anti-Igs to mimic the antigen in its ability to induce transformation and mitosis of treated lymphoid cells.

Lymphocyte transformation occurs in rabbit peripheral lymphocytes when using heterologous antiserum to whole rabbit serum (Sell et al, 1965; Daguillard and Richter, 1970), to specific immunoglobulins (Sell, 1967a) or their subunits (Sell, 1967b). Gell (1968) has observed that no immunoglobulin production occurs when cells are transformed by these reagents.

Homologous antisera directed towards heavy chain or light chain allotypic determinants are also mitogenic for rabbit lymphocytes in tissue culture (Sell and Gell, 1965a; Gell and Sell, 1965; Sell, 1970). Sell and his colleagues (1970a) have ruled out passive absorption of these determinants by extensive investigations.

A second anti-allotypic serum directed against the primary stimulating anti-allotypic serum induces an enhanced degree of blast trans-

formation in rabbits (Sell et al, 1970b). This suggests that the induction of lymphocytic transformation may require the close approximation of two antibody molecules reacting with a given lymphocyte. Mixtures of anti-allotypic sera induce a greater degree of blast transformation than the sum of the responses that can be obtained when using each anti-allotypic serum separately (Sell et al, 1970c). This implies that the expression of allotypic specificities by lymphocytes is not limited to one allele. This data is not consistent with the phenomenon of allelic exclusion by lymphocytes.

By inducing blast transformation of lymphocytes from newborn rabbits by an anti-allotypic serum to the paternal IgG allotype not present in the serum of the lymphocyte donor, Sell and Gell (1965b) have demonstrated that the allotypic specificity is not conferred upon lymphocytes by environmental IgG.

Marcuson and Roitt (1969) have morphologically examined anti-allotype transformed lymphocytes and have observed a cell type with free cytoplasmic ribosomes and no rough endoplasmic reticulum. The probable presence of deposited complexes of antiserum and antigen was observed around and in the transformed cells.

By investigating the appearance of anti-allotypic reactive cells after birth, Kaplan and Thorbecke (1970) have shown that this reactivity appears in peripheral lymphocyte organs during the process of maturation of the immune apparatus after birth.

Lymphocytes from other species including human beings (Oppenheim et al, 1969; Daguillard et al, 1969) and chickens (Skamene and Ivanyi, 1969; Alm and Peterson, 1969) are able to respond mitotically to anti-immunoglobulin reagents although to a lesser degree.

2. In vivo

If immunoglobulin molecules are the receptors for antigen, injection of anti-Igs in vivo would be expected to alter serum immunoglobulin levels as well as the ability of the host cells to respond to antigen by either a humoral response or by cell mediated immunity. Thus one can use in vivo inhibition of these responses as a measure of the functional importance of surface immunoglobulins.

Injection of anti-μ chain antisera into neonatal mice results in suppression of IgM production and also reduces the serum levels of other Igs (Manning and Jutila, 1972a; 1972b). Anti-variable L chain sera injected into newborn animals leads to suppression as measured by a reduction in the rate of synthesis of these Igs which reacted with the injected antiserum (Lawton et al, 1972). Administration of anti-μ antisera to newborn mice at birth depresses the level of antibody forming cells in the secondary lymphoid organs (Lawton et al, 1972).

Injection of anti-idiotypic antibody (Hart et al, 1972) or anti-allotypic antibody (Herzenberg et al, 1967) results in complete suppression of the production of only that particular immunoglobulin. Anti-allotypic suppression in mice is usually short lived when compared to the chronic suppression observed in rabbits (Mage and Dray, 1965; Dubiski, 1967) although utilizing different strains Jacobson and Herzenberg (1972a) have reported chronic suppression in mice. Chronic allotypic suppression is considered to be actively maintained by cells which are resident in the lymphoid organs (Jacobson et al, 1972b).

Administration of anti-μ antiserum to chickens during embryonation together with bursectomy and additional anti-μ at hatching results in agammaglobulinemia with no detectable circulating IgM or IgG (Kincade

et al, 1970).

These in vivo and in vitro studies implicate surface associated immunoglobulins in immunological reactions. There is little doubt that surface associated Ig is the antigen receptor and that it is an antibody to the antigen to which it has been precommitted. These reports also suggest that the presence of surface immunoglobulins is a property of B cells and very few if any T cells display detectable surface immunoglobulin.

IV. STRUCTURAL DIFFERENCES CHARACTERIZING T AND B CELLS

To allocate immunological reactions according to cell types involved requires the development of a system of detection markers for these cells. This section will concern itself with defining such markers on T and B cells. It should be noted that only those widely used surface markers will be discussed.

i. Bone marrow derived cells

1. Immunoglobulin

As previously described, surface immunoglobulin molecules appear to be found predominantly on B cells. Thus detection of the Ig by a variety of techniques would serve as a valid marker for identifying the B cell and also in defining its distribution in an animal's lymphoid system.

Direct demonstration of surface Ig molecules and thus of the identification of B cells is attributed to the visualization of anti-Ig treated cells by various indicator systems. The most direct approach is the use of anti-Igs labeled with either fluorescein (Raff, 1970b;

Pernis et al, 1970) or radioiodine (Raff et al, 1970c; Rabellino et al, 1971; Bankhurst and Warner, 1971). A mixed agglutination technique has also been used with the formation of rosettes (Coombs et al, 1969) and the reverse immune cytoadherence technique utilizes a hybrid antibody erythrocyte detection system (Paraskevas et al, 1970, 1971a, 1971b).

Fluorescein or radioactively labeled anti-Ig antisera have been successfully used on living cells to demonstrate that the cells in the mouse which carry surface Ig are composed mainly of bone marrow derived cells (Raff, 1970b; Raff et al, 1970c; Unanue et al, 1971; Rabellino et al, 1971; Bankhurst et al, 1971; Perkins et al, 1972) although by varying the conditions of the technique, it has been possible to detect thymus derived cells (Bankhurst et al, 1971; Nossal et al, 1972).

The cells which stain positive are morphologically described as lymphocytes (Raff et al, 1970c; Perkins et al, 1972). Whether plasma cells carry surface Ig by this technique is at present controversial (Rabellino et al, 1971; Perkins et al, 1972).

By the use of class specific antisera it has been demonstrated that some B lymphocytes display multiple heavy chains on their surface (Bankhurst and Warner, 1971; Bankhurst et al, 1971; Nossal et al, 1972) although other investigators have failed to detect these multipotent cells (Rabellino et al, 1971).

Similar results have been obtained in the rabbit (Pernis et al, 1970; Jones et al, 1970; Davie et al, 1971), chicken (Rabellino and Grey, 1971; Kincade et al, 1971; Bankhurst et al, 1972) and human (Fröland et al, 1971; Pernis et al, 1971; Abdou, 1971; Grey et al, 1971; Hijmans and Schart, 1972; Fröland and Natvig, 1972b, 1972c; Van Boxel et al, 1972) with the proportion of positive cells differing to some

degree in each species.

Another method to detect the presence of Igs on lymphocytes is provided by the mixed anti-globulin reaction (Coombs et al, 1969) in which rosettes are formed around lymphocytes pretreated with anti-Ig and Ig coated erythrocytes. A high frequency of Ig carrying lymphocytes is found in the rabbit (Chalmers et al, 1959) in contrast to the lower figures for human peripheral blood lymphocytes (Coombs et al, 1969). Again lymphocytes are observed which appear to be multipotent (Coombs et al, 1969).

Another technique similar to that described above is the reverse immune cytoadherence technique (RICA) (Paraskevas et al, 1970, 1971a, 1971b) which utilizes a 5S hybrid antibody to form a bridge between the lymphocyte surface Ig and the protein coated SRBC. Two populations of lymphoid cells, one carrying and one lacking surface Igs, are distinguished by this technique in the lymphoid organs of mice, guinea pigs and man (Paraskevas et al, 1971a, 1971b). Thymus cells are not detected as carrying surface Ig by this technique (Paraskevas et al, 1971b). Mature normal plasma cells as well as plasmacytoma cells lack surface associated immunoglobulin (Paraskevas et al, 1970, 1971a, 1971b) and is in agreement with others (Pernis et al, 1970; Perkins et al, 1972). Utilizing highly specific hybrid antibodies, the majority of immunoglobulin carrying mouse spleen cells were shown to be pluripotential (Paraskevas et al, 1971b; Lee et al, 1971) and agrees well with the results of other investigators (Bankhurst and Warner, 1971; Bankhurst et al, 1971; Nossal et al, 1972) although the absolute numbers are higher utilizing the above technique.

This large volume of existing data not only suggests that surface

immunoglobulin is a valid B cell marker but also demonstrates that a large number of different techniques can be employed to detect this marker.

2. Complement receptor

Lay and Nussenzweig (1968) described a certain population of mammalian lymphocytes which are capable of binding Ag-Ab-C complexes through a membrane receptor for a modified complement component. These cells are termed complement receptor lymphocytes (CRL).

These lymphoid cells have been shown to comprehend most if not all B lymphocytes and do not contain or contain very few thymus derived lymphocytes (Lay and Nussenzweig, 1968; Bianco et al, 1970, 1971; Dukor et al, 1970, 1971; Bianco and Nussenzweig, 1971).

Bianco and Nussenzweig (1971) have demonstrated that although receptors for complement and membrane bound Ig coexist on the same cell, that these markers are located at different sites on the membrane.

The ability of lymphocytes to bind Ag-Ab-C complexes is the basis for the development of a method to purify CRL from a mixed population of cells. Initially rosettes are formed by exposing lymphocytes to SRBC-antiSRBC and complement complexes. Rosettes are separated from free cells by sedimentation in a BSA gradient. Rosette formation is reversed by anti-C3 antibody and the pure CRL are collected by differential flotation in BSA.

Such a receptor has been successfully used to classify the origin of cells during the neoplastic process in lymphoid organs (Shevach et al, 1972).

3. Fc receptor

A receptor for the Fc fragment of antibody or antigen-antibody complexes has recently been described on the surface of mouse B lymphocytes (Paraskevas et al, 1971b; Miller et al, 1971; Paraskevas et al, 1972a, 1972b; Basten et al, 1972a, 1972b, 1972c). This Fc receptor is detected either by a radioautographic technique in which lymphoid cells are incubated with antibody (Basten et al, 1972b) or by the interference of detection of surface immunoglobulin (Paraskevas et al, 1971b, 1972a). This process is complement independent and the failure of anti-mouse Ig F(ab)2 fragments to prevent access of antibody to B cells implies that the Fc and Ig receptor are two separate entities on the B cell membrane (Basten et al, 1972c).

Employing this marker on B lymphocytes, Basten and his colleagues (1972c) have been successful at separating B cells from T cells in normal and immune mice by passage through antigen coated columns after treatment of the cells with antibody. Furthermore, Paraskevas and his coworkers (1972b) have demonstrated that the Fc receptor is occupied on B cells six hours after immunization, thus suggesting a possible biological significance for this receptor.

Thus three widely used markers have been described for distinguishing bone marrow derived cells from thymus derived cells. These techniques have the advantage of being easily applied and appear specific for the detection of B cells.

ii. Thymus derived cells

Although various T cell specific antigens have been described, this discussion will deal only with the surface marker theta (θ) since

this is the most widely used and accepted marker for T lymphocytes.

Employing dye exclusion cytotoxicity tests, Reif and Allen (1963, 1964) demonstrated that an antiserum to a θ alloantigen on thymocytes reacts with thymus cells and a certain proportion of the cells in the secondary lymphoid organs. They subsequently observed that this antigen is under the control of a single locus with two alleles: θ-AKR in AKR, RF and a few related substrains and θ-C3H found in most other inbred strains of mice (Reif and Allen, 1966).

Immunization of AKR with C3H thymocytes or vice versa results in an antiserum which, although not monospecific, detects only anti-θ activity (Raff, 1972). Utilization of this serum has demonstrated that thymocytes have more θ on their surface than do peripheral lymphocytes (Aoki et al, 1969).

Evidence that θ can serve as a marker for T lymphocytes is summarized below:

a) all thymus lymphocytes can be killed by anti-θ serum and complement while only a proportion of lymph node and spleen lymphocytes can be so killed (Raff, 1969).

b) the percentage of θ positive cells is markedly decreased in peripheral lymphoid tissues of mice treated with ALS (Schlesinger and Yron, 1969; Raff, 1969), neonatally thymectomized (Schlesinger and Yron, 1970; Raff and Wortis, 1970), thymectomized as adults, lethally irradiated and reconstituted with bone marrow cells (Raff and Wortis, 1970) or nude mice (Raff and Wortis, 1970). These treated mice are all known to be depleted of lymphocytes in thymus dependent areas (Parrot et al, 1966; Davies et al, 1969; de Sousa et al, 1969).

Thus the evidence described above clearly demonstrates that anti-θ

serum can be used directly to study the distribution of T lymphocytes and can serve as a valid T cell marker.

V. NATURE AND MECHANISMS OF REGULATION BY ACTIVATED T CELLS

The preceding sections demonstrated that T and B cells interact synergistically in many humoral immune responses. Recognition of antigen by B cells is observed to occur through an immunoglobulin associated with the plasma membrane.

Another important question raised by the cooperation data is the nature and mechanism by which T cells cooperate with B cells. It is the purpose of this section to define the nature of the regulatory influence of activated T cells on B cell humoral responses as well as the proposed mechanisms of regulation.

It is well established that humoral immune responses can occur in the absence of detectable T cells to such antigens as pneumococcal polysaccharide (Humphrey et al, 1964; Davies et al, 1970; Howard et al, 1971), polymerized flagellin (Armstrong et al, 1969), E.coli polysaccharide (Andersson and Blomgren, 1971) and polyvinylpyrrolidone (Andersson and Blomgren, 1971). However, many other antigens require T cell participation for induction of an immune response. I will thus be concerned solely with responses that require T cells in an attempt to define the vast regulatory powers that T cells possess in regulating humoral antibody synthesis.

Thymus independent antigens such as those discussed above, are characterized by antibody responses consisting essentially of antibody of the IgM class (Baker et al, 1970; Britton and Moller, 1968). In

contrast, thymus dependent antigens induce antibodies predominantly of the IgG type. These findings strongly suggest that T cells provide some sort of selective pressure for the development of the IgG antibody response. Further support for this concept was provided when Miller and his coworkers (1971) observed a marked shift in the class of anti-hapten antibodies in mice preimmunized to the carrier alone. That is, while nonimmune mice stimulated with the conjugate develop an IgM response, mice preimmunized with the carrier develop a marked IgG response.

Some type of T cell regulation is also observed with regard to the affinity of antihapten antibodies. Gershon and Paul (1971) observed that the affinity of antihapten antibody produced is dependent on the nature of the carrier molecule as well as the number of T cells present in the immunized animal. These results strongly implicate a regulatory role of T cells in the selection of precursor B cells.

Thus evidence has been presented which appears to define two main functions of T cells: 1) modulating in some way the presentation of antigenic determinants to B cell receptors for appropriate immune induction to occur (IgM vs IgG responses), and 2) exerting of some selective pressure either directly or indirectly on the precursor B cell population (low affinity vs high affinity antibody).

Three main possibilities have been entertained in an attempt to define the mechanism of regulation by T cells of B cell function:

i. Antigen focusing

Mitchison (1969, 1971) proposed this theory as a possible explanation for the nature of T and B cell cooperation in humoral immune responses. This theory assumes a passive role for the specific T cell

whereby antigen is recognized, bound to the T cells, transported to areas of the lymphoid organs where B cells are located and then presented to the appropriate B cells at a concentration threshold where subsequent stimulation occurs. That is, T cells concentrate antigen on their surface for presentation to B cells. It is assumed that the T cells concentrate antigen via an IgX receptor (Mitchison, 1969).

Since T cells comprise the major portion of the recirculating small lymphocyte population (Miller and Sprent, 1971a) such a proposal appears attractive. However, such a theory requires T cell specificity for recognition and it is difficult to visualize the large numbers of T cells specific for any given antigen required to interact with specific B cells to effectively induce humoral antibody synthesis.

ii. Carrier antibody

Bretscher and Cohn (1968, 1970) postulated that humoral antibody formation involves the recognition of two determinants of an antigen; one by receptors on B cells and the other by free antibody or antibody carried on another cell. Generation of carrier antibody depends upon a thymus dependent process. Tolerance is the result of interaction of antigen only with the receptor antibody on the B cell.

A variation of this theory postulates that T cells bind antigen through monomeric IgM receptors (Feldmann, 1972). This interaction induces shedding of the complexes which are then picked up by macrophages to form a lattice of antigenic determinants. B cells are subsequently immunized by interaction with this matrix of determinants. Such a scheme is more compatible with current thinking since macrophages are not antigen specific and are relatively common. Thus interaction of specific B cells with macrophages is much more likely to

occur than that of specific T cells with specific B cells as suggested by Mitchison (1969, 1971).

iii. Mediators produced and secreted by T cells

The data to be presented in the main body of this thesis concerns itself with mediators released by T cells. This section, therefore, will be discussed in some detail as to the actual relevance of T cell mediators in regulation of B cell responses.

The hypothesis formulated for T cell mediators advocates that interaction of antigen with T cells results in a non-antigen specific diffusable mediator which exerts a regulatory role on B cells (Dutton et al, 1971b). Two signals are required for induction - one from the interaction of the B cell with antigen and the other from the reaction of the B cell with the soluble T cell mediator.

I will discuss below the various lines of evidence which have been reported in support of this concept. However, it should be noted that controversy exists as to whether the mediators released by T cells are actually specific or non-specific.

1. In vivo evidence for T cell mediators

This section will define various reports which have been published which indirectly suggest a possible involvement of T cell products in vivo in profoundly influencing the response of antibody forming precursors to antigenic stimulation.

Katz et al (1971a, 1971b). "The allogeneic effect". Guinea pigs previously primed with a hapten protein conjugate, when injected with allogeneic lymphoid cells, induce increased levels of antihapten and anticarrier antibodies. Furthermore, challenge of these animals with

a hapten conjugated to a heterologous carrier molecule results in a striking anamnestic response to the hapten.

Katz et al (1971c). The allogeneic effect is the result of a specific immunological attack of donor cells on cells of the host. That is, it is the result of a graft versus host reaction.

Wu and Cinader (1971). "Antigenic promotion". Preinjection of animals with structurally unrelated macromolecules enhances hapten specific antibody responses.

Kreth and Williamson (1971). Utilizing a double transfer system, they demonstrated the allogeneic effect on hapten specific mouse cell clones.

Katz and Osborne (1972). Demonstrated that the allogeneic effect operated in mice and that it could be elicited in an adoptive transfer response in inbred mice.

Osborne and Katz (1972). Demonstrated that the severity of the allogeneic effect is related to the number of allogeneic cells transferred as well as to the dose of heterologous conjugate used for secondary challenge. The magnitude of the effect also depends on the relative strengths of histoincompatibility differences.

The "allogeneic effect" as described above may be the result of (1) a general proliferative response within the host T cell population or (2) a facilitative effect of some sort on antibody forming cell precursors. The second explanation is most consistent with the above data. Furthermore, it has been suggested that these postulated soluble factors are nonspecific, most likely act rapidly and have a short half-life (Katz and Benacerraf, 1972).

2. In vitro evidence for soluble T cell mediators

Hartmann (1970). Addition of T cells primed in vivo to horse red blood cells to an in vitro culture system in the presence of both SRBC and HRBC restores the capacity of B cells to develop primary anti-SRBC responses.

Hirst and Dutton (1970). Addition of allogeneic spleen or thymus cells to cultures of neonatally thymectomized or anti-θ serum treated spleen cells enhances or restores the primary anti-SRBC response.

Kennedy et al (1970). Supernatant obtained from gentle heating of peritoneal exudate lymphocytes is active in permitting B cells to develop antibody responses upon adoptive transfer to irradiated recipient mice. This factor is specific since it is only active if obtained from donors specifically immunized to the antigen.

Dutton et al (1971b). A cell free supernatant obtained from cultures of allogeneic spleen cells has the ability to enhance in vitro anti-SRBC responses of normal and T cell deprived spleen cells.

Ekpaha-Mensah and Kennedy (1971). Enhancement of primary in vitro anti-SRBC responses of normal mouse spleen cells is observed with mixtures of allogeneic lymphoid cells separated on opposite sides of a nucleopore membrane.

Doria et al (1972). A cell free medium of thymus cell cultures is effective in restoration of the primary in vitro anti-SRBC response of spleen cells from neonatally thymectomized mice.

Gorczynski et al (1972). A nondialyzable antigen specific factor is released from cultured thymus cells (cultured in presence of SRBC) which restores the capacity of T cell depleted mouse spleen cells to develop primary in vitro anti-SRBC responses.

Britton (1972). Supernatants from cultured allogeneic spleen cells restores in vitro anti-SRBC responses of nonresponsive B spleen cells.

Schimpl and Wecker (1972). Cell free supernatants from cultures of allogeneic spleen cell mixtures restores in vitro 19S anti-SRBC responses of T cell depleted spleen cells and spleen cells from nude mice.

Rubin and Coons (1972a, 1972b). A cell free factor specifically released from primed thymus or spleen cells nonspecifically augments the immune response to an unrelated antigen. This factor is probably a protein, is nondialyzable and is heat stable.

Robey et al (1972). A soluble factor released from normal bovine thymus accelerates the production of specific hemolysin when administered to intact neonatal mice. This factor is 4S in size, heat stable and is probably a protein.

Feldmann and Basten (1972). Secondary in vitro anti-hapten responses of untreated or anti-θ treated conjugate primed cells is elicited with a heterologous conjugate by addition of heterologous carrier primed T cells together with the hapten primed cells or separated by a nucleopore filter.

Feldmann (1972). Macrophages cultured in the presence of activated T cells and antigen acquire the capacity to specifically induce antibody responses in B cell containing lymphoid populations.

Sjöberg et al (1972). Lipopolysaccharide from E.coli can reconstitute the immune response of spleen cells depleted of thymus derived lymphocytes. The immune response of nude mice is enhanced by addition of LPS.

Andersson et al (1972a). Humoral factors released by normal or

CON A activated thymus lymphocytes during 24 hours in culture, induce B cells to become competent to respond to CON A.

Andersson et al (1972b). Non-specific soluble factors released from a mixed lymphocyte culture can substitute for T cells in the in vitro induction of antibody synthesis.

Hunter and Kettman (1973). Cell free allogeneic supernatants stimulate the response of spleen cells from nude or adult thymectomized irradiated, bone marrow reconstituted mice to thymus dependent antigens.

Rosenthal et al (1973). Active supernatants prepared from sensitized cells with antigen are capable of stimulating gamma globulin synthesis and the antibody response of both sensitized and nonsensitized B cells.

Vann and Varrati (1973). Thymus cells activated to transplantation antigens, when added to spleen cells from cortisone treated mice, restores the PFC production to SRBC. Moreover, a cell free supernatant obtained from above, when added to cortisone treated spleen cells, also restores full PFC responses to SRBC.

Sjöberg et al (1973). T cell depleted spleen cultures, incapable by themselves to respond to SRBC, are reconstituted by CON A. CON A activated T cells can reconstitute the PFC response in T cell deficient cultures.

Vann and Galloway (1973). The in vitro responses of normal spleen cells to SRBC are significantly enhanced by addition of T cells reactive with the spleen cell histocompatibility antigens. Supernatants from cultures of T cells mixed with target cells also enhances the PFC response.

Schimpl and Wecker (1973). A T cell replacing factor from mixtures

of allogeneic spleen cells reconstitutes in vitro 7S IgG immune responses in T cell deprived spleen cell cultures from primed mice.

This large body of evidence is quite convincing in the implication of T cells in governing B cell responses. Furthermore, it appears that T cells are activated by antigens and release either specific and/or non-specific soluble mediators which can effectively replace the T cells in their capacity to elicit or augment an immune response.

VI. T AND B LYMPHOCYTE FUNCTION IN IMMUNOLOGICAL PHENOMENA

T and B cell interactions in generating humoral immune responses has placed great emphasis on the investigations and concepts of other specific immunological phenomena. This section will concern itself with defining different forms of immunity in terms of the involvement of T and B lymphocytes.

i. Immunological tolerance

The inability of an animal to respond to an immunogenic dose of antigen can be termed immunologic tolerance. Although various environmental and genetic factors contribute to the state of tolerance, this section will be concerned solely with the demonstration that both T and B cells can be rendered tolerant but that the kinetics of tolerance induction differs in these two cell types.

1. T cell tolerance

The importance of the thymus in unresponsiveness was first suggested by the observed prolongation of the unresponsive state to certain antigens in mice after thymectomy (Taylor, 1964). Isakovic

and his colleagues (1965) provided the first direct evidence for tolerance induction in T cells. They observed that thymus grafts from bovine gamma globulin (BGG) tolerant rats effectively transferred specific tolerance to thymectomized irradiated recipients given normal bone marrow. If normal thymus cells are given, the immune response is restored.

Mice rendered tolerant to SRBC display a reduced mitotic response to the antigen (Gershon et al, 1968) and the recirculating thoracic duct lymphocytes are specifically tolerant (Miller and Mitchell, 1970). Furthermore, Taylor (1968) demonstrated that T cells of mice rendered tolerant to BSA are unable to cooperate with normal B cells in an adoptive transfer response to irradiated recipients. The inability of carrier specific T cells to cooperate in secondary antihapten responses also serves as a measure of the unresponsive state in T cells (Paul et al, 1970).

2. B cell tolerance

T cell tolerance can be easily achieved and detected as suggested above. However, it is more difficult to establish the existence of specific tolerance in B cells.

Playfair (1969) reported unresponsiveness to SRBC in B cells but not T cells of adult mice injected with SRBC and treated with cyclophosphamide. However, the B cells are observed to be only transiently tolerant. Gershon and Kondo (1970) have reported that B cell tolerance can be achieved by repeated injections of SRBC. Tolerance induction to deaggregated HGG induces specific tolerance in both B and T cell populations of mice (Chiller et al, 1970).

Chiller and his colleagues (1971) observed a marked difference

between T and B lymphocytes with respect to both kinetics of tolerance induction and the dose of tolerogen required. T cell tolerance is induced early after introduction of tolerogen and is maintained for long periods of time. On the other hand, B cells exhibit tolerance at a much later time and recover from tolerance much earlier. Moreover, T cell tolerance occurs at much lower doses of tolerogen than B cell tolerance.

Thus both T and B cells can be rendered immunologically tolerant but the kinetics of tolerance induction in these two cell populations is quite different with regard to both time of induction and dose of tolerogen required.

ii. Immunological memory

When an animal first encounters an antigen, a state of altered responsiveness is induced and is referred to as immunological memory. This allows rapid production of antibodies as well as the production of greater quantities. Moreover, these antibodies are predominantly of the 7S class and are of higher affinity than those produced in the primary response (Eisen and Siskind, 1964).

The demonstration of specificity in both T and B cell populations may also suggest memory in these populations. That both T and B cell populations are capable of expressing memory will be shown below.

That T cells express immunological memory is based upon the following observations:

a) Thymus cells cultured in the spleen of an irradiated host for 7 days, cooperate with bone marrow cells only if they are stimulated with the antigen in both hosts but not if the first host receives a different antigen (Miller and Mitchell, 1967).

b) Utilizing a double transfer system response to antigen, it was demonstrated that thymus cells which are specifically activated in the primary host, can interact with bone marrow cells to produce a significant response. (Mitchell and Miller, 1968; Shearer and Cudkowicz, 1969).

c) Thymus cells stimulated by HRBC cooperate in the production of anti-SRBC antibody only if HRBC are given with the SRBC in an in vitro system (Hartmann, 1970).

d) The in vitro immune response of normal mouse spleen cells to SRBC is reduced dramatically by anti-θ serum and complement treatment. This response can be restored to control levels by educated thymus cells (Chan et al, 1970).

e) Specific elimination of T cells from the immune spleen results in loss of the ability of such cells to transfer the secondary response to SRBC (Takahashi et al, 1970).

f) In vivo indirect PFC response of spleen cells from primed mice is reduced by in vitro treatment of the cells with anti-θ serum in the presence of complement. This response is restored by thymus cells from normal mice (Mitchell et al, 1972).

Although T cell memory is relatively easy to establish, it was thought for many years that B cell memory was non-existent (Shearer and Cudkowicz, 1969; Cunningham, 1969). However, various investigations have established the existence of memory in B cells:

a) Using as T and B cell donors, congenic mice differing only at the loci coding for immunoglobulin allotype, it has been shown that all the IgG anti-SRBC PFC detected in an adoptive secondary response are of the B cell allotype (Jacobson et al, 1970).

b) Both thymocytes and bone marrow cells can adoptively transfer memory in the response to SRBC (Jehn and Karlin, 1971).

c) Elimination of B cells in immune spleen results in definite reduction of secondary responses (Takahashi et al, 1971).

d) The response in irradiated recipients of primed T cells to fowl IgG can only be enhanced by addition of fowl IgG primed B cells and not with unprimed B cells (Miller and Sprent, 1971b).

Thus a considerable amount of evidence has been presented in an attempt to establish the existence of immunological memory in both the T cell and the B cell population.

iii. Cell mediated immunity

The demonstration of T and B cell synergism in B cell funtions, such as humoral antibody synthesis, leads one to wonder about possible interactions in T cell functions - GvH reactions and delayed hypersensitivity.

Although very little evidence has been reported for T-B cell interaction in cell mediated responses, Globerson and Auerbach (1967) have observed that in vitro GvH reactivity occurs only in the presence of both thymocytes and bone marrow cells. However, T-T cell interaction appears to be more dominant in these reactions (Asofsky et al, 1971).

Although it is very hard to draw any conclusions at this time, the available evidence suggests that for cell mediated immunity, T-T cell interaction is more prevalent than T-B cell interaction.

iv. Immunological adjuvants

Man's desire to enhance immune responses to obtain larger quantities of antibodies for experimentation has led to the discovery of

substances known as adjuvants. These substances have the following properties:

a) convert a nonantigenic substance to an effective immunogen (Dresser and Mitchison, 1968)

b) increase levels of circulating antibody or lead to the production of more effective protective immunity (Freund, 1937)

c) increase cell mediated hypersensitivity (Boyden, 1964)

Adjuvanticity has been defined as the property of the antigen itself (intrinsic adjuvanticity) or some other substance administered with the antigen (extrinsic adjuvanticity) to cause induction of an immune response other than tolerance (Dresser, 1961). That is, an antigen may also be an adjuvant but in other cases it may require the addition of a substance which can enhance the immune response to the antigen.

Adjuvants cover a wide spectrum of substances as shown in Table I.

Although this table is far from complete, it serves to indicate the diversity of substances which can serve as adjuvants. It is not the purpose of this review to discuss all adjuvant observations but simply to describe certain fascinating observations in an attempt to demonstrate possible T cell regulation and the involvement of soluble factors in the adjuvant effect.

The ability to manipulate immune responses at will is desirable in such situations as transplantation of organs (stimulation of enhancing antibody) and rejection of tumor tissue (suppression of enhancing antibody). Therefore, the study of adjuvants is not only of academic interest but it is also of practical importance. However, not all adjuvants act in the same manner as exemplified by the observations

TABLE I

Commonly recognized adjuvants

ADJUVANT	REFERENCE
Bacterial endotoxins	Johnson et al, 1956
Freund's complete adjuvant	Freund, 1956
Freund's incomplete adjuvant	Freund, 1956
Alginate	Amies, 1959
Saponin	Johnson et al, 1963
Phospholipids	Weiss and Dubos, 1956
Quaternary ammonium compounds	Gall, 1966
Vitamin A	Dresser, 1968; Jurin and Tannock, 1972
Silica	Spitznagel and Allison, 1970
Bordetella pertussis	Fleming et al, 1948
Corynebacterium parvum	Neveu et al, 1964
Mycobacterium (Wax D)	White, 1967
Beryllium	Unanue et al, 1969
Anionic polyelectrolytes	Gall et al, 1972
Polynucleotides	Braun and Nekano, 1967 Johnson et al, 1968

that Freund's incomplete adjuvant favors viral oncogenesis (Goldner et al, 1965) while complete adjuvant suppresses the appearance of tumors after injection of adenovirus type 12 (Berman et al, 1967). Thus one has difficulty in defining mechanisms of action for adjuvants in general - as what applies to one adjuvant may not necessarily apply to another.

The main body of this thesis is concerned with a possible mode of action of FCA and thus this review will discuss in some detail observations related to the action of FCA. Similarly, important observations for other well known adjuvants will also be discussed in terms of mechanism of action and the cell types affected by the adjuvant.

1. Freund's adjuvants

Incorporation of antigen into a water-in-oil emulsion results in a marked adjuvant effect characterized by a retardation in the local destruction and elimination of the antigen (Halbert et al, 1946). The addition of mycobacteria to this emulsion results in a further marked increase in antibody levels (Fischel et al, 1952). Furthermore, several dramatic changes in the immunological response are observed. There is a striking increase in delayed-type hypersensitivity (Boyden, 1964) and synthesis of different immunoglobulins (White et al, 1963).

That FCA induces immunoglobulin with special properties is suggested by the following reports. Administration of FCA alone into rabbits causes a sharp rise in immunoglobulin, most of which is not demonstrable antibody (Humphrey, 1963). This is also observed in guinea pigs (Binaghi, 1966). Administration of antigen with FCA induces an increase of IgG1 which is unusually large compared with the antibody response (Barth et al, 1965; Binaghi, 1966). Furthermore,

Boyden (1964) observed the presence of cytophilic antibody in the serum of guinea pigs injected with antigen emulsified with FCA and this was lacking when incomplete adjuvant was substituted.

Further supporting evidence arises from the work of Dawe and his coworkers (1965). They observed that serum collected from rabbits treated with FCA and injected together with antigen into normal rabbits results in an antibody response. The magnitude of this response parallels an increase in immunoglobulin concentration. They were, however, unable to remove the enhancing activity by adsorbing the IgG fraction with insoluble antigen (Dawe et al, 1970).

The cell type affected by FCA may be a T cell as suggested by the following observations. Numerous plasma cells and lymphoid follicles in the thymus of rats appear after administration of FCA (Svet-Moldavsky and Raffkina, 1963). Allison and Davies (1971) reported that in mice depleted of T cells, antibody responses to BSA are decreased and the disparity between normal and T cell deprived mice is greater when the primary immunization is made with adjuvant. Reconstitution with a thymus graft results in marked stimulation of antibody formation by the adjuvant. This confirms earlier work by French and his colleagues (1970) in which they observed that FCA induces the development of a large granuloma at the site of injection. In bursectomized irradiated birds, the granuloma is normal in size while thymectomized irradiated birds develop a granuloma which is considerably reduced in size. These authors suggest that FCA interacts with a population of thymus derived cells in the granuloma which interact with bursa derived cells thus accounting for the rise in antibody levels.

Further support for the thymus dependence of the adjuvant effect is provided from the observations that mycobacterial adjuvants induce expansion of the paracortical areas of the draining lymph nodes with the appearance of pyroninophilic blast cells followed by germinal centre formation and medullary plasmacytosis (Taub et al, 1970).

Several theories about the mechanism of action of FCA have been proposed and these may be grouped into two main categories: (1) those effects attributed directly to the physical nature of the adjuvant and (2) those effects which are the result of the interaction of adjuvant with specific cell types. It should be noted that these categories are not necessarily mutally exclusive and that they may cooperate to produce the net result - increased antibody production.

It has been suggested that FCA acts by the protection of the antigen by the oily coating such that it serves as an antigen depot. The parrafin oil attracts mononuclear cells that may be active in antibody production. This is accompanied by a slow release of antigen which results in the stimulation of antibody production in immunocompetent cells (Halbert et al, 1946; Freund, 1956; White, 1967). Lind (1968) has suggested that the adjuvant effect is the result of a more efficient presentation to the immunocompetent cells of the antigen adsorbed onto the oil droplets.

It has also been suggested that FCA induces the mobilization of cells to the site of injection with antibody formation occurring in the granuloma (Askonas and Humphrey, 1955; French et al, 1970).

These theories, although explaining adjuvant action in rather general terms, do not indicate the cell types involved or how they are involved. The second set of theories as described below have attempted

to answer this question.

White (1970) first implicated a particular cell type as the target of FCA when he observed that thymectomized-irradiated birds had a granuloma which was strikingly decreased in size as compared to normal birds. He suggested that FCA interacts with a population of thymus derived cells in the granuloma thus allowing for more efficient interaction with the bursa derived cells. Similarly, Allison and his colleagues observed that potentiation of antibody synthesis against BSA in mice by FCA does not occur if the mice are thymectomized and treated with anti-lymphocyte serum (Allison, 1970; Allison and Davies, 1971).

Although local concentration and persistence of antigen may be necessary for the adjuvant effect, the cell which carries out this particular function may well be the thymus derived cell. These results strongly suggest that adjuvants stimulate the proliferation of thymus derived cells and that this in turn promotes antibody production by the B lymphocytes. The mechanism by which adjuvant stimulated T cells interact with B lymphocytes to promote antibody production is presently unknown.

2. Polynucleotide adjuvants

Increasing attention has been focused on the use of polynucleotides as adjuvants. Nucleic acids and their derivatives act as adjuvants while single stranded polynucleotides and mixtures of ribonucleotides and ribonucleosides have little or no enhancing effect.

Injecting equimolar complexes of the synthetic polynucleotides Poly(A:U) or Poly(I:C) with antigen, Schmidtke and Johnson (1971) observed a decreased induction period and increases in 19S and 7S

antibodies as well as increased immunologic memory to the antigen. This resulted with both soluble and particulate antigens, and the effect is abolished by heat denaturation or RNase treatment.

Addition of Poly(A:U) or Poly(I:C) to antigen treated peritoneal exudate cells increases circulating antibody levels thus suggesting that the macrophage is one cell affected by the adjuvant (Johnson and Johnson, 1971). However, further work by Cone and Johnson (1971) also implicates a T cell in the adjuvant effect. They observed the restoration of immunologic competence of adult mice thymectomized at birth or mice treated with heterologous anti-thymocyte serum by injection with SRBC and Poly(A:U). In addition, injection of Poly(A:U) enables neonatally thymectomized mice to reject allogeneic skin grafts at the same rate as mice with an intact thymus. Furthermore, irradiated mice which received SRBC, excess BM cells and as few as 40,000 thymocytes are stimulated by Poly(A:U) into antibody formation as detected by the RFC assay.

That this is the result of enhancement of the rate of proliferation of antigen-reactive cells of thymic origin has been demonstrated (Cone and Johnson, 1972). Thus exposure of thymus cells to Poly(A:U) in vivo or in vitro prior to injection of BM cells, increases the anti-SRBC RFC response of irradiated mice. Furthermore, immunization of mice with Poly(A:U) and SRBC results in the formation of a population of immunocytoadherent cells enriched in T cells (Marchalonis et al, 1973). Jaroslow and Ortiz-Ortiz (1972) have observed similar results and have concluded that the adjuvant indirectly increases the number of BM derived cells that respond to antigenic stimulation by increasing the effectiveness of the thymus derived cells.

Thus, although polynucleotides appear to function by replacing the normal regulatory influence of T cells on B cell function, confusing reports have arisen. Thus Cone and Johnson (1971) suggest that Poly (A:U) stimulates T cell activity, whereas Campbell and Kind (1971) suggest that it acts directly on B cells, thereby replacing T cell function. To confuse the issue further, Jaroslow and Ortiz-Ortiz (1972) suggest that polynucleotide adjuvants indirectly increase the number of B cells by increasing the effectiveness of the participating T cells.

3. Other adjuvants

This section will direct itself only to those observations which I feel are pertinent to the above discussion and which may have some bearing to the main body of this thesis.

Requirement for T cell function for potentiation of antibody responses has been observed for other adjuvants. Thus, beryllium, a potent adjuvant in normal animals, has no enhancing effect on the antibody response of thymectomized mice immunized with KLH (Unanue, 1970). Allison and Davies (1971) have extended these results for E.coli lipopolysaccharide and B.pertussis vaccine. That is, thymectomized, irradiated, bone marrow reconstituted mice fail to respond to antigen and adjuvant and that one can restore this defect to normal by a thymus graft. Furthermore, Taub and Gershon (1972) reported that the adjuvant B.pertussis potentiates the antibody response in normal mice but not in thymectomized, irradiated and bone marrow reconstituted mice.

In contrast, Jones and Kind (1972) observed that bacterial endotoxins exert their adjuvant effect on B cells thereby replacing the T cell requirement. This is based on the observation that the PFC response to SRBC in irradiated mice given anti-θ treated bone marrow

is significantly enhanced by Salmonella typhosa endotoxin.

Of particular importance is a report by Maillard and Bloom (1972) using B.pertussis as adjuvant. They noted that supernatants of adjuvant primed cells, stimulated in vitro with the specific adjuvant, enhance the primary response of normal cells to SRBC. Anti-θ treatment abrogates active supernatant thus suggesting that T cells are responsible for production of this active factor which then acts on B cells in a non-specific manner.

That adjuvants may also exert their effect to some extent on macrophages has been reported (Unanue et al, 1969). Thus adjuvants taken up by macrophages in vitro and injected into syngeneic hosts, increase the antibody response of the recipient to hemocyanin.

In summary, I think that it is very difficult to draw any general conclusions on the above data. These data suggest that adjuvants affect many cells and have many effects. However, the majority of the data, but not all, suggest that the main cell affected by adjuvants is a T cell. Since these effects modify B cell activity, it can be concluded that T cells have the ability to regulate B cell function and therefore that adjuvants (which appear to regulate or activate T cells) are very important in immunological phenomena.

It is hoped that this literature review has acquainted the reader with the regulatory influence of T cells on B cell function as well as to the various techniques defined for distinguishing these cell types.

MATERIALS AND METHODS

I. MICE

Inbred BALB/c male mice were obtained from North American Laboratory Supply, Winnipeg, Manitoba. C3H and AKR mice were obtained from Jackson Laboratories, Bar Harbor, Maine. The mice were housed seven to a cage and allowed food and water ad libitum.

II. ANTIGENS

1. Mouse IgG and IgF myeloma proteins

These proteins were isolated from ascitic fluid of transplantable mineral oil induced tumors. Ten ml of ascitic fluid equilibrated in 0.005M phosphate buffer pH 8.0 were chromatographed on a DEAE-cellulose ion-exchange column (22x500 mm) packed in the same buffer. Proteins were eluted by a stepwise elution procedure by an increase in the molarity of the buffer to 0.01M and 0.033M while the remaining protein was eluted with 0.5M NaCl. The eluates were collected in 5-7 ml fractions by an automatic fraction collector and individual tube protein content was determined by measuring the optical density at 280 mμ in a Zeiss spectrophotometer. All the tubes under a given peak were pooled and concentrated by ultra filtration in 0.15M borate saline pH 8.0 to a final concentration of 10-15 mg per ml. Each fraction was subjected to immunoelectrophoresis using a rabbit antiserum to whole mouse serum. Such tests indicated the presence of IgG myeloma protein in 0.005M and 0.01M fractions while IgF myeloma proteins resided in 0.01M and 0.033M fractions from ascitic fluid of that particular type. To eliminate

trace contamination of transferrin, each fraction was passed through a Sephadex G-200 column equilibrated with 0.15M borate saline pH 8.0. The first peak contained pure myeloma protein as indicated by a single line on immunoelectrophoresis. The isolated protein was concentrated to 10-15 mg per ml and stored at -20°C.

2. Ferritin

Horse spleen ferritin (Fe) twice crystallized, cadmium free was obtained from Nutritional Biochemical Corp., Cleveland, Ohio.

3. Human fibrinogen

Human fibrinogen (FIB) was obtained from Connaught Medical Research Laboratories, Toronto, Canada.

4. Bovine serum albumin and egg albumin

Bovine serum albumin (BSA) and egg albumin (EA) were purchased from Pentex, Kankakee, Illinois.

5. Chicken red blood cells

Chicken blood was collected in citrate buffer and the red blood cells (CRBC) were washed three times before use.

6. Tuberculin purified protein derivative

Tuberculin purified protein derivative (PPD), Lot 1014-1 was obtained from Connaught Medical Research Laboratories, Toronto, Canada.

7. Bordetella pertussis organisms

Bordetella pertussis organisms were purchased from Connaught Medical Research Laboratories, Toronto, Canada.

8. E.coli endotoxin

E.coli endotoxin (026:B6) was purchased from Difco Laboratories, Detroit, Michigan.

9. Polyvinylpyrrolidone

Polyvinylpyrrolidone (PVP) was obtained from Matheson Coleman and Bell, Norwood, Ohio.

10. Polyadenylic and polyuridylic acid

Polyadenylic (Poly A) and polyuridylic (Poly U) acids were purchased from Miles Laboratories, Kankakee, Illinois.

III. ADJUVANTS

Freund's complete adjuvant (FCA) and incomplete adjuvant (FIA) were obtained from Difco Laboratories, Detroit, Michigan.

MAAF (mycobacteria adjuvant antitumor fraction) was kindly donated by Dr. I. J. Hiu of the Pasteur Institute, Paris, France.

BCG vaccine, Lot 1065-1, 1058-1 was purchased from Connaught Medical Research Laboratories, Toronto, Canada.

IV. RABBIT IMMUNIZATION AND COLLECTION OF ANTISERA

Two or three rabbits were injected intra-muscularly with either mouse IgG myeloma protein, mouse IgF myeloma protein, horse spleen ferritin or egg albumin at a concentration of 1 mg per ml protein per injection per week for a period of five weeks. The antigens were emulsified in complete Freund's adjuvant (Difco). One week after the last injection the rabbits were bled by cardiac puncture and the antibody

activity of each antiserum was checked by immunoelectrophoresis. When a high titre was obtained, the rabbits were bled once a week for five weeks before a booster shot of antigen was given to elevate the antibody level. Fifty ml of blood was obtained at each bleeding, let stand at room temperature for two hours and the serum was separated from the clot by centrifugation at 3000 rpm for 15 minutes. The sera were then stored at -20°C.

V. MOUSE SERA

Normal mouse serum was fractionated on a DEAE cellulose column. The first peak, eluted with 0.01 M phosphate buffer pH 7.5, was further passed on a Sephadex G-200 column and the 7S peak was collected.

Both FIB and BSA in saline or mixed in Freund's complete adjuvant were injected intraperitoneally (IP) in doses of 250 µg in 0.2 ml. Serum was collected at 6 hours (6 HR-MS) or 24 hours (24 HR-MS) after immunization. In those experiments where radiolabelled antigen was used the radioactivity was detected by a Nuclear Chicago Gamma Counter. The sera were fractionated by gel filtration on a Sephadex G-200 column. The tubes were pooled into three fractions, the 19S, 7S and 4S, care being taken to avoid contamination of one by the other, and they were concentrated to the original serum volume applied on the column.

FCA and FIA were emulsified in equal volumes of saline and 0.2 ml of the emulsion was injected intraperitoneally. Sera were collected 6 hours later by heart puncture and will be referred to here as FCA-S and FIA-S respectively. These sera were also fractionated by gel filtration as described above.

FCA was emulsified in an equal volume of saline and 0.2 ml of

the emulsion was injected intraperitoneally into mice which had received 2.5 mg of hydrocortisone acetate 2 days previously (see Materials and Methods, section XVII). Serum was collected 6 hours later by heart puncture.

MAAF (250 μg) (see Materials and Methods, section III) dissolved in saline was injected I.P. into mice and 6 hours later the serum was recovered by heart puncture and will be referred to as MAAF-S. The serum was fractionated by gel filtration on a Sephadex G-200 column as discussed above.

VI. DEAE-CELLULOSE ION-EXCHANGE CHROMATOGRAPHY

The DEAE-cellulose ion-exchange column was packed according to the method of King (1968). Dry DEAE-cellulose ion-exchanger (Carl Schleicher and Schuell Co., Keene, N. H., 0.89 meq/ gm dry wt) was washed by suspending it in 0.5N NaOH and 0.5N NaCl with continuous stirring. After settling for 30 minutes the cloudy supernatant was decanted. The cellulose was resuspended in 1N NaCl. Stirring and decanting were twice repeated and the cellulose was filtered by suction on a Büchner funnel through filter paper. The moist cellulose was resuspended in 1N HCl, immediately filtered by suction and washed with distilled water until the filtrate was at neutral pH. The ion-exchanger was then equilibrated to the desired pH and molarity by suspending and washing with the starting buffer. The thick cellulose-buffer mixture was dispersed in a Waring blender and trapped air bubbles were removed by vacuum. The homogeneous suspension was then poured into the column and allowed to settle to 4-5 cm length at the bottom of the column with the outlet closed. The outlet was opened

to remove excess buffer and more slurry was added as the packed bed rose. A 3-5 cm column of buffer above the bed was left behind to avoid any disturbance of the packed bed with the next addition of cellulose suspension.

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

Sephadex G-100 and G-200 columns were packed according to the instructions provided by Pharmacia Ltd. (Montreal, Canada). The gel was swelled in excess buffer for three days at room temperature, care being taken not to rupture the beads by excessive stirring. Trapped air bubbles were removed by a vacuum pump before packing. The column was mounted vertically and the dead space under the disc and in the tubing was filled with eluant. The outlet was then closed. The thick slurry mixture was stirred to ensure homogeneity and then slowly poured down the wall of the column until the mixture reached the top. The outlet of the G-200 column was kept at the same level with the top of the gel slurry. When approximately ten cm of the bed was settled the outlet was slowly lowered to maintain a pressure equal to one cm. As the packed bed rose, the outlet tubing was lowered to maintain an optimum pressure equal to one-tenth of the packed bed length. After removal of excess buffer, another portion of the gel slurry was added before the previous portion had settled completely in order to prevent the appearance of boundaries. After the column was packed a sample applicator was inserted to protect the upper surface of the bed. The column was allowed to equilibrate for twenty-four hours at room temperature at 10-15 cm pressure at a rate of 20 ml per hour. In order to check the homogeneity of the packing and to determine the void

volume, 5 mg of blue dextran dissolved in 3 ml of buffer were filtered through the column.

In actual experiments, samples were allowed to pass through the column at a rate of 5-10 ml per hour under a pressure head of 2-3 cm. The process of packing the G-100 column was carried out under atmospheric pressure. A piece of filter paper was inserted to the upper surface of the bed for protection. The column was run under a pressure generally greater than atmospheric pressure at a rate of 30-40 ml per hour.

VIII. IMMUNOELECTROPHORESIS

The method used was that of Grabar and Burtin (1964). Glass slides (25x76 cm) were coated with 2 ml of 0.5% melted agar (Difco Noble) in distilled water and dried at 80°C for 4 hours. In the immunoelectrophoretic experiments, 3 ml of 2% melted agar in 0.025M barbital buffer pH 8.5 was layered onto the coated slides. The agar gel was allowed to solidify and the required wells and troughs were cut. The materials to be tested were placed in the wells and the slides were placed in an electrophoretic apparatus and ran at 14 ma, approximately 70-75 volts for three and one half hours. The slides were removed from the apparatus and the troughs were filled with the proper antisera and placed in a moist chamber at room temperature overnight to allow the development of the precipitin lines. The slides were then washed in 0.9% saline for 24 hours, desalted in distilled water for 8 hours and dried overnight by placing filter paper on the slides. The slides were stained with amido black (1gm/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 air dried.

IX. OUCHTERLONY GEL DIFFUSION

Coated slides as described in Immunoelectrophoresis were layered with 3 ml of 1.5% melted agar in 0.15M saline. The gel was allowed to solidify in a moist environment and then wells were cut using a hole puncher (template). The desired pattern of antigen-antibody precipitation reaction was arranged by filling the wells with antigen or antibody solution. The slide was placed in a moist chamber at room temperature overnight to allow the precipitin lines to develop. It was then washed and stained as described for immunoelectrophoretic slides.

X. ANTIBODY PURIFICATION

For the isolation of highly purified antibodies insoluble aggregates of the corresponding antigen as immunoadsorbents have been employed. Two methods were used for the aggregation:

1. Bis-diazotized benzidine (BDB) method

The stock solution was prepared according to the methods given in the Handbook of Experimental Immunology (Herbert, 1967).

Two hundred and thirty mg of benzidine (Hartman-Leddon Co., Philadelphia, Pa.) were dissolved in 45 ml of 0.2N HCl and cooled in an ice bath. One hundred and seventy-five mg of $NaNO_2$ (J. T. Baker Chemical Co., Phillipsburg, N.J.) was prepared, cooled and added to the benzidine over a period of 1 minute. The reaction proceeded for

30 minutes in an ice bath with stirring every 5 minutes. The resulting stock solution was pipetted into 2 ml volumes and dispensed into ampoules which were then sealed, deep frozen and stored at -20°C.

The proteins were aggregated as described by Bernier and Cebra (1965). Fifteen mg of the appropriate antigen were dissolved in 5 ml of 0.1M phosphate buffer pH 6.8 and added to 5 ml of a 1:15 dilution of the stock solution as prepared above. The mixture was allowed to stand at room temperature for 5 hours. The resulting aggregate was washed three times with the buffer, twice with 0.1M glycine-HCl buffer pH 2.5 and neutralized with 0.2M phosphate buffer pH 7.2.

2. Ethyl chloroformate method

The method used for ethyl chloroformate aggregation was that described by Avrameas and Ternynck (1967).

Fifty mg of the appropriate antigen in 0.2M acetate buffer pH 4.5 was stirred and 0.2 ml of ethyl chloroformate (K&K Laboratories Inc., Plainview, N. Y.) was added dropwise over a period of 1 minute. The mixture was stirred for fifteen minutes and the pH was kept between 4.5-5.0 for the next hour. The resulting aggregate was washed three times with the acetate buffer, twice with 0.1M glycine-HCl buffer pH 2.5 and neutralized by washing with 0.2M phosphate buffer pH 7.2.

To ten ml of rabbit antiserum, 50 mg of aggregated antigen was added. The suspension was left for 2 hours at room temperature and then stirred at 4°C overnight. The aggregate was removed by centrifugation and washed three times with buffered saline at 4°C. After the final wash it was suspended in 0.1M glycine-HCl buffer at pH 2.5 and left for 1 hour at 4°C, centrifuged at 10,000 rpm for 15 minutes and the supernatant immediately neutralized. The aggregate was also

neutralized and the process of elution repeated until all antibody was recovered from the serum. The antibody nature of the various preparations was examined by Ouchterlony technique. The concentration of the specifically precipitable protein was found to be between 80% and 90%.

Antibody to horse spleen ferritin was purified by precipitation with ferritin and subsequent acid dissociation as described by Hämmerling et al (1968). To a ten ml volume of rabbit serum sufficient ferritin was added to produce maximal precipitation as determined by a qualitative precipitin test. The specific precipitate was isolated by centrifugation at $4^{o}C$, dissolved in 0.1M glycine-HCl buffer pH 2.5 and left for 1 hour at $4^{o}C$. Subsequently the solution was centrifuged in an L-2 Spinco for 60 minutes at 35,000 rpm using an SW 39 rotor. The supernatant was collected and immediately neutralized. Seventy-five to eighty percent of the protein recovered was specifically precipitable.

XI. PEPSIN DIGESTION OF IMMUNOGLOBULINS

Purified 7S rabbit anti-mouse immunoglobulin, anti-ferritin, anti-egg albumin and normal mouse 7S immunoglobulin were separately digested with pepsin (Worthington Biochemical Co., Freehold, N. J.) according to the method of Nisonoff et al (1960) and Utsumi and Karush (1965).

Digestion was carried out using a pepsin to protein ratio of 2:100 and the incubation period was 5 hours at $37^{o}C$ using a 0.1M acetate buffer pH 4.0. Digestion was stopped by neutralization with 1N NaOH to pH 8.0. The $F(ab')_2$ peaks were isolated by gel filtration

on Sephadex G-200. The 5S peak of the purified antibodies was found to be precipitating against the homologous antigen.

XII. COATING OF SHEEP RED BLOOD CELLS (SRBC)

Formalinized sheep red blood cells were used in all experiments and the method of formalinization was that of Wede (1962). Sheep red blood cells in Alsever's solution were washed three times with 0.15M saline. One volume of 8% SRBC was incubated with an equal volume of 3% formaldehyde pH 7.0 (adjusted by 0.1N NaOH) for 24 hours at 37°C. Cells were washed four times with distilled water, suspended in distilled water as a 10% suspension and stored at 4°C.

From this a 2% suspension was prepared for tanning and coating according to the method of Herbert (1967). Two ml of the 2% suspension was processed as below using 0.15M phosphate buffered saline pH 6.4 in all steps of the procedure. The cells were washed three times, suspended in 2 ml of a 0.0025% solution of tannic acid in buffer (pH 7.2) and incubated at 37°C for half an hour. The cells were washed once more and left at 4°C overnight. The cells were then suspended in 2 ml of buffer containing 0.12 mg ferritin per ml or 0.3 mg egg albumin per ml depending on the type of protein coating desired. The cells were incubated at 37°C for one hour and then washed three times and suspended in 1.5 ml of buffer containing a 0.25% solution of human serum albumin. This resulted in a cell suspension of approximately 2.5%.

XIII. HYBRID ANTIBODY PREPARATION AND THE RICA TECHNIQUE

The method of preparation of hybrid antibody and the RICA technique was that as described by Paraskevas et al (1970, 1971a). This technique utilizes a 5S hybrid antibody with one anti-Ig site and one site specific for another protein, that is horse spleen ferritin or egg albumin. Through the former it reacts with surface associated Ig on lymphocytes and through the latter with protein coated sheep red blood cells (SRBC) thus forming a rosette.

1. Preparation of hybrid antibody

The purified anti-ferritin and anti-Ig antibodies were separately digested with pepsin as previously described (see Materials and Methods, section XI) and the $F(ab')_2$ fragments were isolated by Sephadex G-100 gel filtration. These fragments were mixed and reduced with 2-aminoethanethiol hydrochloride (2-mercaptoethylamine/HCl) (Matheson Coleman and Bell, Norwood, Ohio) to yield univalent F(ab') fragments. Passage of the sample through a AG 50 WX4 cation exchange resin (mesh 100-200, BioRad Laboratories, Richmond, California) removed the reducing substance. The eluate was reoxidized (Nisonoff and Rivers, 1961) by the passage of molecular oxygen through it and the divalent $F(ab')_2$ fragments were separated from any unoxidized fragments by chromatography on a Sephadex G-100 column.

To obtain only the hybrid molecules the reoxidized material was first absorbed with a BDB-aggregated ferritin conjugate and then a BDB-aggregated mouse Ig protein aggregate. Elution from the second aggregate with acid resulted in a preparation containing only hybrid molecules. Ouchterlony gel diffusion confirmed the presence of only

hybrid molecules.

The following hybrid antibodies were used in this study:

- antimouse immunoglobulin - antiferritin (αMIg- αFe)
- antimouse IgG globulin - antiferritin ($\alpha\gamma$ - αFe)
- antimouse IgF globulin - antiegg albumin ($\alpha\emptyset$- αEA)

The hybrid αMIg- αFe reacts with heavy chains of both IgG (7SIgG2a) and IgF (7SIgG1) globulins as well as with mouse light chains. The other two hybrid antibodies, $\alpha\gamma$- αFe and $\alpha\emptyset$- αEA are highly specific for the IgG and IgF globulin classes respectively (Paraskevas et al, 1971b; Lee et al, 1971).

I follow the terminology suggested by Potter et al (1965) because of its simplicity. Thus IgG corresponds to the commonly known IgG2a class, and IgF to the IgG1. The Greek letter γ is used for the heavy chains of IgG class and similarly the letter $\emptyset$ for the heavy chain of IgF class.

2. RICA technique

Suspensions of normal and immunized spleen cells from inbred BALB/c male mice were examined in the experiments. The suspension was prepared by teasing the spleen with forceps in cold Hanks' balanced salt solution (Microbiological Associates Inc., Bethesda, Maryland) after the mouse was sacrificed with ether. Small tissue clumps were aspirated through a 20 gauge needle to free trapped cells. The cell suspension was then sieved through a stainless steel cloth (200 mesh/inch) to remove tissue debris. The cells were washed three times in the cold at 700 rpm for 7 minutes. The total cell count was performed in 2% acetic acid in a hemocytometer.

In situations where cells were treated first before setting up

RICA, the cells were always washed free of the substances so that no contaminants could possibly be left in the tube. This usually involved washing the treated cells three times with cold Hanks' balanced salt solution (see Materials and Methods, Section XXIII).

To $8x10^5$ washed cells in Hanks' solution 30 cmm of 2.5% ferritin or egg albumin coated sheep red blood cells (approximately 100SRBC per spleen cell) were added. To this mixture 50 µg of the required hybrid antibody was added and the tube was well mixed. The samples were incubated at $4^{\circ}C$ overnight. In each experiment, a control without hybrid antibody and a control of normal mouse spleen cells with hybrid was prepared under similar conditions.

To count the cells and rosettes, Bellco slides (Bellco Glass Inc., Vineland, N. J.) with chambers 20 mm square were used. The sample was mixed by gently rolling and introduced into the chamber by a Pasteur pipette. The chamber was covered by a 22 mm square glass coverslip. The total number of spleen cells and the number of rosette forming cells were counted under a phase contrast microscope (Zeiss) using a 40x objective. A count of 1000 spleen cells per sample was performed recording the number of rosette forming cells (RFC) in the preparation. A lymphoid cell surrounded by a minimum of four SRBC was counted as a rosette.

XIV. CYTOTOXICITY TESTS

1. Antisera

a) Anti-theta antiserum (Anti-θ)

This antiserum was prepared in AKR mice against C3H thymocytes as

described by Reif and Allen (1964). Thymocytes ($1x10^7$) were injected intraperitoneally at weekly intervals for six weeks. The serum was collected by heart puncture 10 days after the last injection. It was decomplemented at 56°C for 30 minutes and absorbed with packed red blood cells from C3H and BALB/c mice at 4°C for 30 minutes. The antiserum was separated in aliquots and stored at -23°C.

The antiserum gave a cytotoxicity titre of 1:256 using C3H thymocytes and reconstituted lyophilized guinea pig serum as complement source (Department of Health, Ottawa, Canada). The antiserum was not cytotoxic to bone marrow cells.

b) Anti-bovine serum albumin and anti-fibrinogen (Anti-BSA, Anti-FIB)

These antiserums were prepared as described in Materials and Methods, Section IV. These sera were absorbed with normal mouse spleen cells to remove possible "natural" cytotoxins. All sera were decomplemented at 56°C for 30 minutes and stored in small aliquots at -23°C.

2. Test system

The method used was that of Takahashi et al (1970). The antiserum (1:8 dilution) and guinea pig complement (1:6 dilution) were incubated with $2\text{-}3x10^6$ spleen cells for 45 minutes at 37°C and the number of viable cells was determined by the trypan blue exclusion method. Spleen cells incubated with only the antiserum served as a control.

The number of T cells was determined after exposure of normal spleen cells to 6 HR-MS or its fractions as well as after exposure to either FCA-S alone or with the addition of antigen. Normal mouse serum was used as a control.

Spleen cells exposed to FCA-S in the presence of BSA or FIB were

washed and treated for 45 minutes at 37°C with the specific antiserum (1:8 dilution) and complement. Control experiments consisted of spleen cells exposed to antigen or FCA-S alone and NMS or a mixture of NMS and antigen. See Fig. 1 for the protocol.

The per cent cytotoxicity was calculated from the following formula:

$$\% \text{ cytotoxicity} = \frac{\text{VcAb} - \text{VcAbC}}{\text{VcAb}} \times 100$$

where VcAb = number of viable cells in antibody treated preparations.

VcAbC = number of viable cells in antibody and complement treated preparations.

XV. RADIOLABELLING OF BOVINE SERUM ALBUMIN

The chloramine T method of McCohaney and Dixon (1966) was used to label bovine serum albumin (BSA). This technique is advantageous since only simple equipment is required and a high efficiency of iodination is achieved. No carrier iodine is required and small amounts of protein can be labelled efficiently with little or no denaturation.

One - five mg of BSA were dissolved in 4 ml of 0.05M phosphate buffer pH 7.0. The sample was placed in a small beaker surrounded by ice and stirred. One - three mCuries of I^{125} (Charles Frosst and Co., Montreal, Canada), carrier free was added to the sample with stirring. Fresh chloramine T (100-200 µg) (J. T. Baker Chemical Co., Phillipsburg, N. J.) was added with stirring and left for 5 minutes. The reaction was stopped by the addition of an equal amount of sodium metabisulphite (Matheson Coleman and Bell, Norwood, Ohio) with stirring. Non-protein bound iodide was removed by exhaustive dialysis in the cold

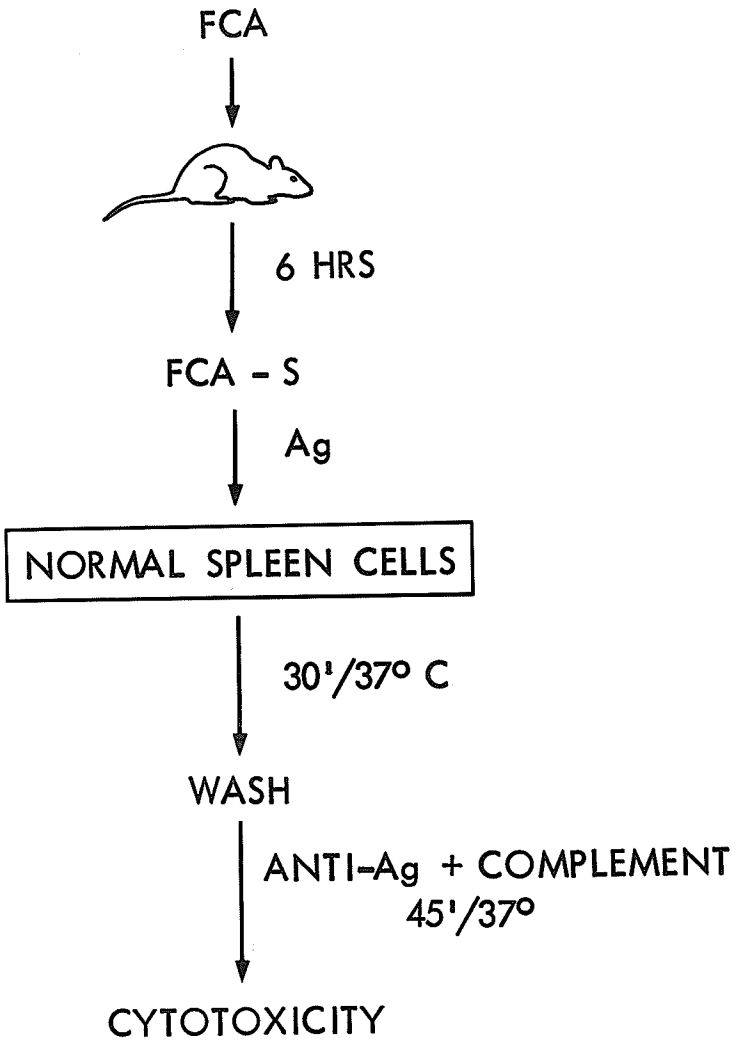


Figure 1: Protocol for detection of antigen on the surface of spleen cells. Ag=BSA or FIB.

against 0.1M phosphate buffer pH 7.0 with several changes over a 12-18 hour period.

The radiolabelled BSA was fractionated by gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals, Montreal, Canada). The bulk of the protein was eluted as a 4S peak but smaller amounts of higher polymers were also present. Only the 4S fraction was used in the experiments. Greater than 90% of the radioactivity was precipitable by 10% TCA and the specific activity among various preparations varied between 20-50 μc per mg protein.

XVI. ABSORPTION OF FCA-S AND 4S FCA-S

Egg albumin, ferritin and fibrinogen were aggregated using the ethyl chloroformate method of Avrameas and Ternynck (1967).

The FCA-S was mixed with any of the aggregates and left at room temperature for one hour under mild stirring. The aggregate was recovered by centrifugation and the supernate serum was saved (FCA-S(A)). The aggregate was washed three times with physiological saline and then suspended in 0.1 M glycine-buffer pH 2.5 for one hour at room temperature. The suspension was centrifuged, the supernate recovered and neutralized immediately and will be referred to here as the eluate (EL) (Fig. 2).

Absorption of FCA-S was also carried out with CRBC ($1x10^8$ per ml of FCA-S). The absorbed serum was used in subsequent experiments but no elution was attempted from the CRBC.

FCA-S was chromatographed on a Sephadex G-200 column and the 4S fraction was absorbed with an EA aggregate as described above for the whole serum.

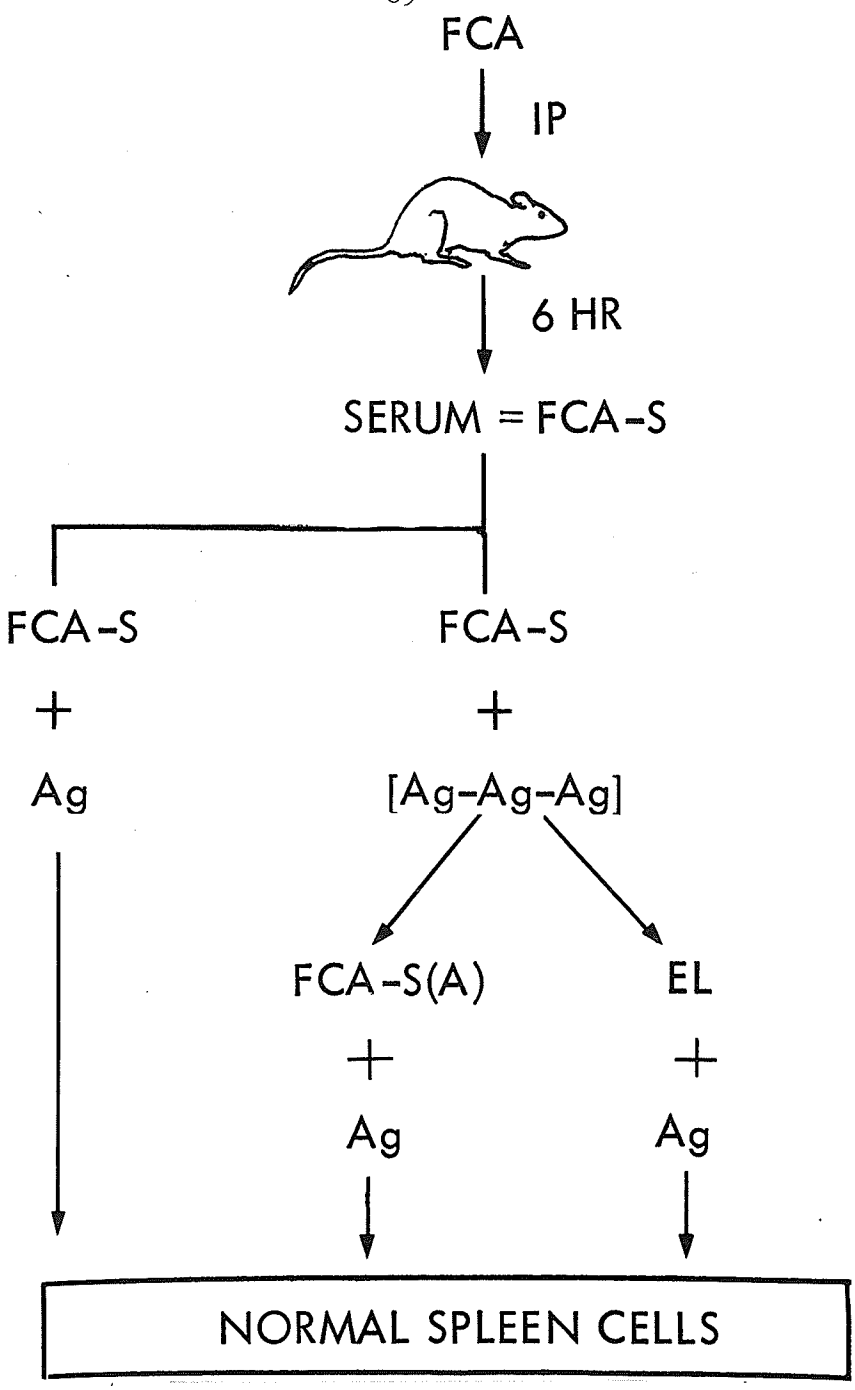


Figure 2: Protocol for treatment of spleen cell suspensions.

XVII. HYDROCORTISONE TREATMENT OF MICE

BALB/c mice were injected intraperitoneally with 2.5 mg of hydrocortisone acetate (Merck Sharp and Dohme, Kirkland, Quebec) two days before killing as described by Blomgren and Andersson (1969).

XVIII. IN VITRO CULTURING SYSTEM OF MOUSE LYMPHOID ORGANS

Whole organs such as spleen, thymus and mesenteric lymph nodes were removed from BALB/c mice and prepared as described in Materials and Methods, Section XXIII. Bone marrow cells were obtained by flushing the femurs with balanced salt solution. Peritoneal exudate cells were recovered by rinsing the peritoneal cavity with balanced salt solution. The cells were allowed to adhere to glass plates for 30 minutes at 37°C. The plates were gently rinsed with balanced salt solution to remove non adherent cells and the adherent cells were harvested by gentle agitation with a rubber policeman. By microscopy, these cells were morphologically macrophages (60-70%). All cells were washed prior to culture.

From 6-120x10^6 cells as described above were suspended in 1.5 ml of balanced salt solution and incubated at 37°C for 45 minutes either alone or in the presence of the following:

1. 100 µg BCG
2. 500 µg BSA in saline
3. 1 mg heat aggregated BSA
4. 500 µg DNP_{60} BSA
5. 300 µg BSA-anti BSA complexes (10xAg excess)
6. 500x10^6 B.pertussis organisms

7. 400×10^6 CRBC
8. 250 µg MAAF
9. 250 µg E. coli endotoxin
10. 200 µg PVP
11. 25 µg POL
12. 500 µg FIB
13. 300 µg Poly (AU)

The cells were subsequently washed three times, resuspended in 1.5 ml balanced salt solution and cultured for 3 hours in an incubator at 37°C. The cells were then centrifuged and the resulting supernatants were neutralized as required. A sample protocol is shown in Fig. 3.

XIX. SHORT TERM CULTURES IN THE PRESENCE OF METABOLIC INHIBITORS

Sodium fluoride inhibits glycolysis by binding with the enzyme enolase to form a magnesium fluorophosphate enzyme complex which is no longer able to catalyze the dehydration of the phosphate ester of a dihydroxy acid.

Actinomycin D is the principal component of the mixture of actinomycins produced by Streptomyces parvullus. This antibiotic is a well known inhibitor of DNA directed RNA synthesis. Thus actinomycins form tight reversible complexes with DNA. The replication of DNA itself is relatively resistant to the action of actinomycins (Reich, 1963).

Cycloheximide or (3 2(3,5-dimethyl-2-oxycyclohexyl)-2-hydroxy methyl glutarimide) is an efficient inhibitor of protein synthesis. Inhibition of protein synthesis occurs as the result of the inhibition

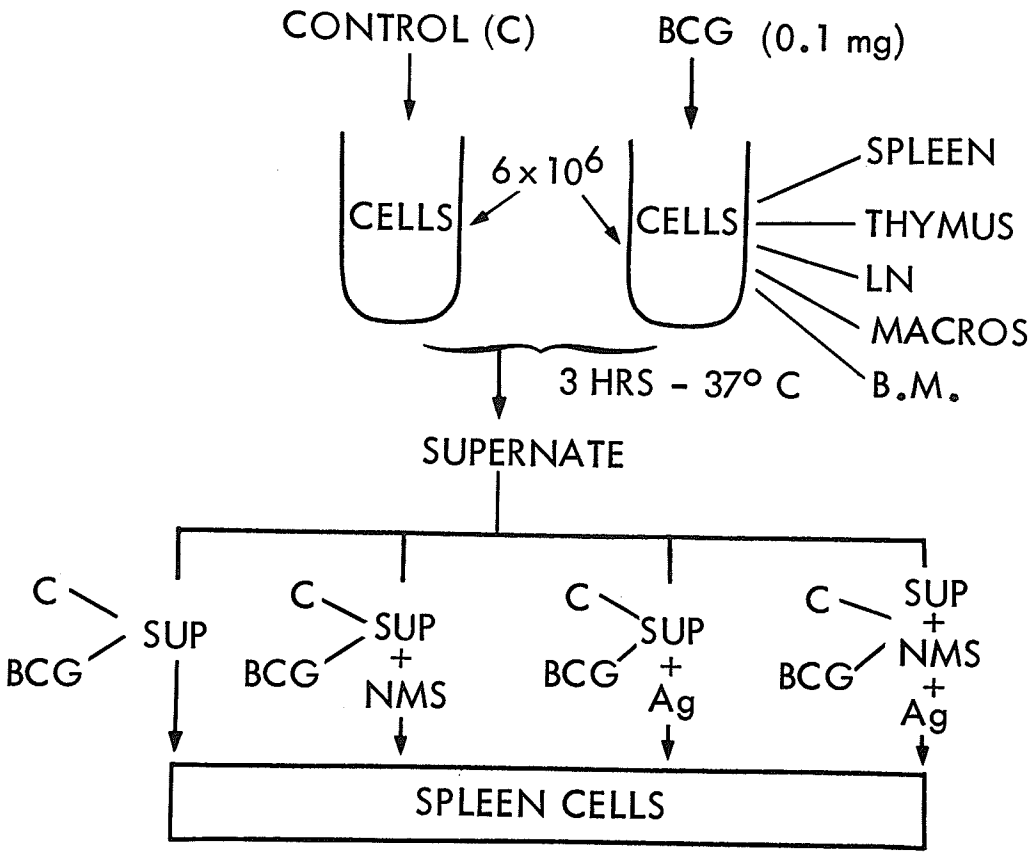


Figure 3: Lymphoid cell culture protocol.

of translocation of amino acyl-tRNA from the acceptor to the donor ribosomal sites (Obrig et al, 1971).

Thymocytes ($6x10^6$) were cultured with 100 µg BCG for 30-45 minutes in the presence of:

1. $2x10^{-2}$M NaF (J.T.Baker Chemical Co., Phillipsburg, N.J.).
2. $4x10^{-4}$M cycloheximide (Sigma Chemical Co., St. Louis, Missouri).
3. 1 µg actinomycin D (Merck Sharp and Dohme, West Point, P. A.).
4. without the above but at 4^oC.

The cells were subsequently washed, resuspended in balanced salt solution containing the above metabolic inhibitors and incubated a further 3 hours. The mixture was centrifuged and the supernatants were neutralized.

XX. FREEZE THAWING

Normal spleen cells ($120x10^6$) were freeze thawed four times, centrifuged and the supernatant collected and neutralized.

XXI. ULTRAFILTRATION OF FCA-S AND ELUATES

Diaflo ultrafiltration membranes (Amicon Corp., Lexington, Massachusetts) are unique anisotropic molecular filters which are manufactured from synthetic polymers. Molecules of given molecular size and dimension will be retained while those below the membrane cutoff level will be passed. These membranes do not denature proteins and are biologically inert. Thus these membranes can be successfully used in biochemical processing and can also be used to provide broad

molecular weight characteristics according to whether or not a substance passes through filters of a given series.

The membrane used in this study is the UM10 membrane (Amicon Corp., Lexington, Massachusetts) with a solute cutoff level of 10,000MW.

Serum was collected from mice 6 hours after intraperitoneal injection of FCA in saline (FCA-S). The serum was either used as is, treated with an equal volume of 0.1M glycine-HCl buffer pH 2.5 or treated as above for 15 minutes and then neutralized. These samples were filtered through UM10 filters.

Eluates which were obtained from FCA-S or 4S FCA-S (see Materials and Methods, section XVI) were subsequently filtered on UM10 filters.

Supernates obtained from cultures (Materials and Methods, Section XVIII) were subjected to the same procedure as described above.

XXII. TRYPSIN AND RNASE DIGESTION AND HEAT INACTIVATION

UM10 filtrates obtained from FCA-S which had been treated with acid and neutralized as described in Materials and Methods, Section XXI or supernates from thymus cells cultured in the presence of BCG (Materials and Methods, Section XVIII) were incubated with 0.17 mg of trypsin (Grand Island Biological Co., Grand Island, N. Y.) for 2 hours at 37°C. The reaction was stopped by the addition of 0.17 mg (4x excess) of Soybean Trypsin Inhibitor (Schwartz/Mann, Orangeburg, N. Y.) for 10-15 minutes at 37°C.

Bovine RNase I (Miles-Seravac Ltd., Maidenhead, England) was heated at 80°C for 15 minutes to destroy contaminating enzymes. To 1 ml of the above was added 50 µg RNase at 37°C for 30 minutes.

The materials described above as well as eluates obtained by

absorption of FCA-S with an EA aggregate (Materials and Methods, Section XVI) were incubated at 56°C for 60 minutes before subsequent use.

XXIII. TREATMENT OF SPLEEN CELL SUSPENSIONS

Spleen cell suspensions were prepared in balanced salt solution from BALB/c mice 6-8 weeks old (Materials and Methods, Section XXIII) and $1x10^7$ cells per ml were exposed for 30-45 minutes at 37°C to 0.4 ml of each of the following:

1. 6 HR-MS, 24 HR-MS or fractions from a G-200 column
2. FCA-S, FIA-S, G-200 fractions and all combinations with addition of NMS, Ag or both (Fig. 2)
3. NMS, Ag or both
4. FCA-S(A) alone or in various combinations with Ag and NMS
5. 6 HR-MAAF-S alone or in combinations with Ag, NMS or both
6. Culture supernatants alone, with Ag or NMS or both (Fig. 3)
7. Ultrafiltration supernates alone or in various combinations with NMS and Ag.

After incubation, the cells were thoroughly washed with balanced salt solution and used in RICA tests at a concentration of $2-3x10^6$ per ml.

In the case of (1) and (2) above, after treatment the cells were washed and incubated with either anti-BSA or anti-FIB antiserum for a further 30 minutes. The cells were subsequently washed and used in RICA tests.

XXIV. STATISTICAL ANALYSIS

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

Test for homogeneity of variances was performed using the F distribution. In these cases where the variances were not equal, the two sample t test for unequal variances was used.

EXPERIMENTAL RESULTS

SECTION A

DETECTION OF EARLY CYTOPHILIC COMPLEXES REACTING WITH T AND B LYMPHOCYTES

I. IMMUNOGLOBULIN CARRYING CELLS IN NORMAL MOUSE SPLEEN

Lymphoid cells displaying surface immunoglobulin on their surface, after reaction with hybrid antibody and indicator cells, form rosettes (Fig. 4) while those cells lacking surface Ig do not and remain as free cells in the suspension. Employing hybrid antibodies of different specificities, one can obtain a profile of the distribution of immunoglobulin classes on the surface of mouse spleen lymphocytes (Table II).

Table II shows that a hybrid antibody which reacts with all classes of mouse Ig (αMIg- αFe) detects 30-35% of the spleen cells as carrying surface immunoglobulin. A hybrid antibody highly specific for the 7SIgG2a (IgG) mouse immunoglobulin ($\alpha\gamma$- αFe) demonstrates that 18-20% of spleen cells carry this immunoglobulin. On the other hand, a hybrid antibody specific for the 7SIgG1 (IgF) mouse immunoglobulin ($\alpha\emptyset$- αEA), detects 28-30% of the normal spleen cells as carrying IgF.

The terms rosette forming cell and immunoglobulin carrying cell are used interchangeably.

II. THE EFFECT OF 6 HR-MS ON Ig CARRYING CELLS OF NORMAL MOUSE SPLEEN

Normal spleen cells were exposed to serum collected 6 hours after immunization (6 HR-MS) for 30 minutes at 37°C, washed and examined by the reverse immune cytoadherence technique (RICA). These results are shown in Table III.

Serum collected 6 hours after injection of FIB emulsified in FCA (6 HR-MS-FIB) induces an increase of the Ig carrying cells (RFC) of

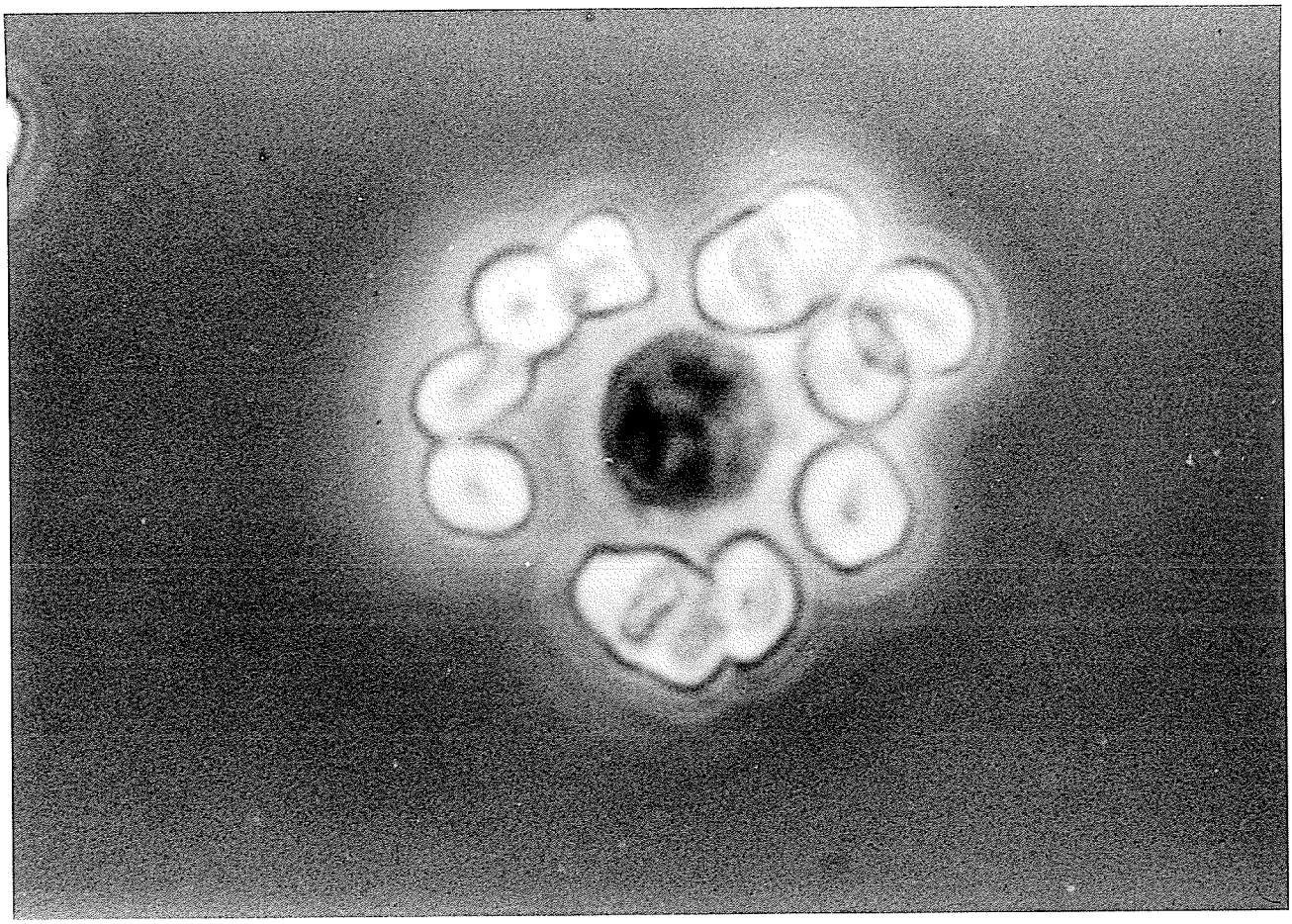


Figure 4: Rosette forming cell in normal mouse spleen as examined by phase contrast microscopy.

TABLE II

Distribution of immunoglobulin classes
on normal spleen cells

HYBRID Ab	CELL TYPE	RFC/1000 SPLEEN CELLS[a]
α MIg-α Fe	Ig	301±3
$\alpha\gamma$- α Fe	IgG(7SIgG2a)	195±7
$\alpha\emptyset$-α EA	IgF(7SIgG1)	287±9

[a]Averages of 30-50 determinations ± S. D.

TABLE III

Effect of 6 HR-MS on mouse spleen cells

	-- B CELLS BY SINGLE RICA[a] -- (PER 1000 SPLEEN CELLS ± S.D.)			% T CELLS
HYBRID:	αMIg- αFe	αγ- αFe	α∅- αEA	
CELLS:	Ig cells	IgG cells	IgF cells	
NORMAL	304±6(5)[b]	189±10(8)	301±10(8)	28±1.7
6 HR-MS-FIB	371±19(7)	266±2(3)	195±2(3)	-
6 HR-MS-BSA	380±15(6)	266±1(3)	203±10(3)	16±1.3
NMS	297±6(4)	-	-	26±2.7
NMS + Ag[c]	316±1(2)	-	-	-
7S FRACTION (6 HR-MS-BSA)	399±7(4)	263±13(4)	199±20(3)	17±0.9

[a] See text for symbols used.

[b] The numbers in parentheses represent the number of animals examined.

[c] Ag = 0.5mg FIB.

normal spleen cells or approximately 70 cells per 1000 spleen cells which is similar to that observed in vivo (Paraskevas et al, 1972c). This represents an increase of 23% above the normal level. The same result was obtained with a serum collected 6 hours after immunization with BSA in FCA (6 HR-MS-BSA). This increase can be accounted for by an increase of cells carrying IgG globulin as detected by $\alpha\gamma$- αFe hybrid antibody (IgG cells). The increase most likely is due to the addition in the pool of Ig carrying cells, of new cells which previously were not detectable. The 6 HR-MS affects also cells which already carry surface Ig. This is shown by a decrease of the cells detectable by $\alpha\emptyset$- αEA hybrid antibody (IgF cells). The cause of this decrease is at present unknown. Previous work (Lee and Paraskevas, 1972) has shown that a cytophilic immunoglobulin which belongs to the IgG class is taken up by spleen cells in vivo. Uptake of this Ig by the spleen cells carrying the IgF globulin could result in loss of the detection of the surface IgF globulin by steric hindrance, or other mechanisms. It was previously demonstrated that when mouse spleen cells were treated with antigen-antibody complexes made of mouse IgG antiferritin and ferritin, the surface associated IgF globulin could not be detected by the specific hybrid antibody (Paraskevas et al, 1972a). Although steric hindrance may be the underlying mechanism, alternate explanations have not been excluded. Normal mouse serum (NMS) or NMS with antigen added have no effect.

Table III shows also that the number of T cells in spleen suspensions exposed to 6 HR-MS, as detected by cytotoxicity tests with anti-θ serum (see Materials and Methods, Section XIV), decreased by about 10-12% of all spleen cells. This decrease is identical to that found

in vivo (Lee and Paraskevas, 1972). Thus 6 HR-MS reproduces in vitro all the changes observed in vivo six hours after antigenic stimulation.

In summary, a cytophilic IgG globulin which is taken up by previously non-immunoglobulin carrying cells is present in the serum 6 hours after antigenic stimulation resulting in an increase of cells detectable by RICA as carrying surface Ig. Concomitantly the T cells decrease by 10-12%. The increase of the Ig carrying cells and the decrease of T cells are closely similar. The slightly larger decrease of the T cell may be due to differences in the techniques employed. The cytophilic IgG globulin is shown here to be taken up also by cells previously carrying Ig (B cells), resulting in a decrease in the number of these cells carrying detectable IgF globulin (IgF cells).

III. FRACTIONATION OF THE 6 HR-MS-FIB

Serum collected 6 hours after injection of FIB emulsified in FCA was fractionated on a Sephadex G-200 column. The fractions were examined for their ability to induce an increase of the rosette count on normal spleen cells. As shown in Fig. 5 the void volume, where fibrinogen is eluted, contains this activity, while the 7S and 4S fractions are completely devoid of such activity. In view of the fact that whole serum shows the presence of a cytophilic globulin of the IgG class (increase of IgG cells, Table III) we assume that this globulin is eluted together with FIB.

In conclusion the cytophilic IgG globulin in this case is not simply a 7S IgG globulin but it is larger in size and is eluted together with FIB. In order to obtain a clearer picture of the relationship of the injected antigen, the 7S IgG globulin and the ability of the serum

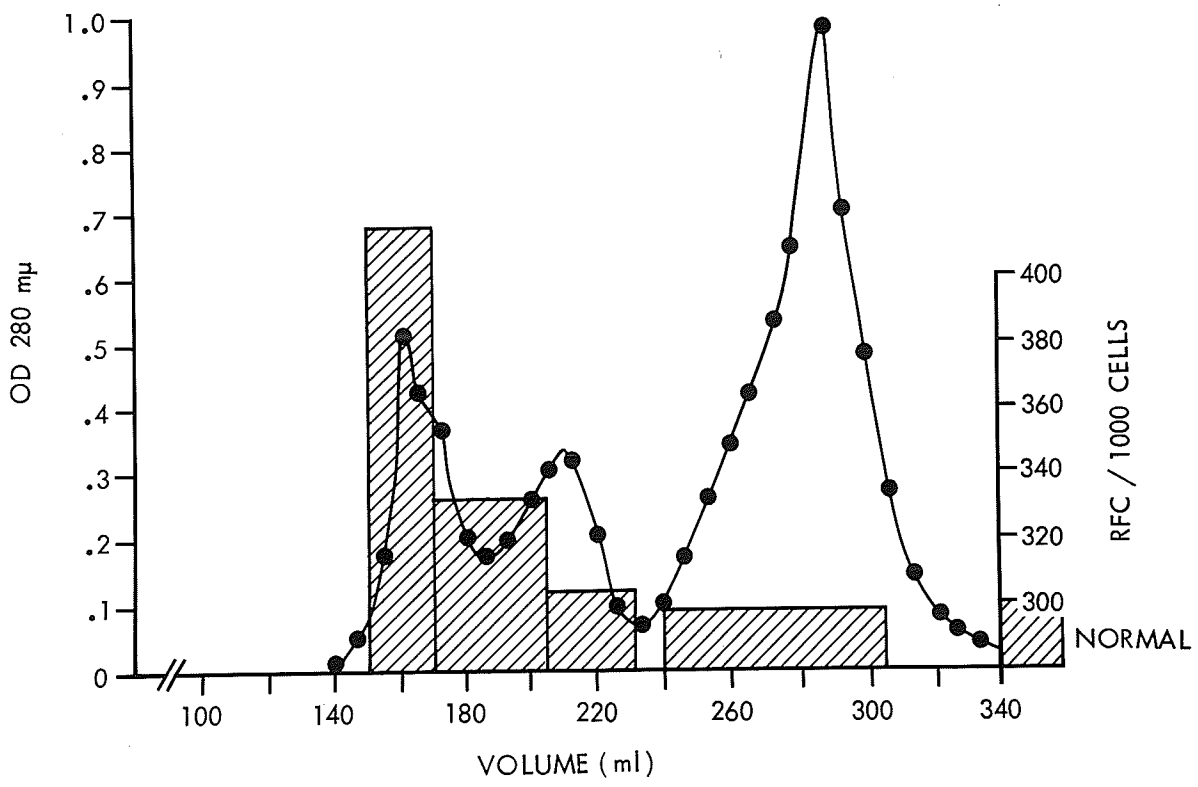


Figure 5: Sephadex G-200 elution pattern of 6 HR-MS-FIB. The hatched bars represent number of rosette forming cells (RFC).

to increase the rosette count, experiments were performed using a smaller antigen (BSA) labeled with radioactive iodine (I^{125}).

IV. CHARACTERIZATION OF THE CYTOPHILIC IgG GLOBULIN IN 6 HR-MS-BSA

Radioactively labeled BSA-I^{125} (see Materials and Methods, section XV) was first purified by gel filtration on a Sephadex G-200 column in order to remove the larger polymers known to exist in such preparations. Only the 4S fraction was used, mixed with FCA for immunization, and the serum collected 6 hours later was fractionated by Sephadex G-200. The eluted fractions were examined for protein and radioactivity and a typical experiment is shown in Fig. 6. Three major protein peaks were eluted, the 19S, the 7S and 4S. The major part of the antigen is eluted with the 4S peak in the same elution volume as the 4S BSA which was used for injection. In addition radioactivity is now eluted in the 7S peak or slightly ahead of it, while very little or no radioactivity is present in the first peak.

The 7S and 4S fractions were examined by Ouchterlony technique against rabbit antisera to BSA and mouse immunoglobulin. The 7S fraction gives a strong precipitin line with antimouse immunoglobulin and a weak line with anti-BSA which fuses with that of the 4S peak (Fig. 7). Autoradiography shows that the line formed between the 7S fraction and the anti-immunoglobulin serum contains radioactivity indicating that BSA in the 7S fraction is complexed with immunoglobulin. The line formed between the 7S fraction and the anti-BSA serum is strongly radioactive and fuses with that formed by the 4S fraction.

Thus at 6 hours the serum contains two major forms of the injected antigen as far as its size is concerned: the bulk is still present as

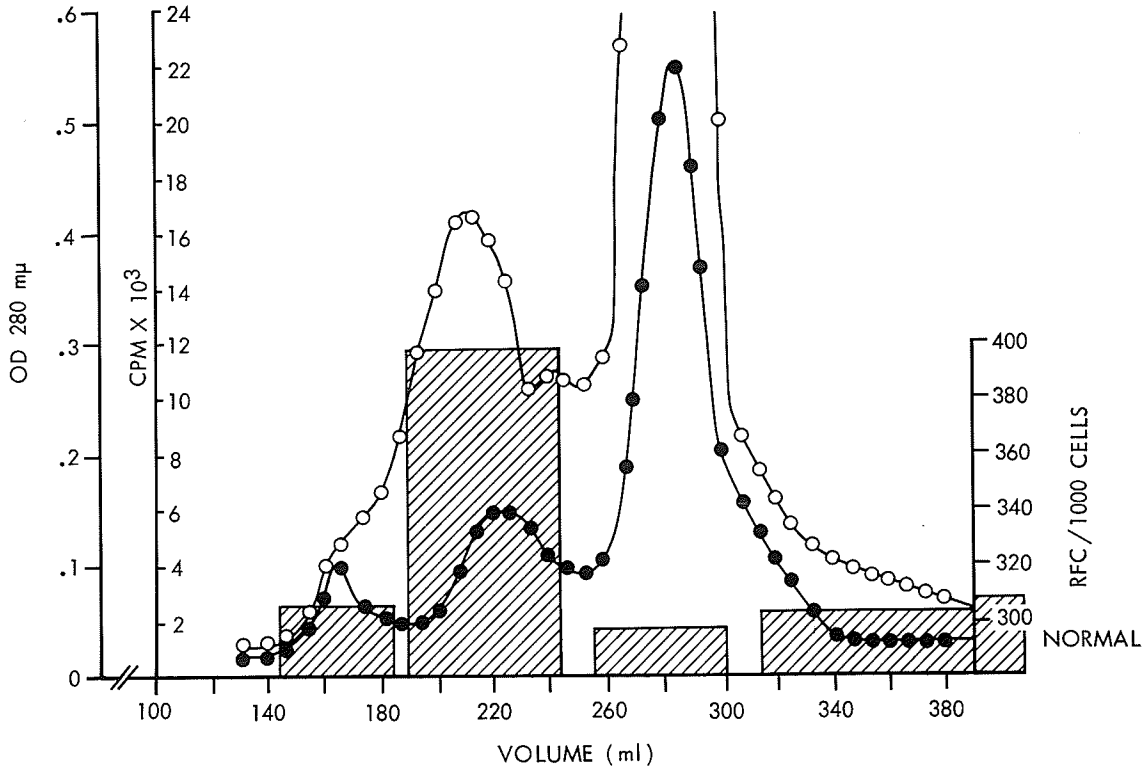


Figure 6: Sephadex G-200 elution pattern of 6 HR-MS-BSA. Open circles = CPM; filled circles = 280 mμ OD. Hatched bars represent number of RFC.

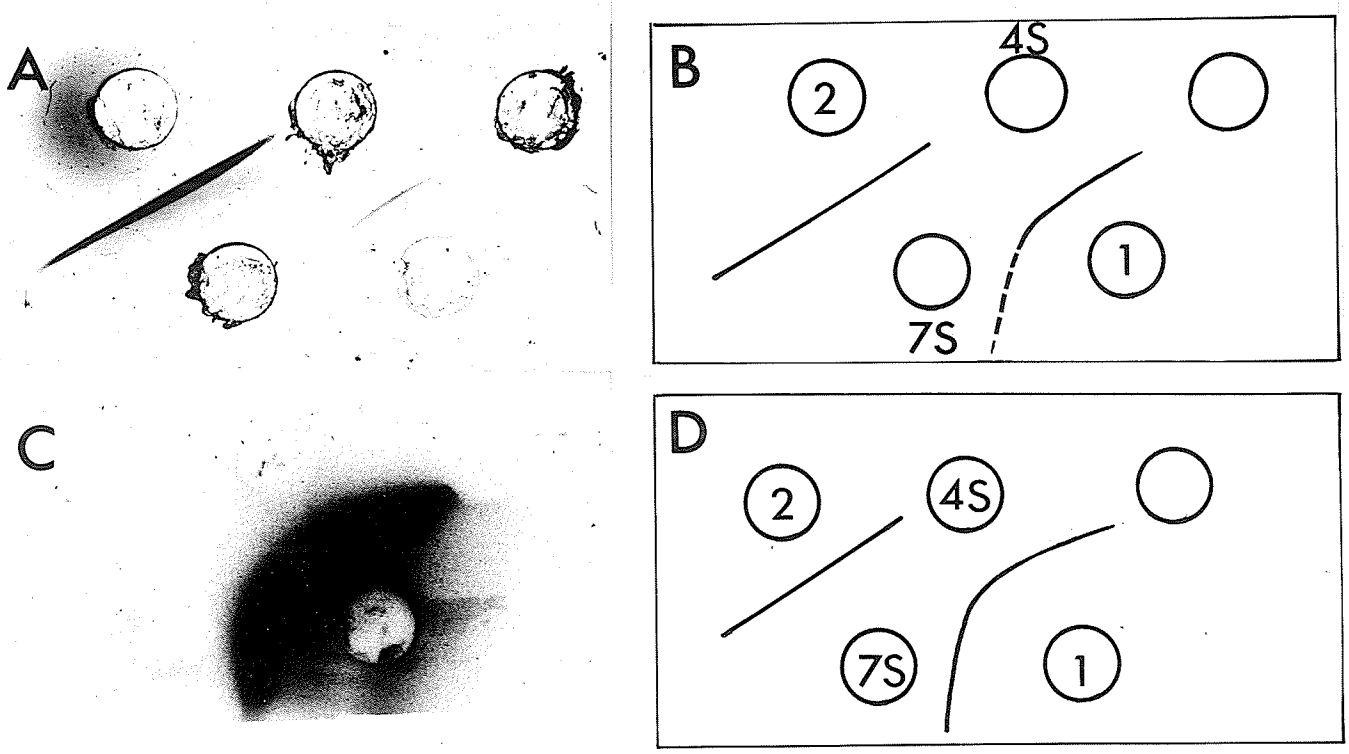


Figure 7: Ouchterlony (A) and autoradiography (C) of the 7S and 4S fractions of 6 HR-MS-BSA. B and D are drawings to facilitate the reading of weak lines. 1 = rabbit anti-BSA, 2 = rabbit anti-mouse immunoglobulin.

4S while a portion is complexed to an IgG globulin and is eluted slightly ahead of the 7S peak. Although this antigenic form is precipitable with anti-BSA, most likely it represents a fragment of the BSA since the sieving properties of the IgG globulin bound to it do not change substantially. BSA complexed to an IgG globulin 5 hours after injection was detected in rabbits by Yuan et al (1970).

V. THE EFFECT OF 6 HR-MS-BSA FRACTIONS ON SPLEEN CELLS

Each fraction from the Sephadex G-200 column was examined for the ability to reproduce the phenomena observed with unfractionated serum. As shown in Fig. 6 only the 7S fraction containing the antigen-Ig complex is able to induce an increase of the Ig carrying cells similar to that of the unfractionated serum. This property is not present in the 19S or 4S peaks. Cells treated with the 7S fraction were also examined in single RICA tests using the $\alpha\gamma$-αFe and $\alpha\emptyset$-αEA hybrids. There is an increase of the cells carrying IgG globulin equal to that produced by the unfractionated serum (Table III). Since this hybrid antibody is highly specific for the mouse IgG globulin class (Lee and Paraskevas, 1971; Paraskevas et al, 1971b) the results indicate that the cytophilic Ig in the 7S fraction belongs to the IgG class. The number of rosettes formed by the $\alpha\emptyset$-αEA hybrid antibody in spleen cells exposed to the 7S fraction decreased (IgF cells, Table III). It is conceivable that the decrease may be caused by a mechanism of steric hindrance due to the uptake of the cytophilic IgG globulin by cells previously carrying IgF globulin, although other mechanisms are also possible. Such blocking of the surface IgF globulin was shown before using mouse IgG anti-ferritin complexed to ferritin (Paraskevas et al, 1972a), and it is

possible that the BSA-IgG globulin complex in the 7S fraction may produce the same result. Finally the 7S fraction reduces the number of T cells detectable in the spleen cell suspension by a percent similar to that of unfractionated serum (Table III). In conclusion all the activities contained in the 6 HR-MS were reproduced by the 7S fraction which contains antigen complexed to immunoglobulin.

VI. FRACTIONATION OF NORMAL MOUSE SERUM MIXED IN VITRO WITH ANTIGEN

Normal mouse serum (NMS) was incubated with BSA-I^{125} for 30 minutes and then applied on a Sephadex G-200 column. The results are shown in Fig. 8. Radioactivity is eluted only in the 4S peak. None of the three fractions were able to induce an increase of the rosette count when examined by RICA.

VII. DETECTION OF ANTIGEN ON THE SURFACE OF SPLEEN CELLS TREATED WITH 6 HR-MS-BSA OR 6 HR-MS-FIB

Spleen cells were exposed to 6 HR-MS-BSA or 6 HR-MS-FIB serum for 30 minutes at 37°C. After washing, the cells were treated for 30 minutes at 37°C with the specific antiserum. The cells were subsequently washed and examined by RICA. Cells examined with no serum and only serum treatment served as controls. The results are shown in Table IV.

Treatment of normal spleen cells with serum collected 6 hours after immunization with FIB or BSA emulsified in FCA results in 377 ± 12 and 368 ± 9 RFC respectively and is comparable to the results shown in Table III. When cells exposed first to 6 HR-MS-BSA are subsequently treated with rabbit anti-BSA, a significant reduction ($p<0.001$) of

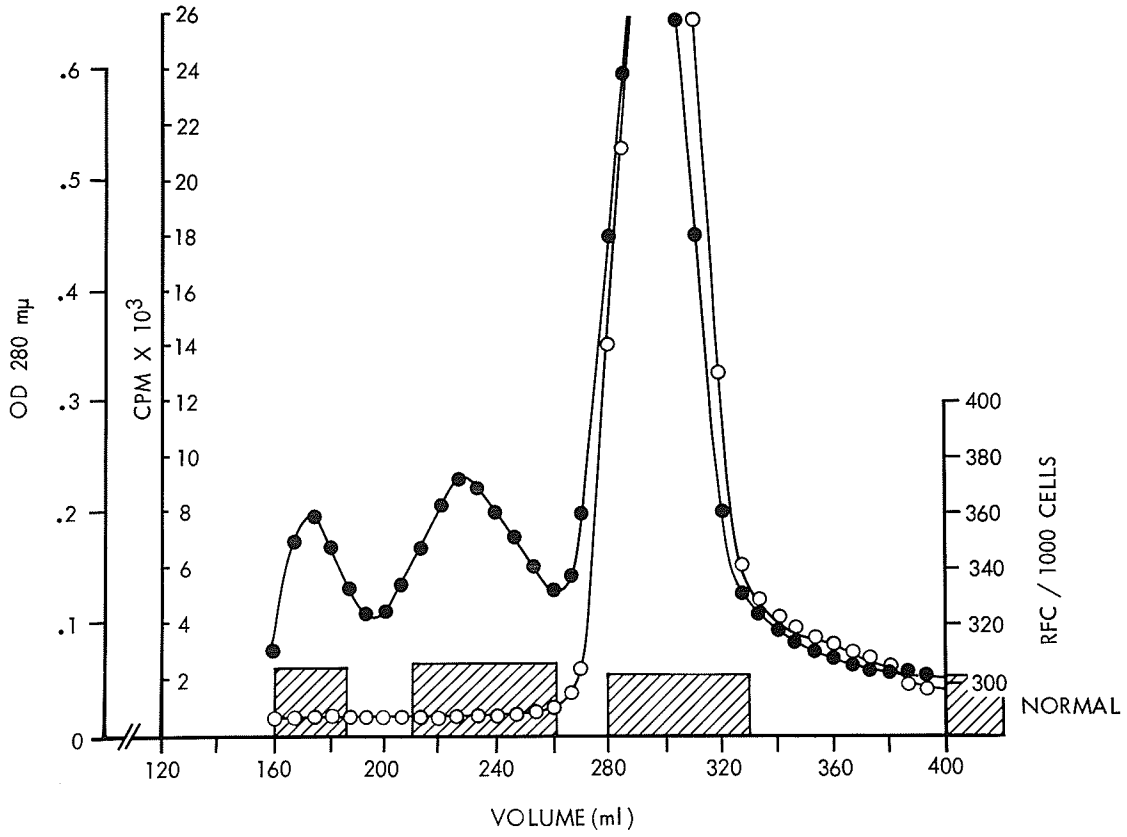


Figure 8: Sephadex G-200 elution pattern of a mixture of NMS+BSA. Open circles = CPM; filled circles = 280 mμ OD.

TABLE IV

Uptake of antigen by spleen cells as detected by inhibition of RICA

	---RFC/1000 SPLEEN CELLS---		
TREATMENT:	NONE	ANTI-FIB	ANTI-BSA
CONTROL	303±4(4)[a]	313±3(2)	303±6(3)
6 HR-MS-BSA	368±9(5)	354±10(4)	251±7(4)
6 HR-MS-FIB	377±12(5)	256±7(3)	361±8(4)

[a]Numbers in parentheses represent the number of experiments.

RFC is observed to 251±7. Such a result is not observed when rabbit anti-FIB is used. Similarly, if 6 HR-MS-FIB treated cells are subsequently treated with anti-FIB and examined by RICA, a reduction is observed to 257±7 RFC which is statistically significant ($p<0.001$). Once again, an antiserum to an unrelated antigen has no effect. Untreated spleen cells are not affected by the antiserum treatment.

These results strongly suggest the presence of antigen on the surface of treated spleen cells. The mechanism by which the antiserum inhibits RICA is probably one of steric hindrance as the rabbit antiserum would not be detected by RICA which detects only mouse immunoglobulin.

VIII. THE 4S FRACTION IN THE 6 HR-MS-BSA

As shown above, 4S BSA not injected in the animal, mixed with NMS in vitro does not give an active 7S fraction. Since 4S antigen already passed through the animal may be altered as shown by physicochemical techniques (Yuan et al, 1970) we have produced mixtures of the 4S peak from 6 HR-MS-BSA with NMS and after 30 minutes incubation at 37°C the mixture was applied on a Sephadex G-200 column. No radioactivity is eluted in the 19S peak and only a small amount (the counts were twice the background) is present in the 7S peak (Fig. 9). The fractions were examined with the RICA technique and as shown in Fig. 9 the 7S peak induces an increase of the rosette count comparable to that obtained with the 7S fraction of the 6 HR-MS-BSA (Fig. 6). These results show another important alteration of the 4S BSA when passed through the animal in addition to the change of the electrophoretic mobility demonstrated by Yuan et al (1970).

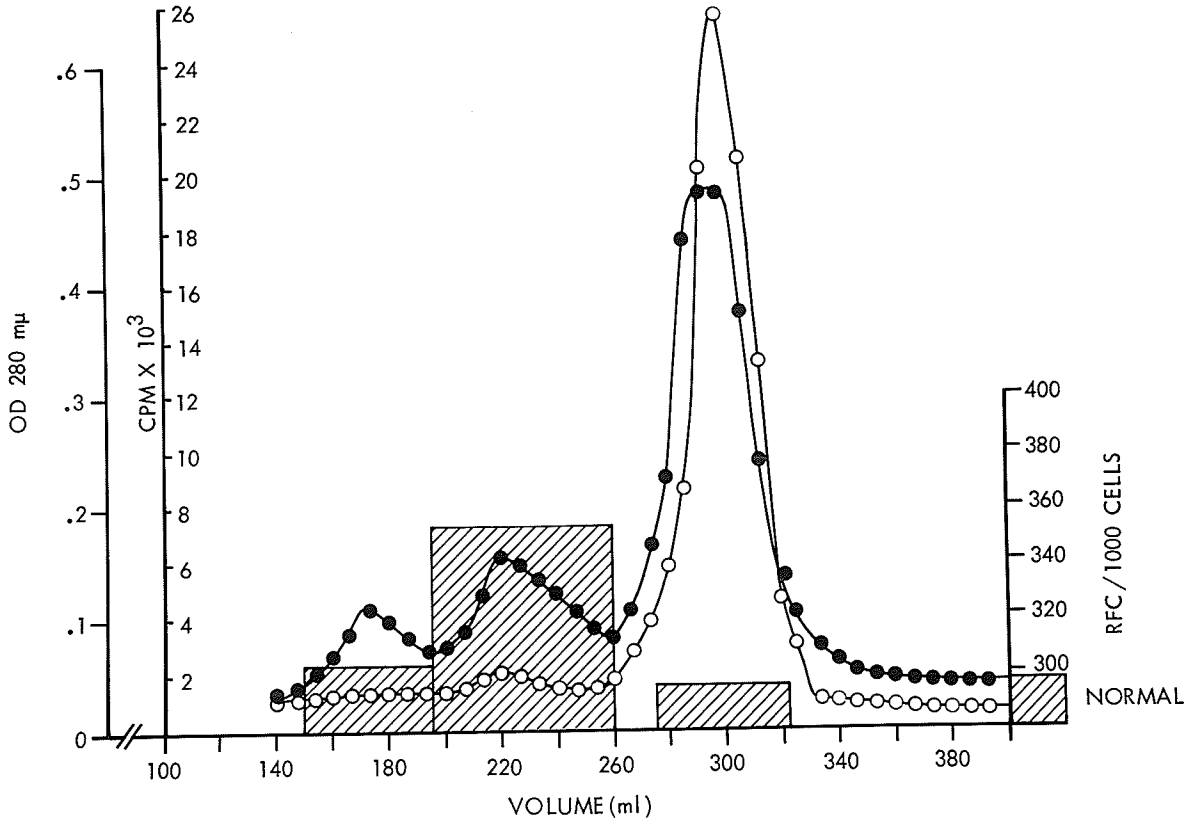


Figure 9: Sephadex G-200 elution pattern of a mixture of 4S fraction from 6 HR-MS-BSA and NMS. Open circles = CPM; filled circles = 280 mμ OD.

IX. FRACTIONATION OF THE 24 HR-MS-BSA

Serum collected 24 hours after injection of BSA-I^{125} emulsified in FCA was fractionated on a Sephadex G-200 column. The fractions were examined for their ability to induce an increase of the rosette count on normal spleen cells. As shown in Fig. 10, no radioactivity is detected in the 7S fraction which completely lacks the ability to increase the RFC of normal spleen cells in contrast to the 6 hour 7S fraction (Fig. 6). This suggests a rapid elimination of the antigen-antibody complex from the circulation.

X. EFFECT OF 6 HR-BSA SERUM ON Ig CARRYING CELLS OF NORMAL SPLEEN

Serum collected 6 hours after injection of BSA-I^{125} in saline was fractionated on a Sephadex G-200 column. As shown in Fig. 11, no radioactivity is detected in either of the 19S or 7S fractions. Radioactivity resides only in the 4S fraction. These fractions were examined for their ability to induce an increase in the rosette count on normal spleen cells. RICA tests performed with these fractions were unable to detect an increase in the RFC count by any of these fractions. Thus it appears that FCA incorporation with the antigen is necessary for the appearance of circulating cytophilic antigen-IgG complexes.

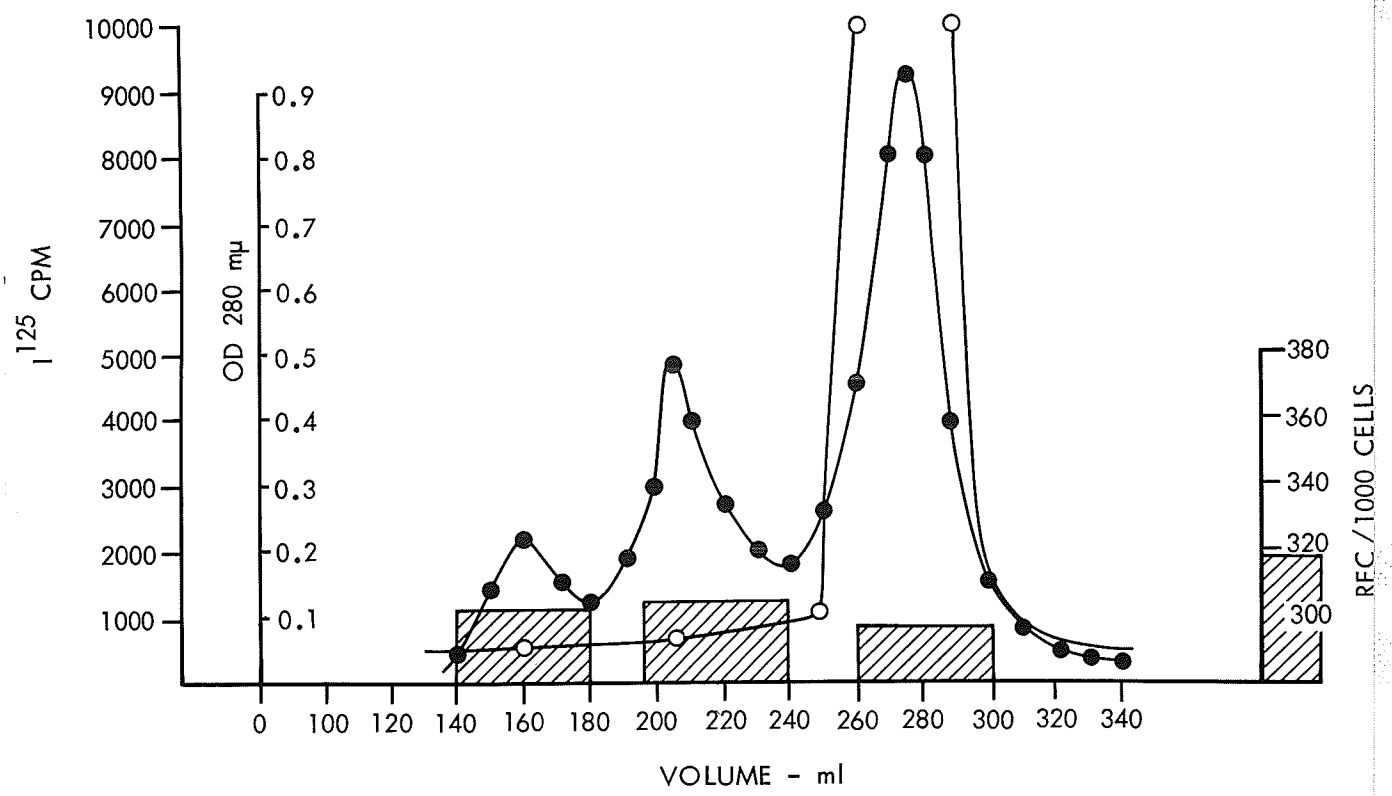


Figure 10: Sephadex G-200 elution pattern of 24 HR-MS-BSA. Open circles = CPM; filled circles = 280 mμ OD.

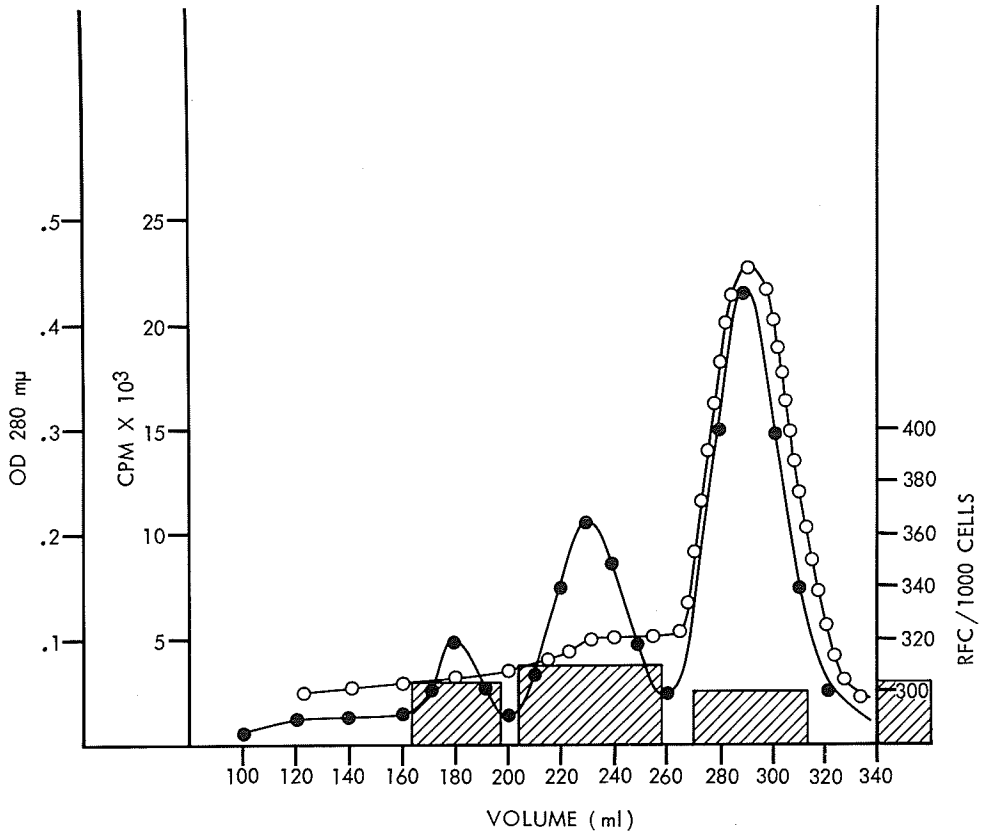


Figure 11: Sephadex G-200 elution pattern of 6 HR-BSA serum. Open circles = CPM; filled circles = 280 mμ OD.

SECTION B

INDUCTION OF SOLUBLE FACTORS BY FREUND'S COMPLETE ADJUVANT

The previous section demonstrated that while antigens incorporated in FCA induce the formation of a cytophilic complex, no such event is observed with soluble antigens in saline. Thus since only FCA is the differing substance in the two preparations, we next attempted to investigate the mechanism by which FCA mediates the formation of such a complex.

I. GENERATION OF CYTOPHILIC Ig IN FCA-S INCUBATED IN VITRO WITH ANTIGEN

Normal spleen cells exposed to FCA-S which was preincubated with any of the three antigens - BSA, EA or FIB - show an increase of Ig carrying cells to 371±27, 379±11 and 373±22 per 1000 spleen cells respectively (Fig. 12) ($p<0.001$). This increase amounts to 23% of the Ig carrying cell population which is present in normal spleen prior to treatment with FCA-S and antigen. FCA-S alone has no effect and none of the various controls used (NMS, antigen and NMS + antigen) show any increase.

II. TITRATION OF FCA-S

To determine whether the increase in RFC observed above was maximal, the FCA-S was titrated by the addition of varying amounts of BSA to the FCA-S before examination by RICA. These results are shown in Fig. 13 and demonstrate that at the dose of antigen commonly used (200-500 μg), maximal RFC formation is achieved. It should be noted that quantities of BSA as low as 5 ng when incubated with FCA-S and

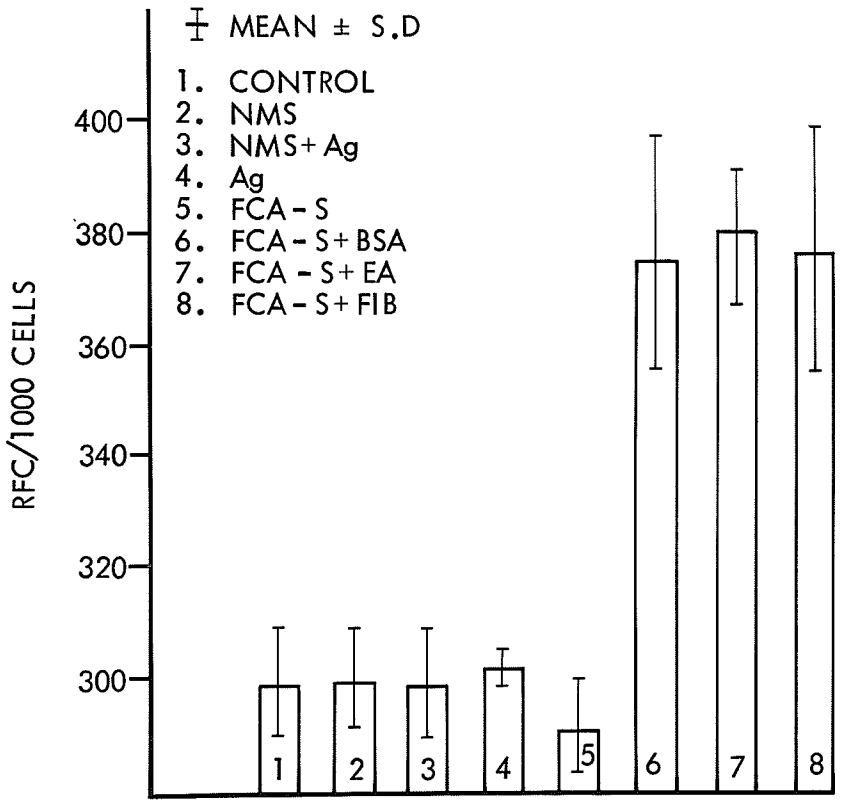


Figure 12: The generation of cytophilic Ig upon incubation in vitro of FCA-S with various soluble antigens. In columns 3 and 4 the results taken with all three antigens are grouped together. In this as well as subsequent figures each column represents a minimum of 4 experiments and as many as 10 experiments.

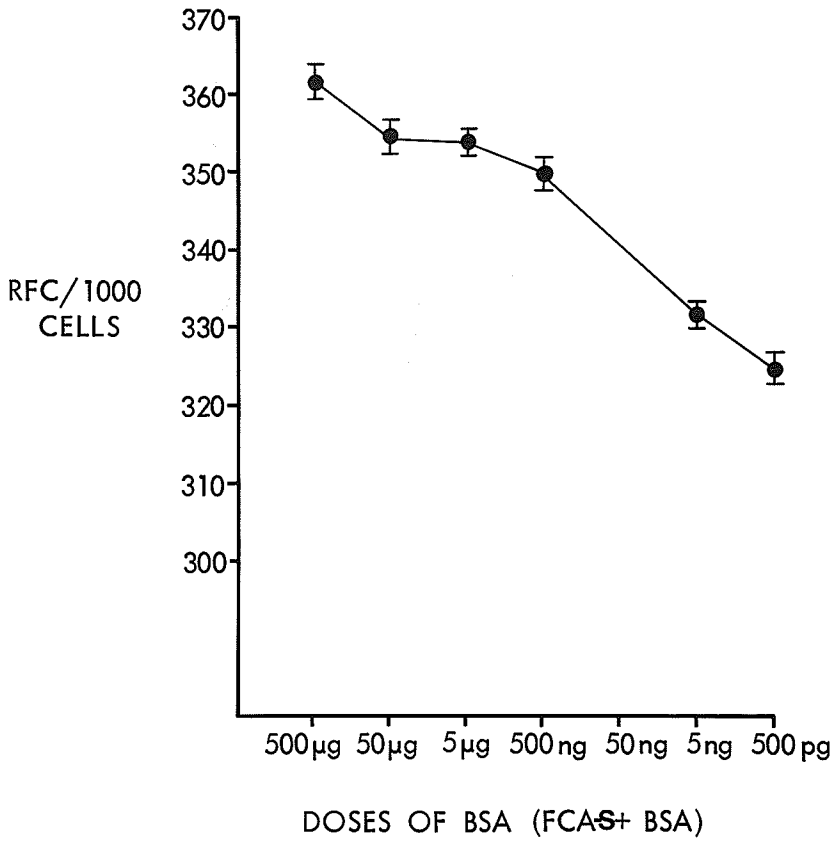


Figure 13: Titration of FCA-S.

spleen cells give significant increases in RFC.

III. ABSORPTION OF FCA-S

Aggregated EA, FIB, Fe and CRBC were used to absorb the FCA-S as indicated in Materials and Methods, section XVI. The absorbed FCA-S(A) was examined as above for its ability to induce an increase of the Ig carrying cells in the presence of BSA. As shown in Fig. 14, this property of FCA-S is lost after absorption. It is important to note that after absorption with any one antigen, (EA, FIB, Fe or CRBC) the serum loses its ability to generate the cytophilic Ig upon the addition of a different antigen. This indicates that the mechanism which generates cytophilic Ig in FCA-S shows no antigen specificity. Addition of NMS or NMS and antigen to the absorbed FCA-S does not restore its ability to generate the cytophilic Ig. A buffer of pH 2.5 was used to elute any material which was possibly absorbed on to the aggregate and the eluate was immediately neutralized. As shown in Fig. 14 and Table V, when BSA is added to the eluate, a cytophilic Ig is induced which is taken up by normal spleen cells, producing a marked increase of the rosette forming cells. This increase is the same as the one detected by whole FCA-S with added antigen. When the eluate is added to the absorbed serum it restores its ability to generate the cytophilic Ig after the addition of antigen. Cells treated with eluate alone do not show any increase of the RFC. Thus although Ig is present in the eluate as shown in an Ouchterlony plate it shows no cytophilic properties except only after the addition of the antigen.

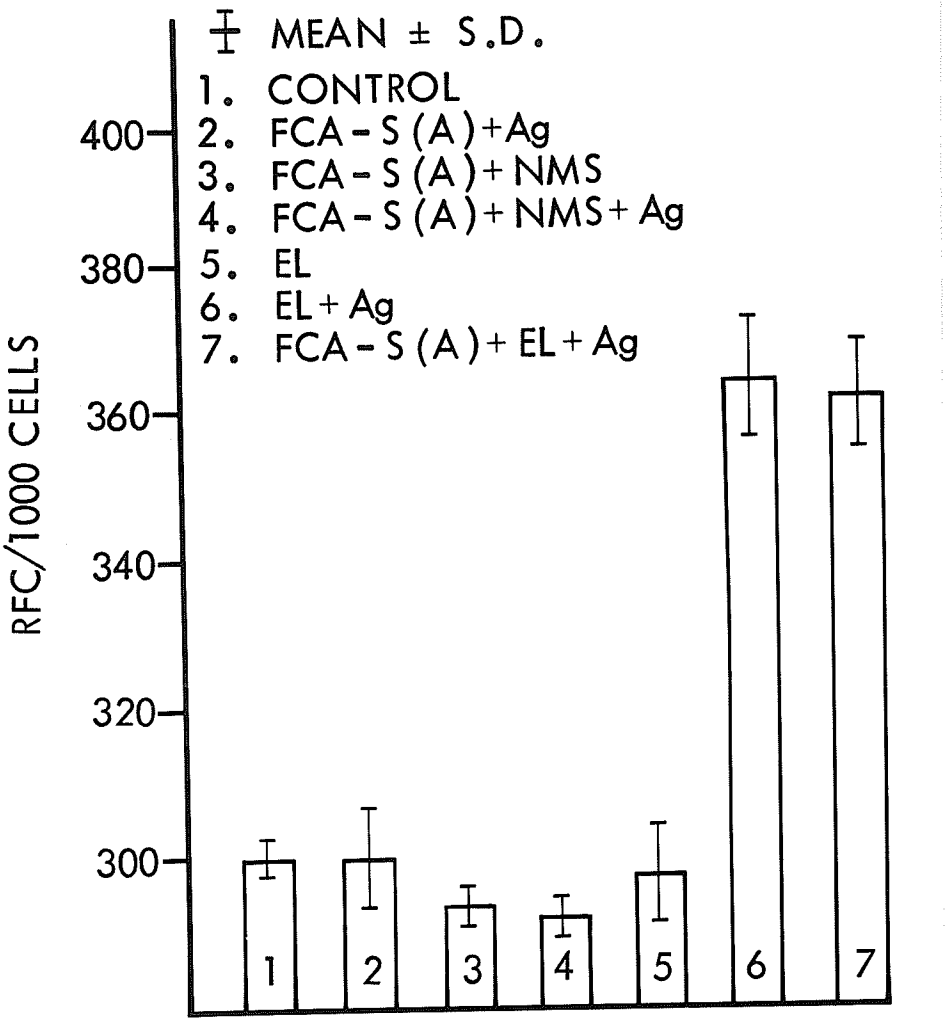


Figure 14: FCA-S loses its ability to form cytophilic Ig after absorption with aggregated antigen. Such activity is present in the eluate recovered upon acidification of the aggregate. EL = Eluate obtained at pH 2.5 from aggregated EA, FIB or Fe. BSA for antigen was used in all experiments in this figure.

TABLE V

Activity of eluates from aggregated antigens
used in the absorption of FCA-S

CELL TREATMENT	RFC/1000 SPLEEN CELLS ± S. D.
None	302±4(3)[a]
EL (FCA-Fe) + BSA	353±8(3)
EL (FCA-FIB) + BSA	357±7(3)
EL (FCA-EA) + BSA	365±12(3)

[a]Numbers in parentheses represent the number of tests performed.

IV. FRACTIONATION OF FCA-S

1. Localization of the FCA-S activity in the 7S fraction

Fractionation of FCA-S on Sephadex G-200 was performed in order to locate its activity. The 19S, 7S and 4S peaks were concentrated to the same volume as the sample applied to the column and the same amount of BSA was added to each fraction. After short incubation, normal spleen cells were exposed to the mixture of the fraction and antigen. As shown in Fig. 15 only the 7S fraction induced an increase of RFC in the presence of BSA but neither the 19S nor the 4S were active. The increase of the RFC produced by the 7S Ig and antigen mixture is the same as that produced by the whole serum and antigen (Fig. 12). None of the fractions alone show any activity.

2. The 4S fraction of FCA-S

The 4S peak either alone or in the presence of antigen was found to lack the ability to produce an increase of the Ig carrying cells in normal spleen cells (Fig. 16). However, since our assay system detects the generation of cytophilic Ig and the 4S fraction is devoid of Ig, it was examined with the addition of NMS (to provide normal Ig) and antigen. Under these conditions an increase of RFC is observed (362 ± 4) which is within the same range as that detected with unfractionated FCA-S ($p < 0.001$). Furthermore, fractions of NMS were used and as shown in Fig. 16 only with the 7S fraction of NMS incubated with antigen and 4S fraction of the FCA-S activity was shown. Finally, 5S fragments from pepsin digested 7S mouse Ig were added to the 4S FCA-S and the mixture was examined in the presence of antigen. No increase of the Ig carrying cells is detected as shown in the last column of Fig. 16.

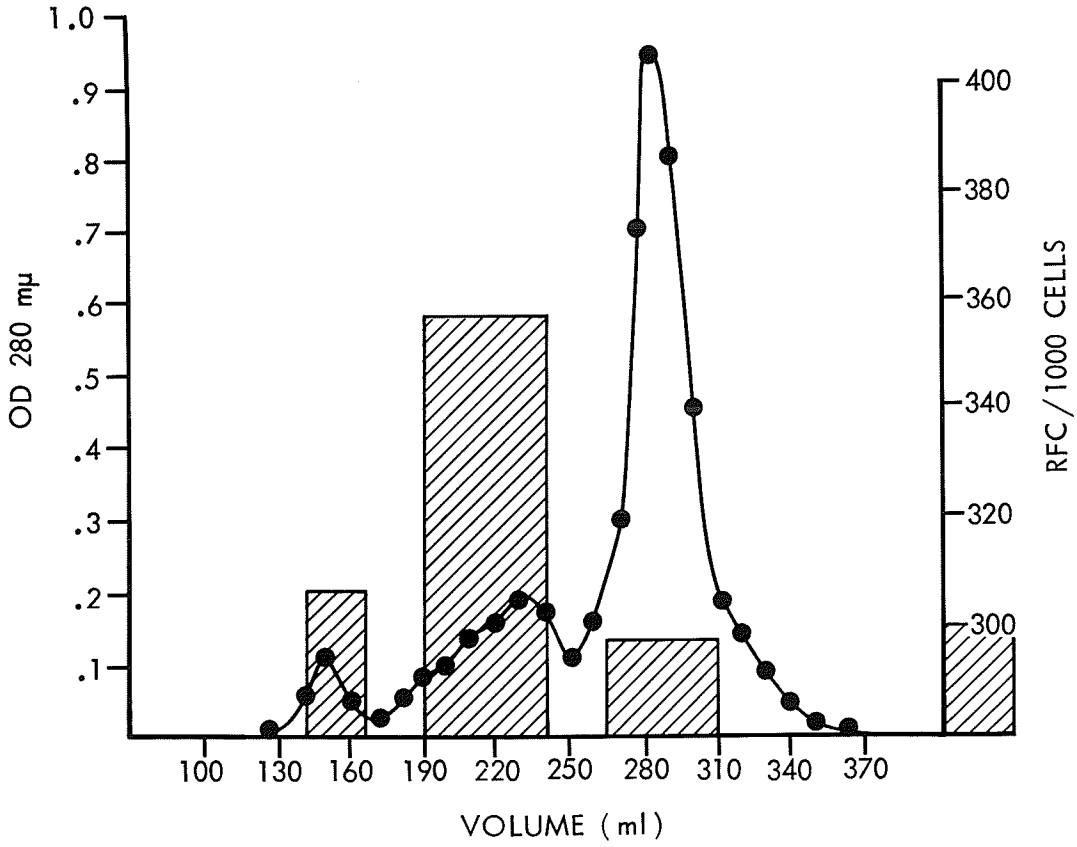


Figure 15: Fractionation of FCA-S on Sephadex G-200. The crossed bars represent number of RFC detected by RICA. The small cross bar on the far right of the figure represents the normal RFC count.

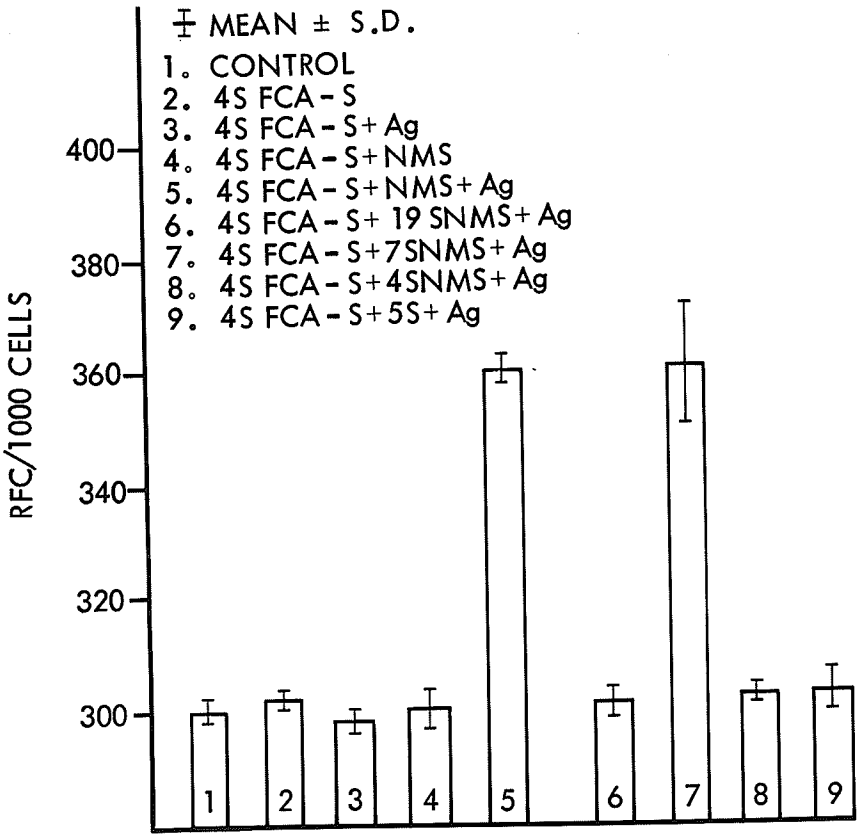


Figure 16: The generation of cytophilic Ig by 4S fraction of FCA-S after addition of NMS (column 5) or 7S fraction from NMS (column 7) and antigen. BSA was used as antigen in all experiments in this figure.

These results suggest that the 4S fraction of FCA-S contains a factor which in the presence of 7S Ig and antigen induces a significant increase of the Ig carrying cells in normal spleen cell populations.

3. The mechanism of action of the 4S factor

In order to understand the mechanism of action of the soluble factor, spleen cells were exposed to the 4S fraction alone and after washing they were subsequently treated with antigen, or NMS, or a mixture of both. In other experiments the spleen cells were first exposed to a mixture of 4S factor and antigen or 4S factor and NMS and after washing they were treated with NMS or antigen respectively. None of these various combinations resulted in an increase of the Ig carrying cells.

The results from these experiments suggest that for the increase of Ig carrying cells, the 4S factor in the FCA-S, 7S Ig and antigen need to be present simultaneously. It is also unlikely that the factor in the FCA-S acts separately on the cell surface with subsequent uptake of normal Ig.

V. THE NATURE OF THE CELL CONTRIBUTING TO THE INCREASE OF RFC

Normal spleen cells were treated with anti-θ serum and guinea pig complement. This treatment eliminates 27-29% of the spleen cells, those considered to be thymus derived. After such treatment, the number of RFC/1000 spleen cells increases to 416±6 (Table VI A). This is only a relative increase due to the elimination of the T cells which by RICA are not detectable as carrying surface Ig. When anti-θ treated cells were exposed to FCA-S to which BSA was added, no increase of the RFC

TABLE VI

Uptake of cytophilic Ig by T cells

A		B	
CELL TREATMENT	RFC/1000 SPLEEN CELLS ± S.D.	CELL TREATMENT	% Θ-POSITIVE CELLS ± S.D.
None	312±12(4)[a]	None	29±1.0(3)
αΘ+C[b]	416±6(2)	FCA-S+FIB	18±0.4(3)
αΘ+C+[FCA-S+Ag[c]]	409±14(4)	FCA-S+BSA	18±3.6(3)
αMIg+C[e]	39±5(2)	EL[d]+BSA	19±0.5(4)
αMIg+C+[FCA-S+Ag[c]]	94±15(4)	FCA-S	28±0.9(3)
		Ag[c]	28±0.6(3)

[a] The number of experiments is shown in parentheses.

[b] αΘ + C = AKR anti-Θ C3H serum plus guinea pig complement.

[c] Ag = BSA.

[d] EL = Eluate obtained at low pH from aggregated EA incubated with FCA-S.

[e] αMIg + C = Rabbit antimouse Ig and guinea pig C.

was observed (409±14, Table VI A). This indicates that elimination of the T cell abolishes the ability of the spleen cells to show an increase of the RFC and suggests that the increase of the Ig carrying cells upon exposure of spleen cells to FCA-S and antigen is due to the uptake of the cytophilic Ig by a thymus derived cell. This is further supported by experiments where the Ig carrying cells were eliminated by a rabbit anti-mouse Ig serum. Such treatment reduces the number of RFC to 39±5 per 1000 spleen cells, but a rise in the number of Ig carrying cells is observed after exposure to FCA-S and antigen (94±15, Table VI A).

Alternately the T cell may not directly take up the cytophilic Ig but T cell action is needed in order for other cells to produce an increase of Ig carrying cells. When spleen cells are exposed to FCA-S and BSA at $4^{\circ}C$, where cell activity is abolished, we observe the same increase of RFC. Furthermore thymocytes which normally form no rosettes by RICA, after exposure to FCA-S and BSA show 50 to 60 RFC per 1000 cells which is statistically significant ($p<0.001$).

These experiments suggest that activation of T cells is not likely to act as the mediating mechanism for the uptake of cytophilic Ig but rather this globulin is taken up directly by the T cell.

It was also shown that exposure of spleen cells to FCA-S with antigen causes a decrease in the number of Θ carrying cells detected by cytotoxicity with an anti-Θ serum by about 11% (Table VI B). Such a decrease was also observed when the eluate and antigen was used. FCA-S alone and antigen alone showed no effect.

Although the mechanism of this phenomenon is unknown it is likely that the uptake of the cytophilic Ig by the T cells renders the Θ antigen unreactive with the anti-Θ serum.

VI. ALTERATION OF SURFACE Ig OF B CELLS

Uptake of cytophilic Ig by the B cells which carry already on their surface Ig detectable by RICA, is more difficult to demonstrate. However, if the cytophilic Ig belongs to one Ig class, when it is taken up by a lymphocyte which carries Ig of a different class, it is possible that it may cause interference in the detection of such surface Ig by a specific hybrid antibody.

Three hybrid antibodies were used in single RICA tests. αMIg- αFe detects all mouse Ig classes; while $\alpha\gamma$- αFe and $\alpha\emptyset$- αEA detect IgG and IgF globulins respectively. The results are shown in Table VII. Cells exposed to FCA-S mixed with FIB or BSA show an increase of RFC by αMIg- αFe hybrid antibody. Since a similar increase is detected by the $\alpha\gamma$- αFe hybrid antibody it is likely that the cells which demonstrate surface Ig after treatment with FCA-S and Ag may do so by acquiring an IgG globulin. The number of RFC by the $\alpha\emptyset$- αEA hybrid in normal spleen cells is 287 ± 9. After exposure to FCA-S in the presence of FIB or BSA, this number is reduced to 242 ± 13 and 257 ± 9 respectively.

Although this difference may appear small, because of the high degree of reproducibility of RICA it is statistically highly significant ($p<0.001$). The same results were obtained with eluate in the presence of BSA. When the fractions of the FCA-S were used in the presence of BSA, the 7S fraction gave an increase of RFC (257 ± 1) by $\alpha\gamma$- αFe hybrid antibody and a decrease of RFC (232 ± 2) by $\alpha\emptyset$- αEA. The decrease of the cells carrying surface IgF globulin is difficult to explain. We have no evidence that the cells which "lose" surface IgF are directly involved with the uptake of the cytophilic IgG. If this is the case, the uptake of IgG globulin may sterically interfere

TABLE VII

Alteration of surface Ig of B cells

CELL TREATMENT	---RFC/1000 SPLEEN CELLS ± S. D.---		
	αMIg- αFe	αγ- αFe	αØ- αEA
None	301±3(7)[a]	195±7(5)	287±9(5)
FCA-S + FIB	378±22(5)	270±23(5)	242±13(5)
FCA-S + BSA	396±15(3)	265±3(3)	257±9(3)
EL + BSA	366±14(5)	257±3(3)	243±22(6)
FCA	285±18(9)	-	-
Ag[b]	303±4(8)	-	-
19S + Ag[b]	305±9(4)	196±4(2)	303±3(2)
7S + Ag[b]	382±25(6)	257±1(2)	232±2(2)
4S + Ag[b]	296±4(6)	205±1(2)	297±1(2)

[a]The number of experiments is shown in parentheses.

[b]Ag = BSA.

with the detection of the surface IgF globulin.

VII. UPTAKE BY SPLEEN CELLS OF ANTIGEN MEDIATED BY FCA-S

1. Inhibition of RICA

Treatment of normal spleen cells with a mixture of FCA-S and BSA or FIB results in an increase of Ig carrying cells to 365±8 and 359±13 respectively (Table VIII). However, treatment of normal cells with FCA-S and BSA followed by rabbit anti-BSA antiserum results in a decrease in the number of Ig carrying cells to 264±5 per 1000 spleen cells. This effect is specific since cells exposed to FCA-S and BSA do not show a similar decrease after treatment with rabbit anti-FIB (357±9). Similarly, treatment of spleen cells with FCA-S and FIB followed by treatment with rabbit anti-FIB results in a decrease of Ig carrying cells to 253±3 per 1000 spleen cells, but once again rabbit anti-BSA antiserum has no effect (365±8).

These results suggest the presence of antigen on the surface of spleen cells treated with FCA-S and antigen. Treatment of such cells with a rabbit antiserum specific for that antigen interferes with the detection of mouse Ig.

2. Cytotoxicity test

Mouse spleen cells exposed to FCA-S in the presence of antigen were tested with an antiserum specific for the antigen used and complement. The number of cells killed was determined with the trypan blue exclusion method. As shown in Table IX, 13.7±2.7% of spleen cells exposed to FCA-S in the presence of BSA are killed by the anti-BSA antiserum. Similarly, anti-FIB antiserum is cytotoxic for 16.9±2.1%

TABLE VIII

Uptake of antigen mediated by FCA-S as detected by inhibition of RICA

	---RFC/1000 SPLEEN CELLS ± S. D.---		
TREATMENT	NONE	ANTI-FIB	ANTI-BSA
CONTROL	303±4(4)[a]	313±3(2)	303±6(3)
FCA-S + BSA	365±8(4)	357±9(3)	264±5(4)
FCA-S + FIB	359±13(3)	253±3(3)	365±8(3)

[a]The number of experiments is shown in parentheses.

TABLE IX

Uptake of antigen by spleen cells as detected by cytotoxicity tests

	---% CYTOTOXICITY ± S. D.---	
TREATMENT	ANTI-BSA	ANTI-FIB
FCA-S + BSA	13.7±2.7(5)[a]	1.91±1.53(4)
FCA-S + FIB	3.16±0.71(4)	16.9±2.1(5)
FCA-S	3.7±1.6(4)	1.2±0.6(3)
BSA	1.0±1.2(4)	-
FIB	-	1.0±1.2(5)
NMS + BSA	0.7±0.4(3)	-
NMS + FIB	-	2.2±1.4(4)

[a]The numbers in parentheses represent the number of tests performed.

of the spleen cells exposed to FCA-S and FIB. Cells exposed to either one of the antigens alone or to a mixture of NMS and antigen show no sensitivity to the corresponding antiserum. Similarly, cells exposed to FCA-S and antigen are not killed by an unrelated antiserum in the presence of complement (1.91±1.53 and 3.16±.71% respectively).

The main question which is raised by these data is whether the cells affected by the FCA-S in the presence of antigen as determined by the cytotoxicity method with an antiserum against the antigen, are the same as the ones detected by RICA. For BSA, the changes detected by RICA are the increase of RFC (75 per 1000 cells) and the decrease of cells carrying IgF (30 per 1000 cells). The sum of the two is 105 cells or 10.5%. This number is somewhat smaller than the 13.7% detected by cytotoxicity; however, the difference may be due to differences in the sensitivity between the two methods.

Similarly for FIB, the sum of changes detected by RICA is 122 cells or 12.2% which is also slightly lower than 16.9% detected by cytotoxicity. If the cells which RICA show to be affected by the FCA-S and antigen are the same as those detected by the cytotoxicity technique, it may be reasonable to assume that the cytophilic IgG may represent a complex of Ig with antigen.

VIII. FIA-S

Serum was collected 6 hours after injection of FIA and was examined in RICA tests after the addition of antigen. As shown in Table X, no increase of the RFC is detected. No cytophilic Ig is detected when the FIA-S is mixed with NMS and antigen. The fractions of the FIA-S from a Sephadex G-200 column were also examined but no cytophilic Ig

TABLE X

Effect of serum from FIA injected mice
on normal spleen cells

TREATMENT	RFC/1000 SPLEEN CELLS ± S. D.
None	300±8.0(4)[a]
FIA-S	297±10.0(6)
FIA-S + NMS	299±2.0(4)
FIA-S + Ag[b]	295±13.5(8)
FIA-S + NMS + Ag[b]	296±4.1(4)

[a]The number of experiments performed is shown in parentheses.

[b]Ag = BSA.

was detected in any of the fractions after addition of antigen (Fig. 17). The 4S fraction was not examined after the addition of NMS (or 7S from NMS) because NMS added to whole FIA-S and antigen did not produce cytophilic Ig.

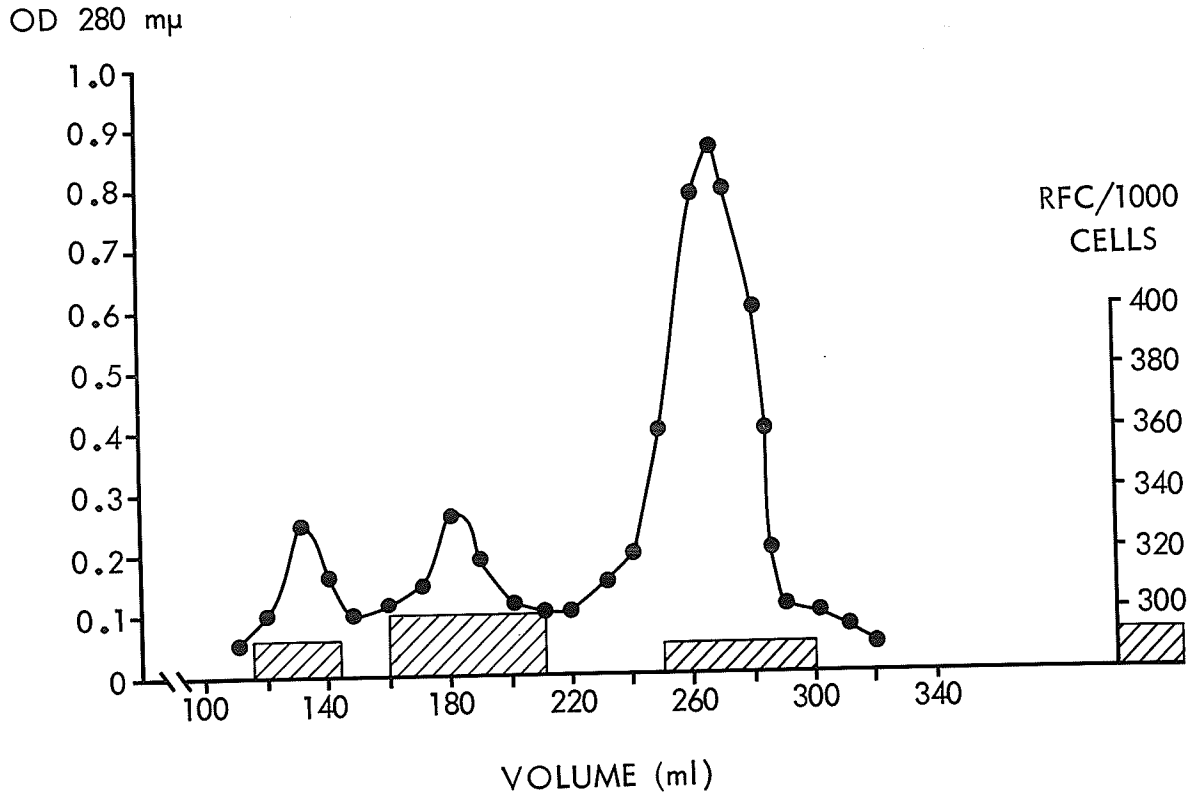


Figure 17: Sephadex G-200 elution profile of FIA-S. The crossed bars represent the number of RFC detected by RICA. The small cross bar on the far right of the figure represents the normal RFC count.

SECTION C

THE ACTIVE PRINCIPLE IN FCA RESPONSIBLE FOR INDUCTION OF SOLUBLE FACTOR

Having defined the ability of FCA to induce a soluble factor which can generate cytophilic immunoglobulin in the presence of NMS and antigen, we next attempted to define the component of FCA which was responsible for this capacity.

I. BCG-IFA-S

Since we have observed that FCA is active at generating cytophilic Ig while IFA is not, it was obvious that the mycobacteria must be responsible for this phenomenon. We therefore emulsified 1 mg/ml BCG into IFA and injected this into mice. Six hours later we collected the serum (BCG-IFA-S) and tested it for the ability to generate cytophilic Ig in the presence of antigen as done previously for FCA-S (Table XI).

It can be seen from Table XI that while BCG-IFA-S itself does not increase the RFC count of normal spleen cells (290 ± 7), addition of antigen results in the generation of cytophilic Ig (367 ± 11, $p<0.001$). These results strongly support the contention that the mycobacteria are responsible for the induction of cytophilic Ig by the whole FCA preparation.

II. PPD-S

PPD is a tuberculin purified protein derivative prepared from a human strain of Mycobacterium tuberculosis grown on a protein free synthetic medium.

TABLE XI

Generation of cytophilic Ig by BCG-IFA-S

TREATMENT	RFC/1000 SPLEEN CELLS ± S. D.
None	304±6(4)[a]
BCG-IFA-S	290±7(3)
BCG-IFA-S + BSA	367±11(5)

[a]Numbers in parentheses represent the number of tests performed.

In an attempt to determine what portion of the mycobacteria may be responsible for inducing cytophilic Ig, we injected mice with PPD and 6 hours later collected the serum (PPD-S). We tested the ability of this serum to increase the RFC count of normal spleen cells in the presence of antigen (Table XII).

Neither the PPD-S nor the PPD-S to which antigen had been added had the ability to increase the RFC of normal spleen cells (303 ± 4 versus 289 ± 16 and 291 ± 14 respectively) thus suggesting that the PPD component is not responsible for the activity of FCA as previously observed.

III. MAAF-S

MAAF (mycobacteria adjuvant antitumor fraction) is a water soluble lipid-free fraction from BCG. It has a molecular weight of 32,000 and is composed mainly of neutral sugars. Hiu (1972) has demonstrated that this substance enhances cell-mediated and humoral immune responses, thus acting as an adjuvant.

Serum collected from mice 6 hours after injection of MAAF was tested for its ability to induce a cytophilic Ig in the presence of antigen. As shown in Table XIII, while the serum itself could not generate cytophilic Ig (310 ± 5) addition of antigen to the serum was active (365 ± 10, $p<0.001$). Incubation of cells with a mixture of MAAF, NMS and antigen had no effect on the RFC of normal spleen cells (304 ± 3).

6 HR-MAAF-S was also fractionated on a Sephadex G-200 column (Fig. 18) and the peaks tested for activity by addition of antigen (BSA) as performed previously with FCA-S. Activity was detected only in the 7S fraction, a situation analogous to that of FCA-S. Furthermore, addition of NMS and Ag to the 4S fraction of MAAF-S resulted in

TABLE XII

Effect of PPD-S on normal spleen cells

TREATMENT	RFC/1000 SPLEEN CELLS ± S. D.
None	303±4(4)[a]
PPD-S	289±16(6)
PPD-S + BSA	291±14(6)

[a]The number of tests performed is in parentheses.

TABLE XIII

Generation of cytophilic Ig by 6 HR-MAAF-S

TREATMENT	RFC/1000 SPLEEN CELLS ± S. D.
None	305±4(4)[a]
MAAF-S	310±5(5)
MAAF-S + BSA	365±10(5)
MAAF + BSA + NMS	304±3(3)
4S MAAF-S + NMS + BSA	383±8(4)

[a]Numbers in parentheses represent tests performed.

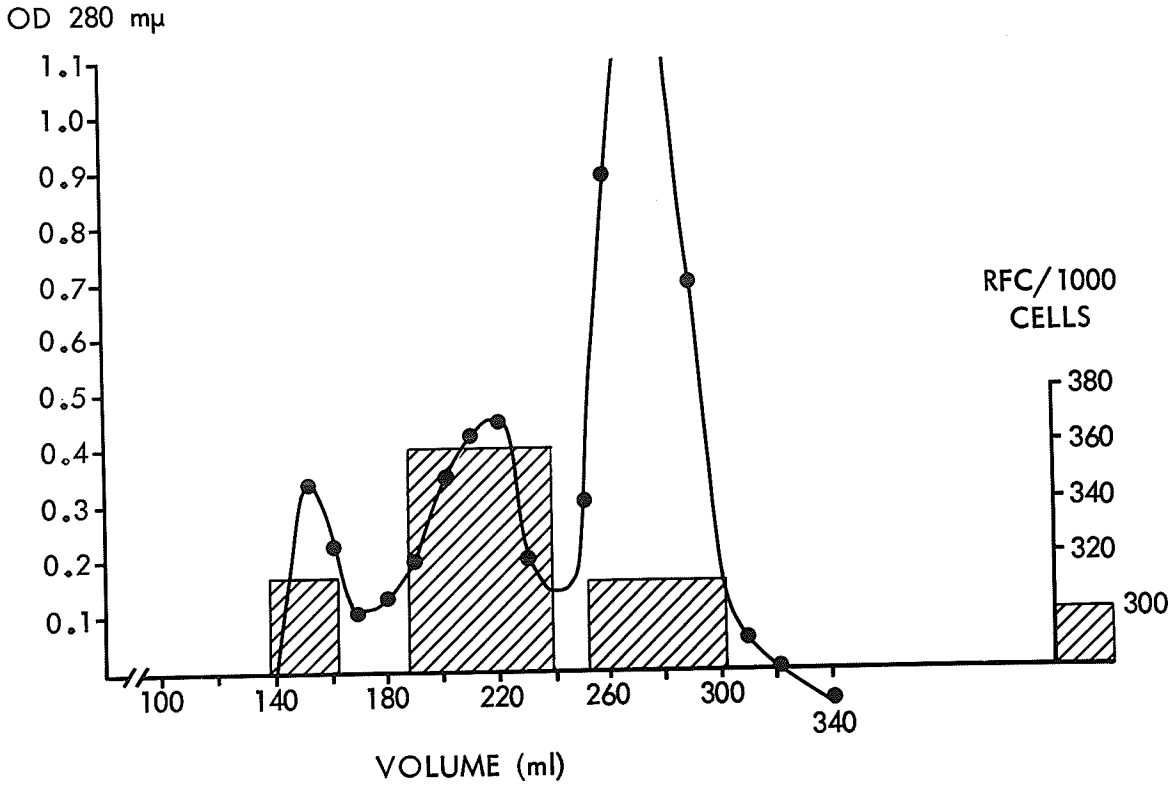


Figure 18: Fractionation of 6 HR-MAAF-S on Sephadex G-200. The crossed bars represent the number of RFC detected by RICA after the addition of antigen. The small cross bar on the far right of the figure represents the normal RFC count.

generation of a cytophilic Ig as detected by an increase in RFC to 383±8 (Table XIII).

Thus MAAF reproduces all the results obtained using FCA. It may be that the activity observed after injection of FCA is due mainly to this small fragment from the BCG in the adjuvant.

SECTION D

CELL ORIGIN OF SOLUBLE FACTOR(S) AND METHODS OF INDUCTION

We have defined above a component of FCA, which has the ability to induce the soluble factor. This soluble factor, in the presence of NMS and antigen, has the capacity to produce cytophilic Ig. Furthermore we have suggested that this may represent a complex of antigen and IgG globulin.

The question arising from these studies was on the origin of this soluble factor. Thus this section will confine itself to defining the cell origin as well as some other substances which can induce its formation.

I. IN VITRO INDUCTION OF SOLUBLE FACTOR(S) BY BCG

We have previously demonstrated that BCG or mycobacteria is the active component in FCA. We therefore constructed an in vitro culture system for induction of soluble factors.

Various lymphoid organs were cultured for 30-45 minutes in the presence of BCG (100 μg), washed and cultured for a further 3 hours at 37^{o}C in an incubator. The mixture was centrifuged and the supernatant collected and neutralized. The ability of these supernatants to induce cytophilic Ig was tested by the addition of NMS, Ag or both to the supernates prior to treatment of normal spleen cells (Fig. 3). Induction of cytophilic Ig was measured as an increase in RFC formation of treated normal spleen cells.

1. In vitro cultured spleen cells

Spleen cells (6-120x10^{6}) were cultured in the presence of BCG as

described above. The ability of these supernatants to induce increased RFC formation (cytophilic Ig) to normal spleen cells was tested by the addition of NMS, antigen or both. These results are shown in Fig. 19.

Supernates obtained from spleen cells cultured in BCG are unable to induce cytophilic Ig to normal spleen cells when tested alone (column 2), or with NMS (column 3). However, addition of either antigen (column 4) or NMS+Ag (column 5) to the supernates results in an increase of RFC formation of normal spleen cells equivalent to that of FCA-S and antigen (Fig. 12). Control experiments in which spleen cells were cultured without BCG were negative at all combinations tested (Table XIV). The ability of supernates from BCG cultured spleen cells to induce cytophilic Ig in the presence of antigen suggests the possibility that some Ig had been shed from the surface of spleen cells during culture, thus abolishing the requirement for exogenous immunoglobulin.

When we cultured anti-Θ treated spleen cells (B cells) with BCG (Fig. 19), no activity was observed in the resulting supernates even upon addition of antigen (column 4) or NMS and Ag (column 5). That is, elimination of thymus derived cells abrogates the ability of the supernates to induce cytophilic Ig. These results strongly suggest that the thymus derived cell (T cell) may be the cell responsible for production of a soluble factor which can, in the presence of NMS and Ag, induce cytophilic Ig.

2. <u>In vitro cultured lymph node and thymus cells</u>

Similar experiments as described above were undertaken by utilizing lymph node and thymus cells in the presence of BCG. These results are shown in Fig. 20. While supernatants obtained from thymocytes are unable to induce cytophilic Ig either alone (column 2), with NMS

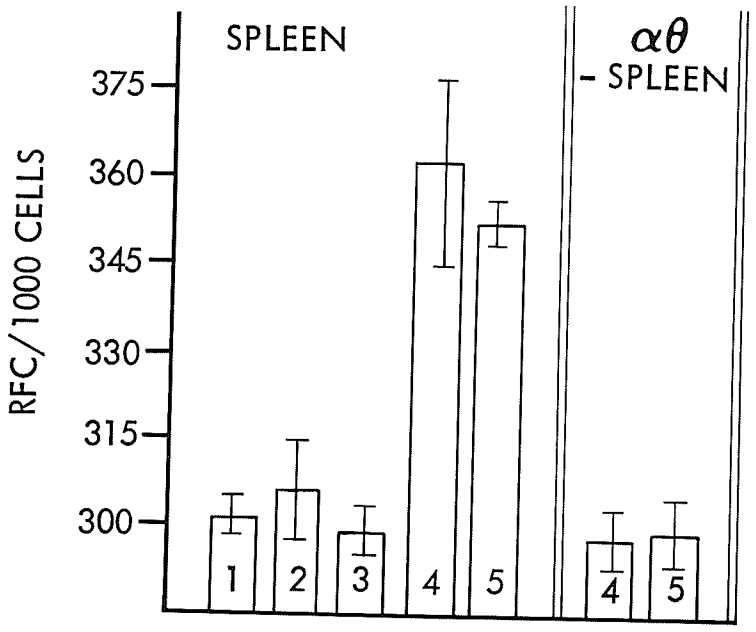


Figure 19: Supernates from cultures of spleen cells with BCG (BCG-S)
$\overline{\underline{\mathrm{I}}}$ = mean $\pm$ S. D. 1. Control 2. BCG-S
3. BCG-S + NMS 4. BCG-S + Ag 5. BCG-S + NMS + Ag

TABLE XIV

Supernates from lymphoid cells cultured in Hank's solution (NORs)

CELL TYPE	---RFC/1000 SPLEEN CELLS ± S. D.---				
	CON	NORs	NORs + Ag	NORs + NMS	NORs+NMS+Ag
LYMPH NODE $6\text{-}30\times10^6$	305±3(2)[a]	298±2(2)	301±7(2)	301±7(2)	300±1(2)
SPLEEN $6\text{-}120\times10^6$	300±2(6)	303±3(3)	300±3(3)	-	297±3(3)
THYMUS 40×10^6	301±1(3)	302±1(3)	299±3(3)	-	305±5(3)
THYMUS 6×10^6	307±13(5)	302±10(3)	303±10(3)	303±14(3)	311±8(3)
MACROPHAGES 6×10^6	299±2(3)	302±7(3)	-	306±9(3)	295±5(3)
BONE MARROW 30×10^6	301±1(3)	299±7(3)	304±4(3)	-	299±1(3)
HYDROCORTISONE TREATED SPLEEN	291±3(3)	299±4(3)	294±4(3)	299±14(3)	297±7(3)
THYMUS 6×10^6	296±7(3)	307±8(3)	289±5(3)	304±9(3)	300±11(3)

[a]The number in parentheses is the number of tests performed.

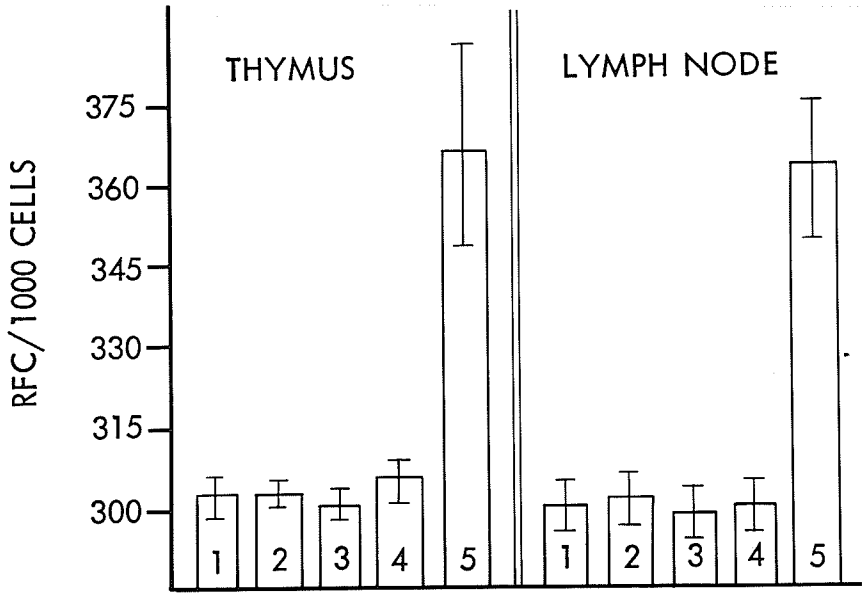


Figure 20: Supernates from cultures of thymus and lymph node cells with BCG (BCG-S). $\overline{\underline{\mathrm{I}}}$ = mean $\pm$ S. D. 1. Control 2. BCG-S 3. BCG-S+NMS 4. BCG-S+Ag 5. BCG-S+NMS+Ag

(column 3) or with Ag (column 4); addition of NMS and antigen results in the generation of cytophilic Ig as detected by an increase of RFC formation of normal spleen cells (column 5). Thymocytes cultured without BCG are inactive at all combinations tested (Table XIV). Lymph node cells show similar results to those described for thymocytes (Fig. 20).

These results clearly establish that thymus derived cells are those cells responsible for production of the soluble factor. As previously described, this soluble factor can, in the presence of NMS and antigen, induce cytophilic Ig which is taken up by normal spleen T and B lymphocytes.

3. In vitro cultured macrophages and bone marrow cells

Macrophages ($6x10^6$) and bone marrow cells ($30x10^6$) were cultured in the presence of BCG as described above. The resulting supernates were tested for the presence of the soluble factor. As shown in Fig. 21, no activity is observed with either cell population at any of the combinations tested. Similarly, cultures of these lymphoid cells in the absence of BCG are also negative (Table XIV).

II. EFFECT OF HYDROCORTISONE ON IN VITRO INDUCTION OF SOLUBLE FACTOR(S)

Mice were treated with hydrocortisone acetate 2 days before removal of the thymus and spleen. Such hydrocortisone resistant cells were cultured in the presence of BCG as previously described. The supernatants thus obtained were tested as before for their ability to generate cytophilic Ig.

These results are shown in Fig. 22. Spleen cell supernatants

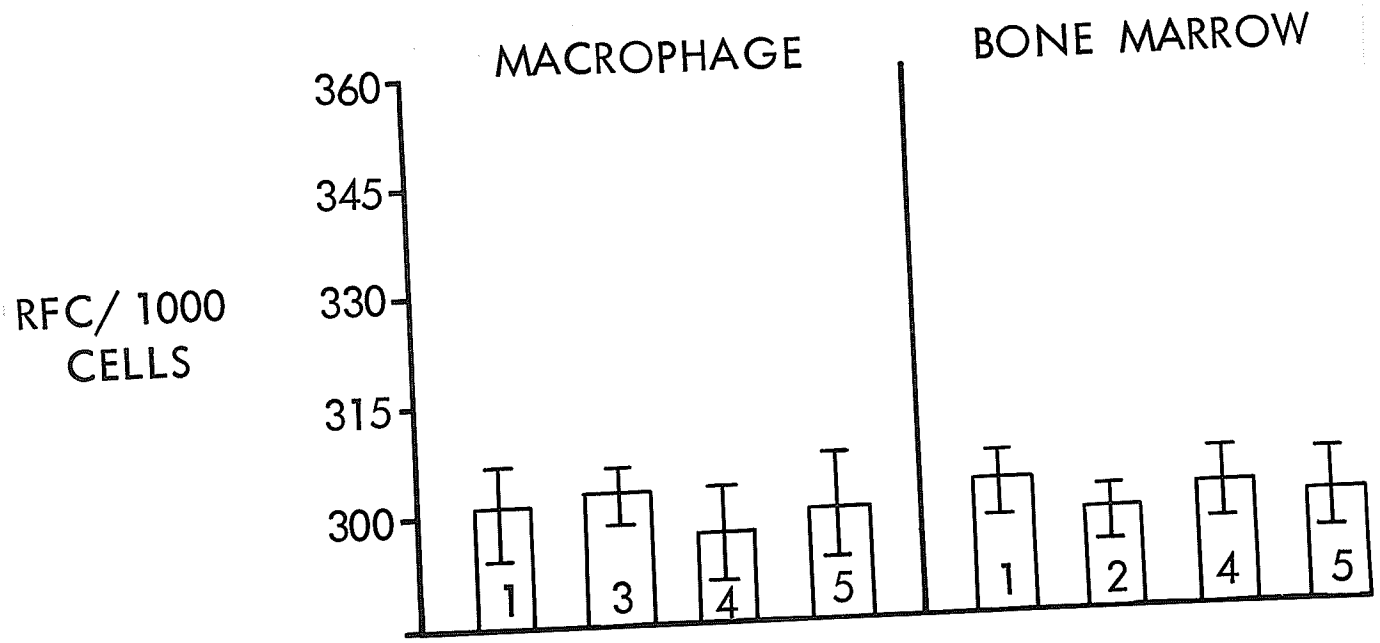


Figure 21: Supernates from cultures of bone marrow cells and macrophages with BCG (BCG-S). $\overline{\underline{\mathrm{I}}}$ = mean $\pm$ S. D. 1. Control 2. BCG-S 3. BCG-S+NMS 4. BCG-S+Ag 5. BCG-S+NMS+Ag

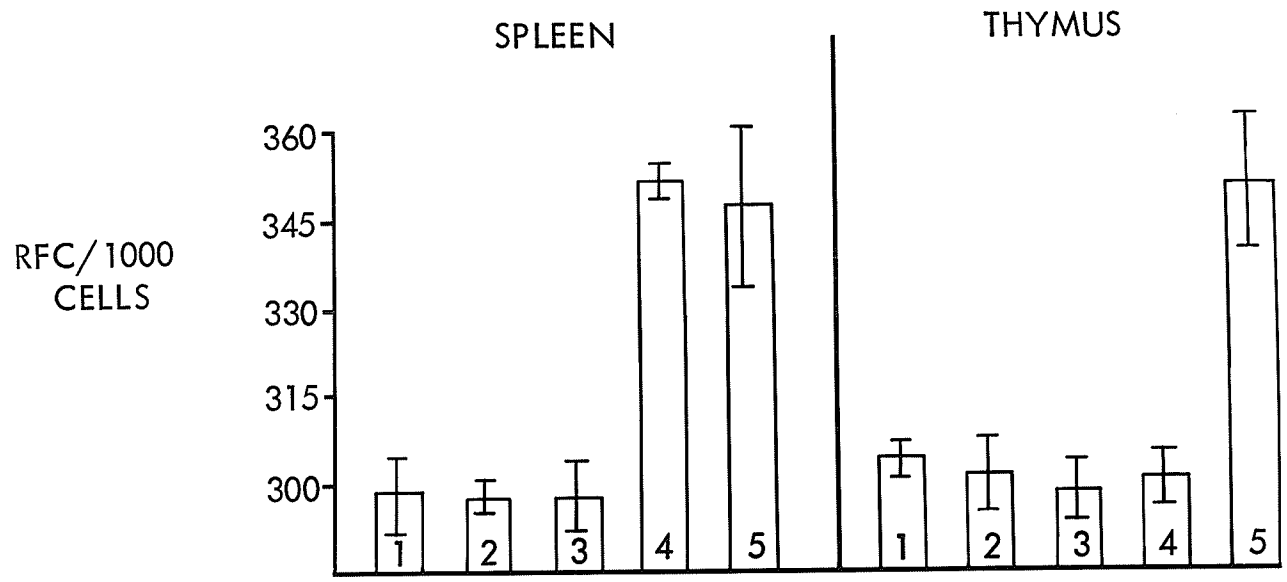


Figure 22: Supernates from cultures of hydrocortisone resistant cells with BCG (BCG-S). $\overline{\underline{\mathrm{I}}}$ = mean $\pm$ S. D. 1. Control 2. BCG-S 3. BCG-S+NMS 4. BCG-S+Ag 5. BCG-S+NMS+Ag

induce cytophilic Ig in the presence of antigen (column 4) or NMS and antigen (column 5), a situation analogous to that of cultures of normal spleen cells with BCG (Fig. 19). Supernates from hydrocortisone resistant thymocytes cultured in BCG are active only in the presence of both NMS and antigen (column 5), a situation identical to that of normal thymus cells cultured in BCG (Fig. 20).

These results suggest that the T cell responsible for induction of the soluble factor is a mature, hydrocortisone resistant cell.

III. IN VITRO INDUCTION OF SOLUBLE FACTOR(S) BY MAAF

In Section C, it was demonstrated that generation of cytophilic Ig not only occurred in the presence of BCG, but also occurred with a small component termed MAAF. To extend these observations further, we cultured spleen and thymus cell suspensions as before in the presence of MAAF. The resulting supernatants were tested for their ability to increase RFC formation of spleen cells in the presence of NMS and antigen.

These results are shown in Fig. 23. The ability of spleen cell supernatants to induce cytophilic Ig either in the presence of antigen (column 3) or antigen and NMS (column 4) correlates well with such ability when cultured with BCG (Fig. 19). Similarly, only the thymus supernatants in the presence of both NMS and antigen can induce cytophilic Ig (column 4, Fig. 23), a situation analogous to that of thymocytes cultured in BCG (Fig. 20).

Thus MAAF can completely reproduce the results obtained with whole BCG in its ability to induce T cells to secrete a soluble factor in an in vitro culture system.

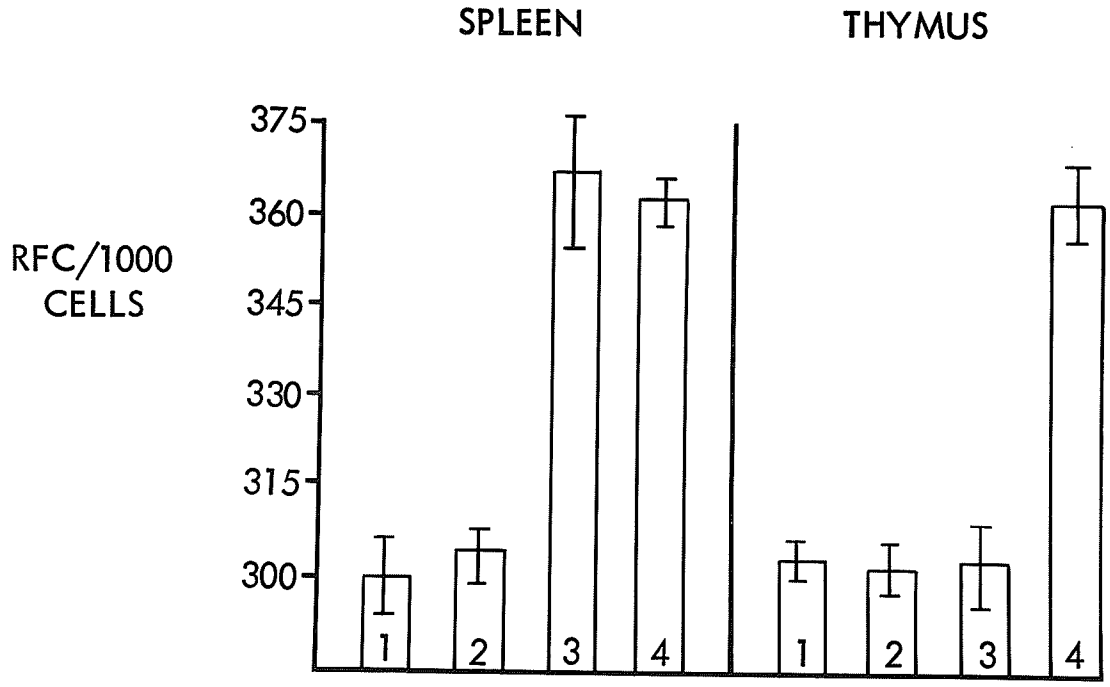


Figure 23: Supernates from cultures of lymphoid cells with MAAF (MAAF-S). $\overline{\underline{\mathrm{I}}}$ = mean $\pm$ S. D. 1. MAAF-S 2. MAAF-S+NMS 3. MAAF-S+Ag 4. MAAF-S+NMS+Ag

IV. IN VITRO INDUCTION OF SOLUBLE FACTOR(S) BY OTHER SUBSTANCES

Having demonstrated that both BCG and MAAF can induce this soluble factor, we were concerned with the question of whether other substances also possessed this capacity.

1. Soluble BSA

Various lymphoid cells were cultured as before in the presence of a soluble preparation of BSA and the supernates were tested for activity by addition of NMS and a different antigen (EA).

As shown in Fig. 24, none of the various combinations tested have any ability to generate cytophilic Ig with supernates obtained from either the spleen, thymus or bone marrow.

2. Bordetella pertussis

Spleen and thymus cells were cultured in the presence of B. pertussis and the supernatants collected and tested for activity. These results are shown in Fig. 25 and demonstrate that no activity is observed with either cell suspension at any of the combinations tested, a situation similar to that obtained with soluble BSA (Fig. 24).

3. Other substances

We studied the ability of a series of other substances to induce a soluble factor from thymocytes. These were tested only by the addition of both NMS and antigen and the results are shown in Table XV.

These results demonstrate that while substances such as LPS, POL, insoluble BSA, Poly AU, BSA- antiBSA complexes and CRBC can induce T cells to produce the soluble factor, other substances such as PVP, $DNP_{60}BSA$ and FIB are unable to do so.

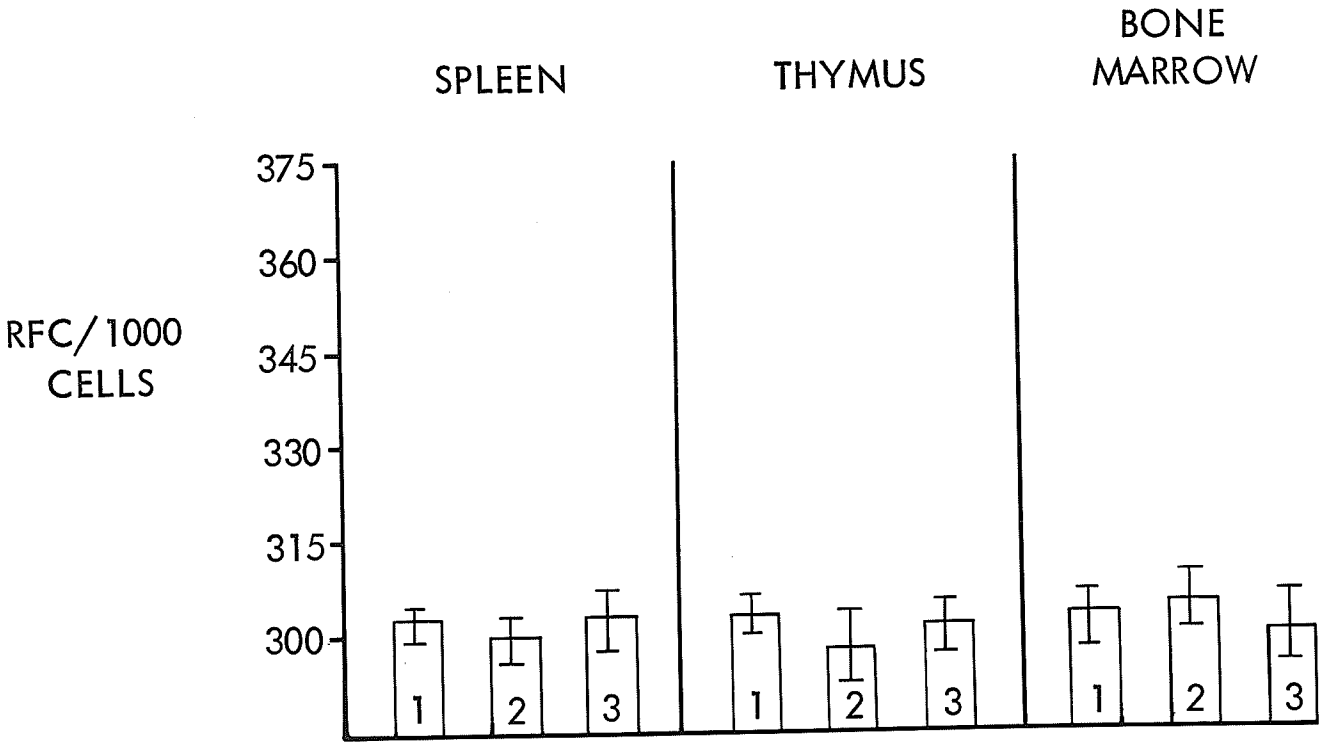


Figure 24: Supernates from cultures of various lymphoid cells with soluble BSA (BSA-S). $\overline{\underline{\mathrm{I}}}$ = mean $\pm$ S. D. 1. BSA-S 2. BSA-S+NMS 3. BSA-S+NMS+Ag

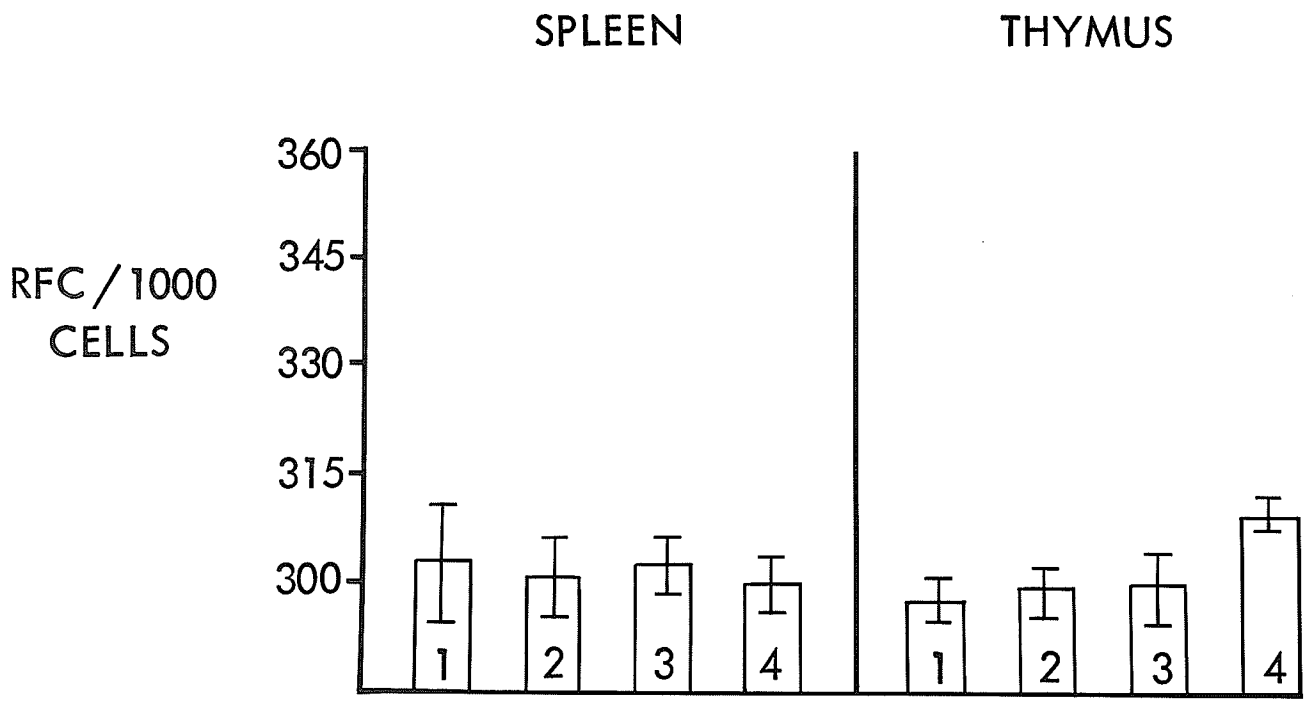


Figure 25: Supernates from cultures of lymphoid cells with B.pertussis (BOR-S). $\overline{\underline{\mathrm{I}}}$ = mean $\pm$ S. D. 1. BOR-S 2. BOR-S+NMS 3. BOR-S+Ag 4. BOR-S+NMS+Ag

TABLE XV

Supernates of cultures of thymocytes with various substances

SUBSTANCE	RFC/1000 SPLEEN CELLS ± S. D.[a]
CONTROL	301±3(3)[b]
LPS-E.coli	360±7(3)
POL	359±7(3)
INSOLUBLE BSA	356±9(3)
POLY AU	385±8(3)
CRBC	360±7(3)
BSA-anti-BSA	355±8(3)
PVP	307±7(3)
$DNP_{60}BSA$	294±3(3)
FIB	304±3(3)

[a]Tested by the addition to the supernate of a mixture of NMS and Ag.

[b]The number of experiments is shown in parentheses.

A list of the substances which give active or inactive supernates in cultures of thymocytes is given in Table XVI.

V. METABOLIC REQUIREMENTS FOR INDUCTION OF SOLUBLE FACTOR

The previous studies suggested that the thymocyte was the cell responsible for production of the active soluble factor. We next attempted to define certain metabolic requirements for induction of the soluble factor.

We therefore cultured thymocytes with BCG in the presence of various metabolic inhibitors. The presence or absence of increased RFC after treatment of normal spleen cells with this supernatant to which NMS and Ag has been added is used again as an assay for the presence or absence of the soluble factor.

As shown in Table XVII, supernates from cultures treated with sodium fluoride, a glycolytic inhibitor, still generate cytophilic Ig (367 ± 21 vs 370 ± 3). Actinomycin D, an inhibitor of DNA directed RNA synthesis also has no apparent effect on the production of the factor (367 ± 21 vs 362 ± 9). However, thymocytes cultured in the presence of cycloheximide, a protein synthesis inhibitor, appear unable to produce a supernatant which can generate cytophilic Ig in the presence of NMS and Ag (367 ± 21 vs 304 ± 3). Similar results were obtained when the culture was carried out at $4^{\circ}C$ (367 ± 21 vs 303 ± 4).

These results strongly suggest that active protein synthesis is required for induction of the soluble factor by the thymocyte.

Thus this section has demonstrated that a thymus derived cell is responsible for production of the soluble factor and that substances

TABLE XVI

Summary of supernates from thymocyte cultures

ACTIVE	INACTIVE
BCG (100 μg)	BSA (500 μg)
CRBC ($6x10^6$)	$DNP_{60}BSA$ (500 μg)
POL (25 μg)	FIB (500 μg)
LPS-E.coli (250 μg)	PVP-40000 (200 μg)
POLY-AU (300 μg)	B.Pertussis ($5x10^8$)
BSA-HEAT-AGGR (1 mg)	
BSA-ANTI-BSA (300 μg)	
MAAF (250 μg)	

TABLE XVII

Cell metabolism and induction of soluble factor(s) by BCG (Thymocytes)

INHIBITOR	---RFC/1000 SPLEEN CELLS ± S.D.---		SITE OF ACTION
	CONTROL	BCG-S+NMS+Ag	
NaF (2×10^{-2} M)	305±3(3)[a]	370±3(3)	Glycolysis
CYCLOHEXIMIDE (4×10^{-4} M)	302±6(3)	304±3(3)	Protein Synthesis
ACTINOMYCIN D (1 µg)	311±3(3)	362±9(4)	RNA Synthesis
4°C	307±4(4)	303±4(3)	Pinocytosis
NONE	301±1(3)	367±21(3)	

[a]The numbers in parentheses represent the number of tests performed.

other than BCG can induce its formation. Furthermore, the metabolic requirements for its production have been described.

SECTION E

PROPERTIES OF THE SOLUBLE FACTOR

In the previous sections we have demonstrated that FCA-S contains a soluble factor which is the product of a T cell or thymocyte. Moreover, this factor is elicited by a number of substances when thymocytes are cultured in vitro. This factor, in the presence of immunoglobulin and antigen, induces the formation of cytophilic Ig. This section will concern itself with defining some physicochemical properties of this soluble factor.

I. FILTRATION AND ACIDIFICATION OF FCA-S

Fractionation of FCA-S on Sephadex G-200 led to the isolation of two active factors: one in the 7S fraction which required only Ag for generation of cytophilic Ig (Fig. 15) and one in the 4S fraction which required both NMS and Ag for activity (Fig. 16). We suggested that perhaps the 7S factor was simply 7S immunoglobulin bound to the factor found in the 4S fraction. This suggested that the 4S factor represented the active factor in excess as compared to the available 7S immunoglobulin. On this basis we have assumed that the factor induced as a consequence of adjuvant action is that factor found in the 4S fraction of a G-200 fractionation of FCA-S.

To study further the properties and size of this factor we passed FCA-S (either at pH 7.5 or 2.5) through UM-10 Diaflo ultrafiltration membranes (solute cutoff of 10,000 molecular weight) and collected the supernatant (SUP-10, that which was retained above the filter) and the filtrate (FIL-10, that which passed through the filter). These fractions

were preincubated with Ag or NMS plus Ag before incubation with normal spleen cells. These cells were then tested by RICA for an increase of RFC (Fig. 26).

After filtration of FCA-S at neutral pH (7.5) the supernatant fraction in the presence of Ag induces the formation of cytophilic Ig (300±5 vs 359±11). This is to be expected as the 7S fraction of FCA-S is too large to pass through the filter and as previously demonstrated requires only Ag to induce cytophilic Ig (Fig. 15).

Since immunoglobulin would not pass through the filter, the filtrate fraction (FIL-10) was tested after the addition of both NMS and Ag. In this situation no activity was demonstrated (300±5 vs 300±2). Thus no additional factors smaller than 10,000 M.W. could be demonstrated in FCA-S.

These experiments have provided further evidence suggesting that the factor induced by FCA which is responsible for cytophilic Ig formation is larger than 10,000 molecular weight.

On the other hand, when FCA-S is acidified (pH 2.5) before filtration, one observes a shift in activity (Fig. 26). The supernatant fraction retains its ability to induce cytophilic Ig upon addition of Ag (358±10 vs 359±11). However, addition of NMS and Ag to the filtrate fraction now results in formation of cytophilic Ig (300±2 vs 354±15).

These findings may suggest that the 4S factor is composed of subunits held together by noncovalent bonds. Acid treatment results in dissociation to molecules of a size sufficiently small enough to traverse the filter, which still retain biological activity.

The retained activity of the supernatant fraction can be explained if one assumes that the binding of the 4S factor to 7S Ig results in

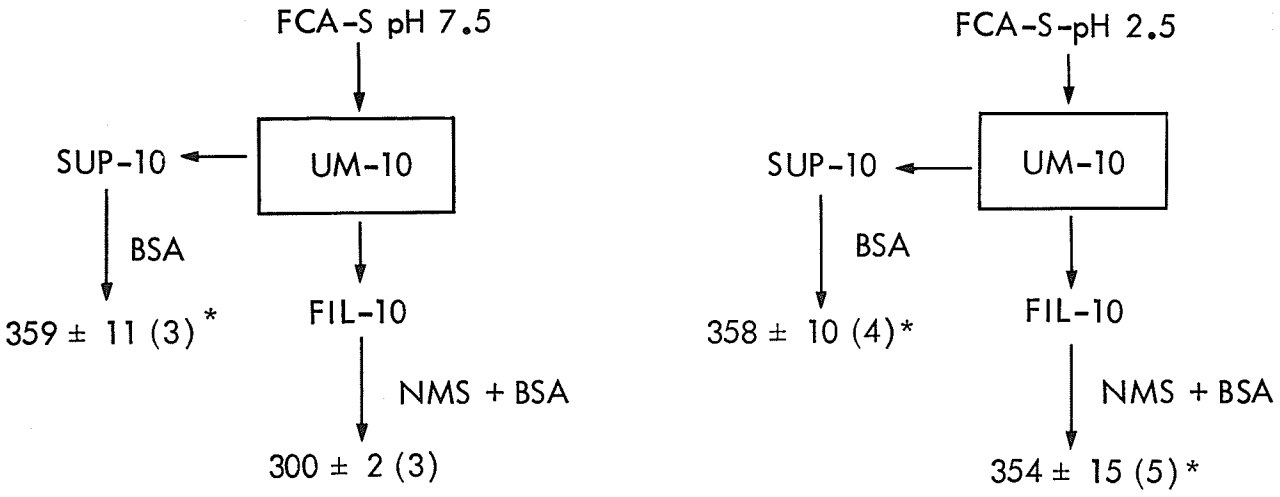


Figure 26: Effect of acidification of FCA-S.

stabilization of the factor such that it is more resistant to acid dissociation. It is also conceivable that not all of the 4S molecules are dissociated by acid and the activity observed is due to the combination of 4S factor, 7S Ig and Ag.

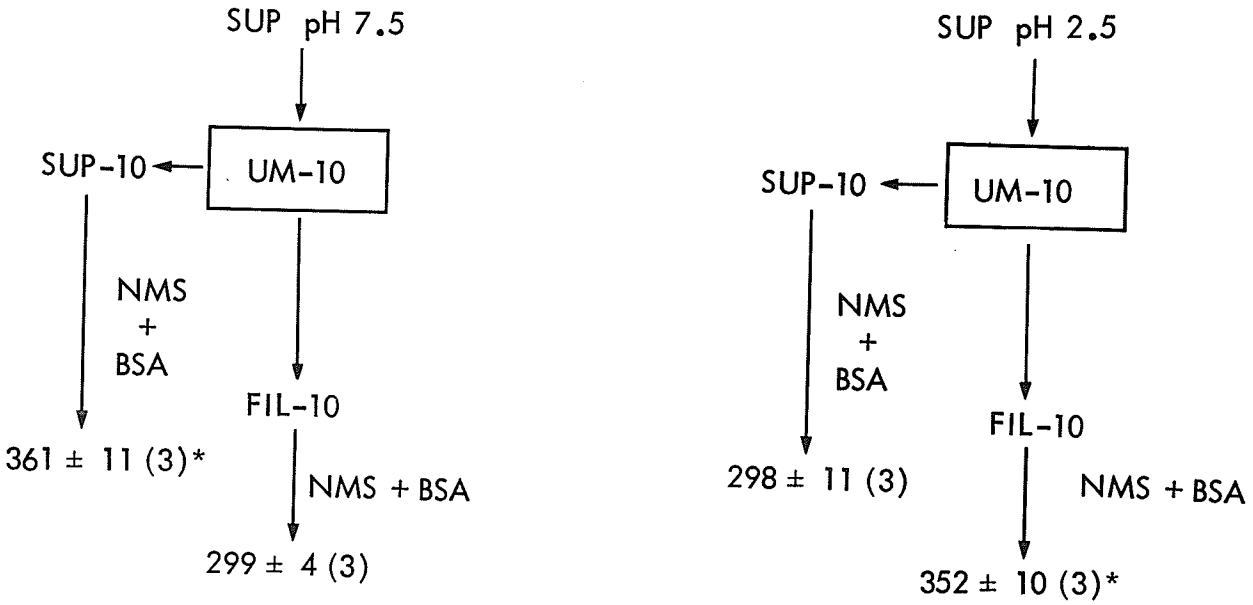
II. FILTRATION AND ACIDIFICATION OF THYMOCYTE BCG CULTURE SUPERNATES

In the preceeding section (D) we demonstrated that cell free supernatants obtained from thymocytes cultured in vitro with BCG could, upon addition of NMS and BSA, generate cytophilic Ig which could be detected by the uptake by spleen B and T cells.

In an attempt to further demonstrate the similarities between this factor and the factor in FCA-S, we passed these culture supernates (at pH 7.5 and 2.5) through UM-10 filters and tested both fractions (SUP-10 and FIL-10) as previously described. Since no Ig was present at any time in these supernatants they were tested by the addition of both NMS and Ag as shown in Fig. 27.

Supernates passed through UM-10 filters at pH 7.5 show similar activity as that described for FCA-S (Fig. 26). Thus the supernatant remaining after filtration at pH 7.5 can generate cytophilic Ig in the presence of NMS and Ag (297 ± 3 vs 361 ± 11) while the filtrate is inactive (297 ± 3 vs 299 ± 4, Fig. 27).

When the same experiments were repeated at pH 2.5, the supernatant lost its activity (297 ± 3 vs 298 ± 11, Fig. 27) while the filtrate now became active (297 ± 3 vs 352 ± 10). These results strongly suggest that this factor can be broken down by acid to subunits of less than 10,000 molecular weight without destroying its ability to generate cytophilic Ig.



UNTREATED CELLS = 297 ± 3 (3)

*$p < 0.01$

Figure 27: Effect of acidification on thymocyte BCG culture supernatants.

There is an apparent difference between these results and those obtained with FCA-S. Acidification of FCA-S did not reduce the activity of SUP-10 (359±11 vs 358±10, Fig. 26) while acidification of BCG culture supernatants completely abrogated the activity of SUP-10 (361±11 vs 298±11, Fig. 27). It is possible that the presence of 7S Ig in the former situation confers resistance to dissociation by the acid as previously suggested.

III. OTHER PROPERTIES OF THE SOLUBLE FACTOR

Since both supernates from thymocytes cultured with BCG and FCA-S appear to display similar properties, we attempted to define other characteristics of this factor. Such properties as heat stability, trypsin sensitivity and RNase sensitivity were investigated. These results are shown in Table XVIII.

Both Thy-BCG supernate and FCA-S pH 2.5 filtrate induce cytophilic Ig in the presence of NMS and Ag (358±3 and 363±15 respectively). Heating of these supernates at 56°C for 30 minutes had no effect on activity (359±10 and 352±16 respectively). Similar results were obtained with RNase thus suggesting that RNA is not part of this factor. However, treatment of these supernates with trypsin abrogates the activity (300±6 and 302±6 respectively), thus suggesting a possible major protein component to this factor.

Thus this factor appears to be heat stable, insensitive to RNase and sensitive to trypsin.

In conclusion, this section has defined certain physicochemical characteristics of the soluble factor. Moreover, it has strongly

TABLE XVIII

Properties of soluble factor

TREATMENT	RFC/1000 SPLEEN CELLS ± S.D.
CONTROL	297±7(3)[a]
THY-BCG SUPERNATE	
SUP + NMS + BSA	358±3(3)
SUP^{56} + NMS + BSA	359±10(3)
SUP^{TRY} + NMS + BSA	300±6(3)
SUP^{RNase} + NMS + BSA	358±4(3)
FCA-S pH 2 UM-10 FILTRATE	
FIL + NMS + BSA	363±15(3)
FIL^{56} + NMS + BSA	352±16(3)
FIL^{TRY} + NMS + BSA	302±6(3)
FIL^{RNase} + NMS + BSA	361±5(3)

[a]The number of experiments is shown in parentheses.

suggested that the factor in FCA-S in many respects is the same as that in cell free supernates recovered from thymocytes cultured in vitro with BCG. Table XIX is a summary of the properties of the soluble factor described in this section.

TABLE XIX

Summary of properties of soluble factor

ORIGIN	
Thymocyte or T cell	
PHYSICOCHEMICAL	
SIZE	4S
M.W. pH 7.5	$6\text{-}7 \times 10^4$
pH 2.5	10,000
TRYPSIN	SENSITIVE
RNase	INSENSITIVE
HEAT	STABLE
BIOLOGICAL	
Complexes Ig with Ag?	

DISCUSSION

Immunocompetent lymphocytes arise via two pathways of differentiation. This results in the generation of two specific cell types - the bone marrow derived cells (B cells) and the thymus derived cells (T cells). The B cells are concerned with humoral antibody formation whereas T cells are responsible for mediation of cellular immunity. However, T-B cell interaction is recognizable for humoral reactions involving thymus dependent antigens. T-B cell interaction has also been described for other immunological phenomena.

Since Freund (1937) described the potentiation of the immune response by mycobacteria, a variety of substances known as immunologic adjuvants have been found to induce the same effect. Freund's complete adjuvant containing mycobacteria suspended in mineral oil is the most widely used. Its mode of action remains obscure although several suggestions have been made. It is possible that it may result in retention of antigen at the site of injection with antigen being slowly released (Halbert et al, 1946), or it may result in a more efficient presentation to the immunocompetent cells of the antigen absorbed on the oil droplets (Lind, 1968). On the other hand, adjuvants were shown to stimulate nonspecifically the proliferation of the reticuloendothelial system (White et al, 1955; Rupp et al, 1960), to produce enlargement of the paracortical areas of regional lymph nodes (Taub et al, 1970) and cause an influx of lymphocytes into such nodes (Dresser et al, 1970).

It has also been suggested that adjuvants exert their enhancing effect on antibody formation through their uptake by macrophages (Unanue et al, 1969; Spitznagel and Allison, 1970). More recently the

adjuvant effect was shown to depend on the presence of the thymus derived cell (Allison and Davies, 1971; Taub and Gershon, 1972). The importance of the T cell was also shown by the decrease, in thymectomized chickens injected with Freund's complete adjuvant, of the size of the granuloma which usually is formed at the site of injection (White, 1970). These results suggest that the adjuvant effect may be the result of some type of amplification of T-B cell interaction in such a way that the antibody response is potentiated. That is, since T cells are required for the adjuvant effect and since T cells are required for some antibody responses, one can logically conclude that adjuvants probably potentiate humoral antibody synthesis by amplifying T-B cell interaction through some effect on the T cell.

Dresser (1961) has defined adjuvanticity as the property of the antigen itself (intrinsic adjuvanticity) or some other substance administered with the antigen (extrinsic adjuvanticity) to cause induction of an immune response other than tolerance. Such a property is illustrated in Fig. 28 and suggests that the adjuvant may be an integral part of the antigen.

This brief outline of existing data implicate the T cell as a cell of importance for elicitation of the adjuvant effect. Furthermore, immunogenic preparations may themselves contain adjuvant properties, or in some cases antigens may require other substances for this purpose.

This discussion will be divided into a series of sections each one pertaining to an important point raised by the data in this thesis. It is hoped that these divisions will make the discussion more easily read and understood as well as to emphasize the important findings described here.

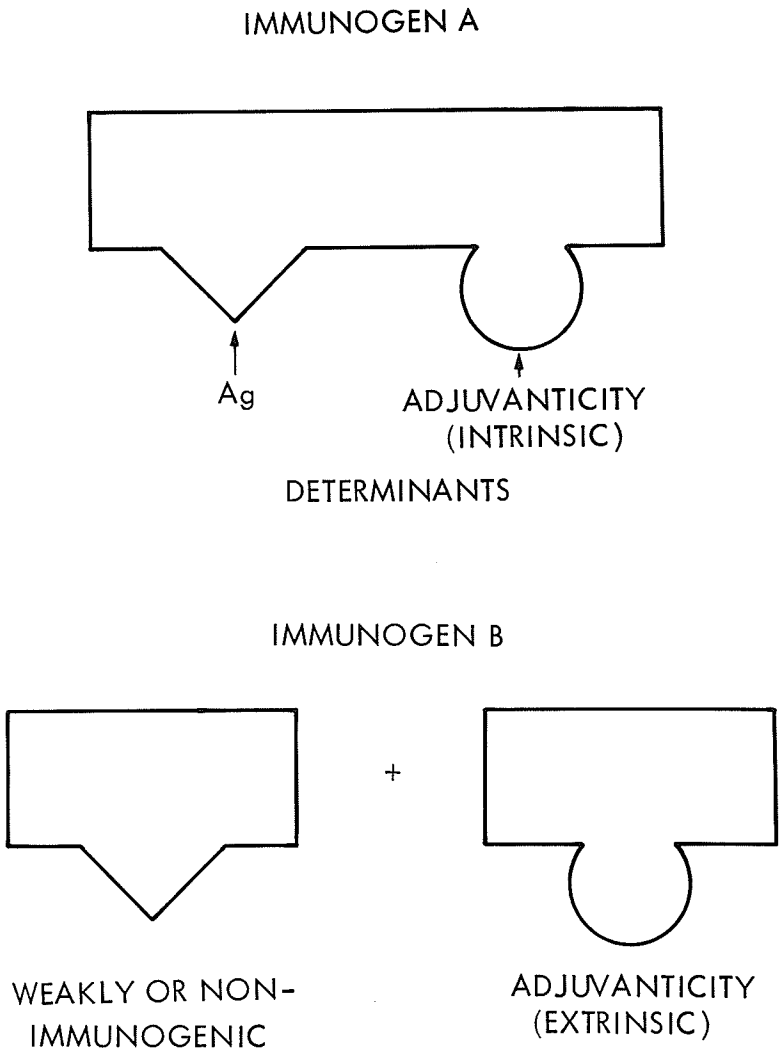


Figure 28: Adjuvanticity - intrinsic versus extrinsic.

I. ADJUVANT DEPENDENT CYTOPHILIC COMPLEXES

In studies of the changes of immunoglobulin (Ig) carrying cells during the primary response it was found that 6 hours after immunization the number of cells detected by RICA as carrying Ig increased by 20-25% of the preimmunization level (Paraskevas et al, 1972c). This increase was shown to be due to the uptake of a cytophilic immunoglobulin of the IgG class, by a cell previously non-detectable by RICA as carrying surface Ig (Lee and Paraskevas, 1972). The cell was identified as a T cell and the T cells decreased 6 hours after immunization in the spleen to the same extent as the number of the new cells which were added to the pool of the immunoglobulin carrying cells. At the same time it was shown that the number of cells which carry surface Ig before immunization (B cells) decreased. Whole unfractionated 6 hour serum reproduced in vitro all the phenomena which take place in the spleen. Thus it produced an increase of the total number of Ig carrying cells (Table III). This increase was accounted for by an increase of the IgG cells which indicated that the cytophilic Ig belongs to the IgG class. The number of T cells in the spleen cell suspension exposed to the 6 hour serum decreased by the same percentage as in vivo. After exposure of the spleen cells to the 6 HR-MS the cells which carry Ig on their surface before immunization showed a decrease. This was detected by a drop of the cells carrying IgF globulin in single RICA tests using the $\alpha\,\emptyset$- αEA hybrid antibody (IgF cells). The mechanism of this phenomenon is at present unknown but two possibilities are entertained. Either the surface immunoglobulin is lost or its detection is blocked by the uptake of the cytophilic IgG globulin. It has been shown before that the uptake by the spleen cells of antigen-antibody

complexes made of mouse IgG antiferritin and ferritin results in lack of detection of surface IgF globulin (Paraskevas et al, 1972a). The whole 6 HR-MS-FIB induced an increase of the number of IgG cells which indicated that the cytophilic Ig was from the IgG class (Table III). When the fractions from a Sephadex G-200 were examined, the activity was located in the void volume where fibrinogen is eluted but not in the 7S fraction (Fig. 5). This suggested that the cytophilic IgG globulin must be larger than the usual 7S IgG and thus possibly complexed to fibrinogen. In order to obtain further evidence, BSA, which is smaller than 7S, was used and the 6 HR-MS-BSA was fractionated. Only the 7S fraction now showed the ability to increase the rosette count using either αMIg- αFe or $\alpha\gamma$- αFe hybrids (Table III and Fig. 6). This fraction contained radioactively labeled antigen which by autoradiography was shown to be complexed with Ig (Fig. 7). We do not have direct evidence whether the activities detected by RICA in the 7S fraction are associated with the BSA-Ig complex or with a 7S IgG which has been otherwise altered. We favour the first possibility since in the fractions of 6 HR-MS-FIB the 7S IgG fraction does not have any activity although cytophilic IgG existed in the whole unfractionated serum. Since the activity existed in the place where FIB is eluted it strongly suggests that the cytophilic IgG has markedly different molecular sieving properties, perhaps induced by the formation of a FIB-IgG complex. Furthermore, inhibition of rosette forming cells by a rabbit antiserum to the antigen strongly suggests that antigen also is bound to the cells. An alternative to this interpretation is that antigen has bound to the cells at a site other than that where the IgG has bound but sufficiently close enough for steric hindrance to occur.

Such an interpretation does not seem feasible if one is an advocate of the clonal selection theory due to the prerequisite of only small numbers of antigen binding cells. The results presented here are in agreement with the findings of Yuan et al (1970) who have been able to show that BSA is complexed with IgG in the serum of rabbits 5 hours after injection. They found, however, that in the rabbit the BSA-IgG complex was eluted in the void volume of a G-200 column which indicated that whole BSA was complexed to the IgG globulin. Since in our experiments the radioactively labeled BSA which is complexed to IgG did not significantly alter the sieving properties of this IgG, we assume that it most likely represents a small fragment of the injected BSA which, however, is still precipitable by anti-BSA. The differences between our results and those of Yuan et al (1970) may reflect a species difference in handling of the antigen. In the case of fibrinogen we cannot decide whether unaltered (whole) FIB or a fragment, is combined to the IgG globulin.

The conclusion from both experiments suggests that the activities which are detected by RICA are not associated with the antigen alone (4S peak of 6 HR-MS-BSA does not have activity, Fig. 6) and that it is not an otherwise altered 7S IgG (7S peak of 6 HR-MS-FIB does not have the activity, Fig. 5). The 7S fraction of 6 HR-MS-BSA has the ability to decrease the number of IgF cells and the number of T cells. Thus all activities are associated with the same active component and the evidence presented strongly implicates a complex of antigen with IgG. Complexes of antigen and 7S Ig which were cytophilic for spleen cells were also detected by Ivanyi (1970) in chickens 3 days after antigenic stimulation. Simple incubation of NMS with 4S BSA which has not passed

through the animal did not result in the appearance of any activity which indicated that the handling of the antigen by the animal is important for the creation of the cytophilic IgG. In striking contrast, the 4S BSA present in the 6 HR-MS-BSA has some important differences from the 4S BSA which has not been passed through the animal. Thus when it is mixed with NMS and the mixture is fractionated, the 7S fraction possesses the ability to produce an increase of the rosette count (Fig. 9). The amount of radioactivity in the 7S fraction was only twice that of background and although perhaps insignificant, it nevertheless indicated a shift of the BSA from the 4S position. Yuan et al (1970) have also shown that some of the BSA in the 4S fraction is altered in terms of electrophoretic mobility. Furthermore, it appears that the limiting factor in the formation of the active complex is the amount of 7S IgG available.

Bretscher and Cohn (1970) have suggested that antigen is presented to the lymphocyte complexed to a carrier antibody. Our results may support such a theory. It is conceivable that the complex may play a central role in the highly complex phenomena of the interactions of various cells in the early stages of antigen recognition, since as shown here and previously (Lee and Paraskevas, 1972), it interacts with both T- and B-cells.

A receptor for Fc was previously described on the surface of the spleen B-cells (Paraskevas et al, 1971b; Paraskevas et al, 1972a). This receptor interacts with the Fc of antibodies in antigen-antibody complexes or Fc fragment from papain digested antibodies. Such a receptor has also been described by others (Basten et al, 1972a). It was shown that neither antigen nor antibody alone could effectively bind

on such a receptor. It is possible that this receptor may be instrumental in binding the 6 HR-MS complex. It was also shown that 6 hours after immunization the Fc fragments do not react anymore with the Fc receptor as in normal cells (Paraskevas et al, 1972b), which indicates that the Fc receptor is undetectable on the cell surface. Whether the Fc receptor is "occupied" or whether the lack of detection results from membrane pertubations of an unknown nature is at present merely speculative.

Fractionation of serum obtained from mice immunized for 24 hours with BSA-I^{125} emulsified in FCA (24 HR-MS-BSA) indicates that the complexes present at 6 hours are not present after 24 hours of immunization. This suggests a rapid elimination of the antigen-antibody complexes from the circulation.

If the formation of this cytophilic complex and 4S active component were a property of the antigen, then the serum collected from mice 6 hours after injection of BSA-I^{125} in saline should show a similar effect to the 6 HR-MS-BSA. RICA tests performed after treatment of normal spleen cells with 6 HR-BSA serum were unable to demonstrate a cytophilic globulin. Fractionation of this serum on a Sephadex G-200 column also confirmed the lack of detectable complexes (Fig. 11). Because of the necessity for incorporation of FCA with soluble antigen for a cytophilic complex to be induced, it is suggested that cytophilic complex induction is a property of the adjuvant and not the antigen, especially for an antigen which has no significant intrinsic adjuvanticity.

II. ADJUVANT INDUCED SOLUBLE FACTOR(S)

Soluble antigens require adjuvant (FCA) to elicit the 6 hour increase as discussed above. It was therefore logical to assume that this was due to the adjuvant and that the adjuvant may accomplish this cytophilic complex formation through induction of soluble factors.

FCA-S pre-incubated with any antigen induced an increase of about 70 new RFC per 1000 spleen cells (Fig. 12). This increase is statistically significant and amounts to 20-25% of the Ig carrying cell population present in the normal spleen. Moreover, titration of the FCA-S with various doses of antigen showed that maximal rosette formation (uptake of cytophilic Ig) was obtained at the dosages of antigen commonly used (Fig. 13). It is equally important to note that quantities of antigen as low as 5 ng when incubated with FCA-S and spleen cells gave statistically significant increases of RFC, thus suggesting that only very small quantities of antigen are required for formation of cytophilic Ig.

It should be noted that the mechanism by which FCA-S increases the RFC is non-specific since any antigen is effective in the same serum sample. This is further supported by the fact that absorption with one of the antigens in aggregated form or with CRBC can eliminate the property of the FCA-S to induce the increase of RFC by all other antigens. Material recovered by acid elution from the aggregated antigen had the property of the original serum upon addition of antigen to induce the same increase of RFC. In addition the eluate was able to reconstitute this property to the absorbed FCA-S (Fig. 14). The eluate contained Ig as shown by Ouchterlony technique.

In order to understand the underlying mechanisms involved we have

fractionated the FCA-S by gel filtration on a Sephadex G-200 column. When all fractions were first pre-incubated with BSA and then examined in the RICA system only the 7S fraction contained the activity present in the FCA-S (Fig. 15). Since normal mouse serum has no similar activity we conclude that some of the 7S Ig in FCA-S is "altered". The fractionation of the FCA-S, however, revealed that in addition to the activity present in the 7S fraction, the 4S fraction contained a factor which could induce the increase of RFC upon addition of NMS or its 7S Ig fraction and antigen (Fig. 16). Thus we were able to generate in vitro "altered" 7S Ig which in the presence of antigen induced the increase of the RFC. It was also shown that the 4S factor does not act separately on the cells which then absorb Ig in the presence of antigen. It is reasonable to conclude that for the expression of full activity, that is the increase of RFC, all three components, the 4S factor, normal Ig and antigen, must be simultaneously present. Conclusive evidence at present is lacking for the mechanism of interaction between these three components in the generation of the increase of RFC.

The results from inhibition of RICA by antiserum directed against the antigen suggest that the antigen is on the surface of the cell (Table VIII). It is conceivable that the antigen is bound to the cell at a site close to the Ig receptor and thus is sterically hindered by the rabbit antiserum, but this seems unlikely. It is more likely, in view of the fact that all three components are required together, that the antigen is bound to the Ig in the form of a complex and is then bound to the surface of the cell possibly via the Fc receptor as previously discussed. Uptake of antigen by the spleen cells was also shown by cytotoxicity tests using an antiserum to the antigen (Table

IX). The number of cells which are shown to take up the antigen is closely similar to the total number of cells which were shown by RICA to be affected after their exposure to FCA-S and antigen. It is conceivable that cytophilic Ig may represent a complex of the Ig and the antigen mediated by the factor present in the FCA-S but other alternatives cannot be excluded.

The inability of pepsin digested 7S Ig to produce an increase of the RFC when it was incubated with the 4S fraction and antigen is expected since the Fc fragment is necessary for the uptake of cytophilic Ig on the cell surface. It is also possible that the Fc fragment in the Ig molecule may provide the reactive site which upon interaction with the 4S factor and antigen generates cytophilic Ig.

The experiments designed to identify the nature of the cell which is responsible for the increase of RFC showed that when the T cells were abolished by treatment with an anti-θ serum and complement no increase of RFC was detected. This indicates that either the T cells directly take up the cytophilic Ig or only T cell action is necessary. Since the increase of the RFC was obtained even at $4^{\circ}C$ the second possibility is unlikely. Furthermore, about 5% of the thymocytes showed the presence of surface Ig after exposure to FCA-S in the presence of antigen, which suggest that it is a thymus or thymus derived cell which directly takes up the cytophilic Ig. That the T cells are affected when spleen cells are exposed to FCA-S and antigen was also shown by the decrease in the number of θ carrying cells detected by an anti-θ serum. In normal BALB/c spleen about 25 to 30% of all the cells are killed upon treatment with the anti-θ serum and guinea pig complement. When the spleen cells were exposed to FCA-S and BSA the number of θ carrying

cells decreased by 10-11%. We do not know the mechanism for the lack of detection of the θ antigen. Loss of the antigen on the basis of activation of the T cell is unlikely since, as we have demonstrated, the spleen cell changes take place in the absence of such activation. It is also possible that the uptake of the cytophilic Ig by the T cell may sterically interfere with the detection of the θ antigen.

If the increase of RFC represents a shift of cell populations from the non-RFC (θ carrying) to the Ig carrying, then one would expect that the decrease of θ carrying cells to be equal to the increase of the Ig carrying. The decrease of θ carrying cells is slightly larger than the increase of Ig carrying cells (10-11% vs 7-8% of all spleen cells respectively). The difference may be due to technique and it is possible that although some T cells may take up enough cytophilic Ig to block the detection of θ antigen by cytotoxicity techniques, this may not be enough to form a rosette in order to be detected as Ig carrying. The observations reported here further extend similar observations made in vivo. An increase of Ig carrying cells has been detected in the spleens of mice after injection of various antigens in FCA for 6 hours (Paraskevas et al, 1972c) while at the same time the number of T cells decreased significantly (Lee and Paraskevas, 1972). The same shifts in these two lymphocyte populations were observed by treating, in vitro, normal spleen cells with serum collected from mice 6 hours after injection of antigen in FCA (Paraskevas et al, 1972c; Lee and Paraskevas, 1972).

These studies have also shown that the cytophilic Ig in the 6 hour serum is taken up by T cells in the normal spleen cell suspension (Lee and Paraskevas, 1972). Such sera contained antigen complexed with

7S IgG and it is very likely that the cytophilic IgG represents antigen-antibody complexes.

B cells have also been shown to be affected by the FCA-S and antigen. This was shown with the use of specific hybrid antibodies (Table VII). The hybrid antibody $\alpha\emptyset$- αEA which is highly specific for IgF globulin detects about 290 RFC per 1000 spleen cells. After exposure to FCA-S and antigen there is a decrease of 45-50 RFC per 1000 spleen cells. This decrease is statistically significant ($p<0.001$) and represents about 15% of all cells carrying surface Ig. We have no evidence of the mechanisms of the lack of detection of surface IgF, but the use of the specific hybrid antibody $\alpha\gamma$ - αFe may be of some relevance in this aspect. This hybrid antibody is highly specific for the IgG class and detects 195$\pm$7 cells per 1000 spleen cells carrying Ig on their surface.

After exposure of the spleen cells to FCA-S and FIB or BSA the number of RFC increases to 270$\pm$23 and 265$\pm$3 per 1000 spleen cells respectively (Table VII). This increase is statistically significant and is similar to the increase detected by the hybrid αMIg- αFe. It is reasonable to say that the overall increase of the RFC is due to the uptake by T-cells of an IgG globulin.

The same cytophilic Ig, taken up by IgF carrying cells, may cause a decrease of the detection in the surface IgF either because of steric hindrance or for other reasons.

It is interesting that Dawe et al (1970) have shown that the enhancement of FCA on antibody response in rabbits could be passively transferred by a serum Ig fraction which belonged to the IgG class. They were, however, unable to remove the enhancing activity by absorbing

the IgG fraction with insoluble BSA. The discrepancy with our results may be due to species differences or the fact that the serum in their experiments was collected 7 weeks after repeated injection of FCA while we collected the serum only 6 hours after one injection. No cytophilic Ig could be detected in sera collected after injection of FIA upon addition in vitro of antigen (Table X). This strongly suggests that the mycobacteria in FCA may be responsible for these differences. Alternatively if soluble factors are also induced by FIA the time of their induction may be different.

III. PRODUCTION OF SOLUBLE FACTOR(S) BY MAAF AND OTHER SUBSTANCES

Thus FCA can induce the formation of a soluble factor which, in the presence of NMS and antigen can generate cytophilic Ig while FIA does not appear to possess this ability. Since mycobacteria are the only differing substance in these preparations, it was reasonable to assume that this effect was due to the mycobacteria and thus could be easily tested.

Incorporation of BCG into FIA resulted in a preparation which, when injected into mice, acted like FCA and induced a soluble factor as detected by cytophilic Ig complex formation in the presence of antigen (Table XI). As with FCA, antigen is required to demonstrate the generation of the cytophilic Ig. Thus these results strongly support the contention that the mycobacteria are responsible for generation of a soluble factor which in turn can induce cytophilic Ig formation in the presence of Ig and antigen.

Having defined the component responsible for this soluble factor formation we next attempted to pinpoint this activity by testing

various fractions of BCG. PPD is a tuberculin purified protein derivative prepared from a human strain of Mycobacterium tuberculosis. Serum collected from mice injected for 6 hours with this component did not contain an active soluble factor as measured by its inability to generate cytophilic Ig in the presence of antigen (Table XII). Thus the PPD did not reproduce the ability of whole BCG.

Hiu (1972) from the Pasteur Institute has isolated a water soluble lipid-free fraction from BCG by a complicated extraction technique. He has demonstrated that this fraction, MAAF (mycobacteria adjuvant antitumor fraction), enhances cell-mediated and humoral immune responses thereby replacing BCG as the adjuvant. Dr. Hiu kindly donated some of this material for our work and it was tested in a manner similar to that of PPD. It was evident that while the MAAF-S itself was inactive, addition of antigen resulted in the generation of cytophilic Ig (Table XIII) as was observed with FCA-S. Fractionation of MAAF-S demonstrated that the activity resided in the 7S fraction, a situation analogous to that of the FCA-S fractionation.

These observations suggest that MAAF, a 32,000 molecular weight fragment of BCG can effectively replace FCA in its ability to induce the active soluble factor. It is strongly suggested from these results that the soluble factor produced by FCA may be due to the action of a subcomponent of the mycobacteria called MAAF. The mechanism by which this induction occurs is unknown at this time.

As discussed, BCG and MAAF were able to induce T cells to produce this soluble factor. We therefore addressed ourselves to the possibility that other substances also possessed this capacity.

It was observed that while soluble BSA did not have this capacity,

BSA anti-BSA complexes and insoluble BSA did possess this capacity (Table XV). Chicken red blood cells, POL and LPS from E. coli also possessed this ability while PVP, FIB and B.pertussis were inactive.

Although there are always exceptions to the rules, it appears that the more particulate a substance is, the more likely it is to be competent to activate T cells into production of active factor. However, this list of substances is not sufficiently large enough to justify categorization of active versus inactive substances on the basis of size and is mentioned simply as a possibility which has been entertained.

IV. THE CELL RESPONSIBLE FOR PRODUCTION OF SOLUBLE FACTOR(S)

The previous section defined an activity associated with a small component of mycobacteria. Our next concern dealt with the cell type responsible for production of the active soluble factor.

In order to define the cell of origin we cultured, in vitro, various lymphoid organs in the presence of material known to elicit this factor in vivo (BCG or MAAF) and examined the cell-free supernatants for activity.

Thus when spleen cells were cultured in the presence of BCG and the supernatants appropriately tested, activity was demonstrated (Fig. 19). It should be noted that addition of antigen alone was sufficient, thus suggesting the presence of Ig in the preparation. The turnover rate of surface Ig on the lymphocyte has been calculated to be less than two hours (Wilson et al, 1972), and thus culture for three hours could conceivably result in sufficient quantities of free Ig which could serve as the exogenous immunoglobulin source. Cultures of various

lymphoid organs in balanced salt solution were negative, thus suggesting the necessity for an initiation event for elaboration of this factor. In contrast to the above, when spleen cells enriched in B cells (anti-Θ treated spleen cells) were cultured with BCG, no activity was observed in the resulting supernates. This suggests that a T cell or Θ carrying cell may be responsible for induction of this soluble factor.

Supernates from BCG cultured lymph node cells were active only in the presence of both NMS and antigen (Fig. 20). This is to be expected if one considers that the large majority of lymph node cells are thymus derived and thymus derived cells do not carry surface Ig. Thus an exogenous source of immunoglobulin is necessary to reconstitute activity. Furthermore, supernates from BCG cultured thymocytes were active only in the presence of both NMS and antigen (Fig. 20). These results strongly implicate the T cell as the cell responsible for induction of the soluble factor.

Supernates from in vitro cultures of macrophages and bone marrow cells with BCG were unable to generate cytophilic Ig in the presence of NMS and antigen, thus suggesting that induction of the soluble factor is restricted solely to the thymocyte or T cell. It is possible, however, that longer incubation times may result in the production of factors in these organs, but such alternatives were not examined.

Hydrocortisone acetate is known to eliminate immature cells of both primary and secondary lymphoid organs (Blomgren and Andersson, 1969). That is, 95% of the thymus cells, 50% of the bone marrow cells and 80% of the spleen cells are eliminated (Claman et al, 1971). We used this observation to determine what type of T cell was being

activated by BCG to produce the active factor. Hydrocortisone resistant spleen and thymus cell suspensions were cultured in vitro with BCG and the supernates were shown to be active, a situation similar to that of the normal suspensions (Fig. 22). These results strongly suggest that the T cell responsible for production of this active factor is hydrocortisone resistant and is thus a mature T cell. Raff and Cantor (1971) have subdivided T cells into T1 and T2 cell types. T1 cells are immature cells which are sensitive to corticosteroids while T2 cells are those that have encountered antigen and are mature non-sensitive cells. According to this classification, the T cells responsible for induction of the soluble factor belong to the T2 series.

As described previously, MAAF could reproduce the effects of FCA in terms of circulating factors in the serum after injection into experimental animals. To extend these observations we examined supernatants from MAAF stimulated spleen and thymus cells and observed that all of the activities observed with BCG were reproduced by MAAF (Fig. 23).

These results strengthen previous convictions that MAAF is the most likely candidate for the activity observed with BCG or FCA.

In an attempt to define metabolic requirements for production of this factor, we monitored the effects of various metabolic inhibitors on the production of active soluble factor.

Sodium fluoride is an inhibitor of glycolysis. It binds to enolase, thus preventing the dehydration of the phosphate ester of a dihydroxy acid. Incubation of thymocyte cultures in the presence of this inhibitor did not affect the ensuing supernatant from generating cytophilic

Ig in the presence of NMS and antigen, thus suggesting that glycolysis is not a prerequisite for production of the active factor (Table XVII).

Cycloheximide, an inhibitor of protein synthesis, completely abrogated the ability of BCG to activate thymocytes to produce the soluble factor. These results suggest that protein synthesis is required, thus implicating the synthesis of new proteins. This is further supported by the observation that supernatants obtained from freeze thawed thymocytes were inactive and presumably did not contain the active factor or contained it at concentrations too low to be detected. Incubation at $4^{o}C$ also prevented development of active supernatants. Since low temperatures result in minimum metabolic activity by the cell, such an observation would be expected in view of the requirement for active protein synthesis.

Actinomycin D, an inhibitor of DNA directed RNA synthesis, did not impair the production of active soluble factor, thus suggesting the non-involvement of RNA synthesis.

These results suggest that the soluble factor produced by T cells as a result of BCG stimulation relies on protein synthesis, but that the RNA needed for this translation is present and does not have to be transcribed. Furthermore, the glycolytic pathway is not a requirement in this system.

V. CHARACTERIZATION OF THE SOLUBLE FACTOR

As this study would not be complete without some characterization of this factor we attempted to define certain characteristics or properties of this factor.

The detection of soluble factors in the 4S fraction of a G-200

column fractionation of FCA-S suggested that this factor had a sedimentation value of 4S. This coincides with a molecular weight of 60-70,000. Such a molecule will not pass through a UM-10 ultrafiltration membrane as the solute cutoff level is 10,000. However, treatment of the soluble factor (4S fraction of FCA-S) with acid at pH 2.5, resulted in a preparation which now passes through the membrane but which still retains its ability to generate cytophilic Ig in the presence of NMS and antigen (Fig. 26). Similar results were obtained when thymocyte BCG culture supernatants were acidified and passed through the ultrafiltration membranes.

We interpret these results to suggest that the soluble factor consists of a polymer of subunits held together by noncovalent bonds. Acid, which breaks noncovalent bonds, dissociates these subunits into free units which still retain their capacity to generate cytophilic Ig in the presence of NMS and antigen.

We have further demonstrated that this factor is heat stable, insensitive to RNase but sensitive to trypsin. This suggests a possible major protein component to this factor. It is interesting to note that these properties are very similar to those described by Rubin and Coons (1972a, 1972b) for a soluble factor released from primed spleen and thymus cells which nonspecifically augments the immune response to unrelated antigens.

The question which arises from the presentation of this data relates to the relevance of these observations to the immune response. Unfortunately, further investigations are required before this can be confidently answered.

The data presented here suggest that FCA acts by the interaction

with a mature T cell, resulting in the production of a soluble factor. This factor binds antigen and 7S immunoglobulin (by an unknown mechanism), thus forming a complex which is cytophilic for T and B cells. What occurs from here on is not known. It may be that the binding of this complex to T cells provides the stimulus for T cell proliferation. This may result in induction of delayed hypersensitivity or more efficient cooperation with B cells. This could result, indirectly or directly, in increased antibody production.

Whether the phenomenon described here may underlie the common pathway for both aspects of the immune response is unknown, but it is anticipated that future investigations will clarify this point.

This work, like many others, poses more questions than it has answered. It is hoped, however, that this work will help revitalize investigations in the area of adjuvants; an area which is becoming of increasing importance as man strives to manipulate the immune response as a method of therapy for treatment of immunologic disorders.

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