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The role and characterization of C3 receptors in the immune response

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The Role and Characterization of C3 Receptors in the Immune Response

by

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ABSTRACT

It has been shown that depletion of serum complement in mice abrogates the T-dependent antibody response in these animals. While C3, the third component of the complement system, has been hypothesized to play a role in initiation of the immune response, this has not been clearly substantiated. The present study provides important data bearing on this question.

First we developed a hemolytic assay for mouse C3, allowing us to measure production of this component, and a C3-destroying substance, by mouse spleen cells in culture. Then specific indicator particles for cells bearing C3b receptors and C3d receptors were developed, allowing us to separate different mouse spleen cell sub-populations, based on their surface complement receptors. These different sub-populations were then compared, in primary and secondary immune responses, and by polyclonal activation. The effects of adding C3 or anti-C3 were also assessed, and the stability of the receptors during culture was examined.

We found that in response to the T-dependent antigen, horse RBCs, lymphocytes bearing only C3b receptors produced only IgM antibody, while C3d-receptor cells produced both IgM and IgG. Cells lacking C3 receptors produced mainly IgG. In the polyclonal mitogen and antibody responses to lipopolysaccharide and to purified tuberculin protein (PPD), C3d-receptor cells responded well to both substances, while C3b-receptor cells and cells lacking C3 receptors respnded weakly. C3b-receptor cells, however, were the only ones which responded to the T-independent antigen, DNP-Ficoll. C3d receptors were relatively stable during culture, but cells bearing only C3b receptors at the

initiation of culture were found rapidly to express C3d receptors as well due to effect of 2-mercaptoethanol.

In the cell cultures used to study antibody production in vitro, C3 (secreted by the cultured cells) was apparently limiting for antibody secretion. The IgM response of both C3b-receptor and C3d-receptor cells was enhanced by addition of semi-purified mouse C3 to the culture, but this had no effect on IgG production or on the limited IgM response of cells lacking C3 receptors, and did not affect the response to polyclonal activators or a T-independent antigen. Anti-C3 antibody completely shut down the IgM response of cells bearing C3 receptors, but did not affect cells lacking C3 receptors.

We hypothesize that C3 receptors are B cell differentiation markers, and that their stimulation promotes IgM antibody secretion. Our data are consistent with the following progression: C3b receptors = IgM secretion > C3b and C3d receptors = IgM and IgG secretion > C3d receptors = IgM and IgG secretion > no C3 receptors = mainly IgG secretion.

ABBREVIATIONS

A cells	Accessory cells
AFC	Antibody forming cells
BCDF	B cell differentiation factor
BCGF	B cell growth factor
C'	Complement
C1, C2 etc.	The first, second, etc. component of C'
C3b-INA	C3b-inactivator
Con A	Concanavalin A
CR	Complement receptor
DNP	Dinitrophenyl
EAC1, EAC4, etc.	Sensitized sheep erythrocytes bearing the first, fourth, etc. component of C'
EBSS	Earle's balanced salt solution
e.m.	Effective molecule
EDTA	Ethylene dinitrilotetra-acetic acid, disodium salt
FCS	Fetal calf serum
Fl	Fluorescein
GVB^{++}	Gelatin-veronal buffer with calcium and magnesium
HBSS	Hank's balanced salt solution
HRBC	Horse red blood cells
HuE	Human erythrocytes
I-A	Immune-adherence
Ig	Immunoglobulin
IL-1, IL-2	Interleukin 1,2
LPS	Lipopolysaccharide
PFC	Plaque forming cells

PPC	Plaques per culture
PPD	Purified protein derivative
PBS	Phosphate buffered saline
SRBC	Sheep red blood cells
TRF	T-cell replacing factor

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INTRODUCTION

The importance of complement as a mediator of allergic reactions and inflammation is well recognized. In contrast to this effector role, the possibility that complement might also play a part in the induction of the immune response was suggested by two sets of observations: first, the detection by Gajl-Peczalska et al. (1969) of C3 and complement fixing immune complexes within germinal centers of lymphoid follicles; and second, the demonstration by Bianco and his colleagues of receptors for fixed C3 on the surface of B lymphocytes (Bianco et al., 1970). The first experimental evidence of such a role for complement was the suppressive effect of C3 depletion by cobra venom factor on thymus-dependent antibody production in mice (Pepys, 1972). This suggested that complement might participate in cooperative antibody production (Pepys, 1974), and there has since been further exploration of this concept at the experimental and theoretical levels.

Thus, the view that complement should be considered merely a serum molecular system whose association with cells is secondary to complement activation in serum, is insufficient. The purpose of the research presented in this dissertation was to try to answer the question: "What roles do C3 and its receptors on lymphocytes play in the induction of the B cell immune response?" The approaches taken to answer this question were: 1) to develop a hemolytic assay for measuring functional mouse C3 so that amounts of C3 or C3 inactivating enzymes synthesized in culture could be assayed; 2) to make specific indicator particles for the C3b and C3d receptors on cells so that each cell type could be separated from whole spleen cell suspensions

and tested in various systems, such as the primary and secondary *in vitro* immune responses to horse erythrocytes and the polyclonal responses to lipopolysaccharide and purified protein derivative of tuberculin; and 3) to assess the effects of semi-purified C3 and anti-C3 on the immune responses of these various cell subpopulations in culture.

SURVEY OF THE LITERATURE

A. B Lymphocyte Immune Recognition and Induction

Immunoglobulin (Ig) molecules as recognition structures on the surface of lymphocytes were postulated by the clonal selection theory (Jerne, 1955; Burnet, 1957), and were first experimentally demonstrated by Moller (1961). Clonal selection requires a lymphocyte to synthesize and express on its surface receptors of a single antigen binding specificity. This postulate has been supported by the findings that surface-bound Ig molecules show allelic exclusion (Sell, 1970), and that subsets of cells among the lymphocytes cover different antigenic specificities. It was expected that binding of antigen at the cell surface would initiate reactions leading to replication and maturation of the cell. Different clones of antigen specific lymphocytes responded to different antigens, and were found to retain their original specificity during clonal expansion (Dutton and Eddy, 1964).

This simple concept of antigen recognition was no longer tenable when it was found that three different types of cells -- T lymphocytes, B lymphocytes and macrophages -- had to cooperate in order to induce many humoral immune responses (Miller and Mitchell,

1967). Mitogens were later discovered which could activate a large proportion of B cells in a polyclonal fashion (Andersson et al., 1972a), by circumventing the need for antigen binding to surface Ig molecules. B cell mitogens also bypassed the need for T cell help, acting directly on B cells and inducing them to replicate and to mature to Ig secretion (Andersson et al., 1972b). Antigens bound to such mitogens therefore elicited T cell-independent B cell responses. Many B cell mitogens, such as lipopolysaccharide (LPS), lipoprotein, and the purified protein derivative of tuberculin (PPD), are found in the outer layers of bacteria.

Taken together, these findings suggested that B cells must cooperate with other cells, either with T cells and macrophages within the system or with mitogen-bearing bacteria coming from without, to be stimulated to replication and Ig secretion. The action of B cell mitogens clearly indicated that B cells received these stimuli through receptors -- so-called mitogen receptors, which had to be structurally different from Ig molecules. This became apparent in C3H/HeJ mice, which were found to be genetically defective in their response to LPS (Sultzer and Nelson, 1972), while responding normally to lipoprotein, PPD and T-dependent antigens. This genetic defect indicated also that receptors for LPS had to differ structurally from those for lipoprotein, PPD and T cell help. The effects of external B cell mitogens also made it plausible that T cell help could, at least in part, be effected by antigen-unspecific, polyclonally stimulating factors (Schempl and Wecker, 1972; Waldman and Munro, 1973). Therefore, antigens had to associate in a functional way with mitogens or other factors in order to become immunogenic.

The piecing together of the story of immune induction was further complicated when it became evident that antigen-specific T cell-macrophage-B cell cooperation was H-2 restricted (Katz et al., 1973; Rosenthal and Shevach, 1973). It is now clear that such H-2 restriction operates at two levels. At the first level, helper T cells recognize antigen in association with Ia determinants on macrophages. This leads to activation of the helper T cells and to the production of helper factors by either the T cells or the macrophages. At the second level, the activated T cells, the antigen and the factors produced in the first stage of interaction act on B cells. At this second stage B cells have to express surface Ig (to recognize antigens); Ia molecules (to be recognized by the helper T cells); and receptors for the factors produced in the first stage.

The antigen culture system developed by Mishell and Dutton (1967) has permitted experimental manipulation of cell types and factors involved in the immune response under carefully regulated conditions. Such experiments have greatly increased the understanding of the functional role of each of the required cells and/or factors needed in the generation of antibody responses.

B. Theories of Immune Triggering of B Cells

Immunological triggering of (bone marrow derived) B lymphocytes can be defined as the molecular process which drives the cell from its initial resting state into a metabolically active phase, resulting in clonal proliferation, differentiation and subsequent secretion of Ig. Contact with antigen plays a major role in the triggering process as is evident from the selectivity of the antibody

response. This binding of antigen to Ig receptors may directly deliver the initial signal for B cell activation via cell membrane perturbation related to redistribution and steric changes of Ig molecules; and it probably renders the B cell susceptible to a variety of second signals and thus be necessary but not sufficient for cell activation.

Many hypotheses have been formulated to explain the mechanism of B cell triggering. The matrix hypothesis (Feldman et al., 1973) postulates that multivalent interaction of antigen with B cell receptor molecules brings about a triggering stimulus whereas monovalent interaction is ineffective. T cell helper function is envisaged as a mechanism for organizing a nonpolymeric antigen into a matrix structure capable of interacting in multivalent array with specific sites on B cells. The matrix is thought to be formed on a macrophage surface by means of cytophilic IgX (specific helper factor) complexed with antigen. The importance of the macrophage in the control of B cell triggering has received much experimental support. The Ig character of the specific helper factor appears unlikely in view of the results of Taussig and Munro (1974). The matrix hypothesis derived its strongest support from the in vitro behavior of the monomeric and polymeric forms of flagellin antigen. However, in vivo studies using the same antigen system have provided conflicting results (Langman et al., 1974).

The two signal theory for B cell triggering was formulated by Bretscher and Cohn (1968). In this theory, Signal 1, occurring on contact of haptenic determinants with B cell receptor sites, induces a tolerogenic state unless counteracted by Signal 2, provided by

interaction of a carrier determinant with specific T cell-derived helper factor. The hypothesis has been further extended (Watson et al., 1973) to suggest that Signal 1 leads to production of cyclic AMP which in turn inactivates the cell, this event being transferred into a differentiation pathway in the presence of Signal 2, postulated to release cyclic GMP. The two signal hypothesis is successful in rationalizing the known capacity of nonimmungenic carrier molecules to bring about hapten-specific B cell tolerance. However, the model does not accunt for the action of T-independent antigens or the observed dose dependence of the in vitro induction of immunity and tolerance to polymerized flagellin (Diener and Feldman, 1972).

In their studies with T cell replacing factor (TRF), Schimpl and Wicker (1975) have postulated a three signal system in B cell triggering. They have shown that soluble factors are regularly produced upon stimulation of T cells and that they show no specificity for the test antigen used, nor for the H-2 antigens that stimulated their production. Removal of adherent cells was shown to have no effect when carried out 14 hr after the commencement of allogeneic cultures but before a measureable TRF activity could be detected in the supernates. TRF showed the peculiar trait of having a better effect when added later during the culture period (24-60 hr), without delaying the peak response. Even as late as 84 hr after initiation of the culture, TRF still exerted considerable biologic activity, leading to a plaque response 24 hr later. These authors suggest that in Stage 1, a resting mature B cell makes contact via its Ig antigen receptors with appropriate antigen determinants. This first signal does not by itself lead to proliferation but renders the cell receptive to

subsequent events and is the stage of selection. In Stage 2, the B cell receives an additional stimulus via part of its membrane other than the specific Ig antigen receptor. This second signal gives the actual stimulus for the ensuing proliferation of the cell. The various stimuli which can initiate this signal are likely to be channeled into some final common pathway. If the antigenic determinants which initiate Signal 1 are part of the same structure which evokes Signal 2, then Stages 1 and 2 are jointly initiated and clonal expansion ensues. Sheep RBCs (SRBC) fulfill these requirements. Even in the presence of sufficient antigen and after several cycles of division a third signal is required in order to bring about active synthesis and secretion of antibodies. In the case of T cell dependent antigens, this third stage is initiated by helper T cells, via a soluble and antigen non-specific factor. After onset of antibody production, the B cell continues several more cycles of division until it reaches the end stage of differentiation, that is, becomes a plasma cell. The stages of B cell activation are meant to indicate that events are sequential. This is clearly so for Signal 3, which can be received only after Signals 1 and 2. This activation system allows for three types of antigens: a) haptens and those T-dependent antigens which can give only Signal 1; b) T-dependent antigens which can give both Signal 1 and Signal 2; and c) T-independent antigens which can give all three signals. Only use of the second type of antigen can reveal antigen non-specific mediators such as TRF, thus showing the need for a distinct third signal.

The last major hypothesis of B cell triggering is the "one non-specific signal" of Moller and Coutinho (1975). This theory states

that B cells are activated by nonspecific signals delivered to the cells by surface structures which are not the Ig antigen receptor. The antigen receptors in their view have an important passive focusing function which permits selective binding of the antigen to the cells and thus these receptors are responsible for the characteristic specificity of the immune response. The actual triggering event is caused by signals delivered either by the antigen itself (but not by its antigenic determinant) in the case of T-independent antigens, or by factors released from accessory cells such as T cells and macrophages in the case of T-dependent antigens. A signal must lead to reprogramming of the cell, revealing the immunological capacity of the responding cell at its particular stage of differentiation, e.g., induction of DNA synthesis or antibody synthesis or both. Thus, antigen binding to Ig receptors is an event, but not a signal because, by itself, binding does not change the resting state of the cell. The nature of the triggering receptor on B cells remains unknown. These authors feel the receptor is not the combining site of the Ig receptor or the receptors for complement or the Fc piece of the IgG molecule. It is also not known whether subsets of B cells have the same receptor or whether there are different receptors for different polyclonal activators. The receptor is present on B cells from all animal species so far studied, indicating that it has been preserved during evolution.

Each of the above hypotheses is successful in rationalizing certain aspects of B cell behavior. However, at the present time, it is impossible to specify the exact nature of the membrane perturbations which direct the cell towards the various pathways of

proliferation, differentiation, tolerance or cell lysis.

C. B Cell Differentiation

The aspect of B cell differentiation which begins with the reaction of antigen with surface Ig receptors and ends with the production of antibody secreting cells comprises a complex train of events. Important aspects of this process are the chemical signals, positive and negative, and their corresponding receptors, whereby the steps in the process are regulated. Over the last several years, much data has been accumulated concerning the soluble factors produced by polyclonal activation of T cells by Con A. Farrar and Hilfiken (1982) found that the antibody response to heterologous red blood cells was non-specifically boosted by Interleukin-1 (IL-1), Interleukin-2 (IL-2), T-cell replacing factor (TRF) and colony stimulating factor (CSF), all generated in a T-cell culture stimulated by Con A.

Jaworski and co-workers (1982) proposed a model of events leading to antigen specific B cell triggering: antigen primed helper T cells elaborate various factors, one of which is Helper factor (Hf), an antigen specific, Ia-bearing factor with a minimum molecular weight of 50,000. This factor is specific both in terms of its binding to antigen and in terms of its functional activity in inducing a humoral response. Focused by its antigen specific receptor to the antigen binding B cell, Hf interacts in an H-2 restricted fashion with the cell surface membrane to induce the cell to proliferate. Whether or not the proliferation signal requires that Hf interact simultaneously with the Ig receptor-antigen complex and with other determinants on the cell surface membrane has not yet been determined. The

differentiation into IgM secreting cells does not occur unless additional non-antigen specific signals are given by Accessory cells (A cells). A cells secrete soluble products which in the presence of Hf promote the differentiation of B cells into antibody forming cells (AFC).

Two factors have been isolated from Con A stimulated T cell supernates which appear to be B-cell specific: B cell growth factor (BCGF), and B cell differentiation factor (BCDF). Nakaniski and coworkers (1983) have isolated two BCDF molecules. B-115-TRF is required within the first 24 hr of a 96 hr culture and EL-TRF functions in the last 24 hr. The actions of both are apparently antigen non-specific and both appear to function predominantly as differentiation stimuli. Pine and coworkers (1983) have purified BCGF and found its molecular weight to be less than 20,00. They have identified two BCDF molecules, which show specificity for either IgM or IgG secretion. The specific cell secreting these factors, the receptor on the surface of B cells for these factors, and the ways these factors interact in culture still have to be elucidated.

D. Synthesis of Complement Proteins

The sites of production of complement proteins and the cell types involved have been studied in primary cultures and in established cell lines. The newly synthesized proteins are identified by hemolytic function and/or by their reaction with specific antibodies after metabolic labeling with radioactive amino acids. Taking advantage of common structural polymorphisms of the third, sixth and eighth complement components and properdin factor B, it was

shown that more than 90% of each of these proteins is synthesized in the liver (Alper and Rosen, 1976). Evidence for extrahepatic synthesis of C3, and three subcomponents of C1, C4, C2, C5, and factors B and D has been obtained by Whaley (1980). These findings, along with studies of C3 synthesis by monocytes, confirmed the earlier suggestion by Thorbecke et al. (1965) that many primate tissues were capable of synthesizing C3, including the liver, spleen, lymphoid cells and other tissues of the reticuloendothelial system as well as a few epithelial organs such as the gut and mammary tissue.

To date, it is not clear whether peripheral blood lymphocytes are also capable of synthesizing some complement components. Sundsmo (1980) has preliminary evidence for synthesis but not secretion of several complement components by peripheral blood lymphocytes. He suggests that peripheral blood monocytes synthesize and secrete C2, C3, C4, C5, factors B, D, P, C3bINA and B1H as antigenically recognizable products; and that they produce functionally active C2, B, P, D and C3bINA, but not C3, C4, C5, C6, C7, or C9 under conventional tissue culture conditions.

The concentration of functional complement proteins in the plasma is controlled by modulating the rates of biosynthesis, distribution and catabolism. Hormones influence serum levels of complement, the late-acting complement components (C3 to C9) in serum from male mice being 8 to 10 times more abundant than in females (Churchill et al., 1967). Only one tissue culture system is available for study of hormonal control of complement protein production. This is a well-differentiated rat hepatoma cell line which synthesizes C3 at up to nine-fold higher rates in response to hydrocortisone (Struck et al.,

1975).

Factors other than hormones can also lead to increased synthesis of complement components. In monocyte cultures little synthesis of C2 or factor B is observed during the first 3 to 6 days in vitro, after which a marked increase in the rate of C2 production is noted (Einstein et al., 1976). This increase, coincident with morphological evidence of monocyte maturation, can also be induced in one day old cultures by soluble mediators from antigen stimulated lymphocytes (Littman and Ruddy, 1977). Many synthetic functions of peritoneal macrophages are modulated by injection of starch, casein, thioglycolate and other inflammatory stimuli. Fey et al. (1980) have performed systematic studies on resting and elicited macrophages in order to provide some quantitative estimate of the effect of inflammation on complement production. Guinea pig peritoneal macrophages elicited by starch injection secreted C4 at a 5 to 8 fold greater rate than resident peritoneal cells. This increased rate results from a change in the rate of synthesis by individual cells, not a change in the proportion of complement-producing macrophages. Matthews et al. (1979) have also noted a negative feedback control by C4-related peptides on C4 synthesis in peritoneal cells. Genetically determined deficiencies of nearly all the components of complement are known and have been extensively reviewed by Alper and Rosen (1976).

E. Complement Receptors on Cells

The existence of binding sites for complement component C3 was first described on human peripheral leukocytes by Nussenzweig and his colleagues (Lay and Nussenzweig, 1968, Bianco et al., 1970),

although Nelson showed long before this that similar receptors were present on primate erythrocytes and non-primate platelets (Nelson, 1953) (see below). Since then, specific membrane receptors for bound C3, C4, C5, C1q and B1H have been shown to be expressed on several different leukocyte types. The majority of the complement receptor bearing lymphocytes belong to the B-lymphocyte population (Dukor et al, 1971); however, some complement receptor lymphocytes have been identified as T lymphocytes (Arnaiz-Villani et al., 1974; Wilson, Tedder and Fearon, 1983). In addition, mast cells and various leukocyte types bear membrane receptors for C3a, C4a, and C5a. And it has recently been shown that neutrophils bind a small acidic fragment of C3, known as C3e, and that the injection of this fragment into rabbits intravenously induces a brief leukocytosis involving neutrophils (Ghebrehiwet and Muller-Eberhard, 1979).

It has been found that there are three different C3 receptors called CR1, CR2 and CR3, each one reacting with a different C3 degradation product (Ross et al., 1982, Ross et al., 1983 and Gaither et al., 1983). The first, CR1, is specific for both C4b and the C3c portion of C3b, but is unreactive with both C3bi (formed by cleavage of C3b with the enzyme C3b-inactivator and its cofactor B1H), and the C3d fragment of C3 (formed by proteolytic degradation of C3bi). CR1 is found on B cells, some T cells (at lower concentration), macrophages and other antigen-presenting cells, neutrophils, primate erythrocytes, non-primate platelets, and glomerular epithelial cells (Iida et al, 1982).

Fearon (1980) recently succeeded in isolating CR1 from human erythrocytes, and found the isolated receptor to be a glycoprotein

containing only a single polypeptide chain. Labeled CR1 receptor was found to bind fixed C4b, as well as C3b, but not C3bi nor C3d. Anti-CR1 inhibited C4b and C3b dependent rosette formation equally, but had no effect on C3d dependent rosettes (Dobson et al., 1981). This confirmed earlier studies showing that C4b and C3b were bound by the same complement receptor (Ross and Polley, 1975), and that CR1 was antigenically distinct from CR2 (Ross et al., 1973).

CR2 binds to C3d and to the C3d region of C3bi and is unreactive with C3b and C4b. It is expressed on B lymphocytes and is probably absent from monocytes, macrophages and neutrophils. Isolation of CR2 from lymphocytes has yielded a glycoprotein with similar specificity to that of membrane bound CR2. Labelled soluble CR2 bound to C3d but not C3b, and anti-CR2 inhibited C3d-dependent rosette formation, but not C3b-dependent rosettes (Lambris et al., 1981). Thus, CR2 is completely distinct from CR1 in both structure and specificity. Although the majority of normal B cells express both CR1 and CR2, B cells that express only one or the other of these two types of receptors can be readily demonstrated both in tonsils (Ross et al., 1975) and peripheral blood (Ross and Polley, 1978).

Recently, it has been shown that both monocytes/macrophages (Whaley, 1980) and B lymphocytes (Ross and Dobson, 1980), synthesize and secrete B1H and C3b-INA. This enables these cell types to convert surface C3b to C3bi, which would then react with CR2 and CR3 instead of CR1. This explains several previously published studies in which complex-bound C3b was shown to bind to either CR2 or CR3 receptors. In the presence of anti-C3b-INA, C3b binds only to CR1 and not to CR2 or CR3 (Lambris et al., 1980).

Much less information is available about the CR3 receptor. It was previously thought that enzymatic digestion of C3b by C3b-INA produced C3c and C3d fragments, and the initial C3b breakdown product, C3bi, was unrecognized (Ross and Polley, 1976). C3b-coated erythrocyte complexes treated with purified C3b-INA were thought to contain bound C3d exclusively, whereas they actually contained only C3bi, and no C3d (Carlo et al., 1979). This made no difference in assays for CR2 receptors, as they subsequently were found to bind C3bi as well as C3d fragments. C3bi-bearing indicator complexes can be converted to pure C3d-bearing complexes by treatment with a wide variety of proteases including trypsin, plasmin, elastase and thrombin. Such true EAC3d complexes, though fully reactive with CR2, were found to be completely unreactive with myeloid cells and monocytes-macrophages that were previously thought to express CR2 type receptors (Dobson et al., 1981). Thus it seems likely that myeloid and monocyte-macrophage cells express CR1 and a third type of receptor, CR3, that is distinct from CR1 and CR2. CR3 is specific for a site within C3bi that is distinct from the C3d region binding site for CR2 (Ross and Rabellino, 1979). It appears possible that the CR3 binding site might reside within the C3e region of C3bi, since granulocytes apparently bind C3e fragments and may have a C3e region-specific receptor (Ghebrehurst and Muller-Eberhard, 1979). B cells may also express CR3 in addition to CR1 and CR2; however, this has been difficult to ascertain since both CR2 and CR3 bind to C3bi. Future studies that examine inhibition of B cell C3bi dependent rosettes with anti-CR1 and anti-CR2 should answer this question.

Specific receptors for C1q have been demonstrated on B

lymphocytes and monocytes and are absent from other blood cell types (Tenner and Cooper, 1980). B1H receptors have recently been identified on human B lymphocytes and the expression of this receptor on other blood cell types has yet to be defined (Dierich and Schult, 1980). At this time, B1H receptors are the only B cell membrane receptor for which a function can be readily demonstrated *in vitro*. Engagement of B1H receptor with either aggregated or complex bound B1H induces the release of endogenous C3b-INA from B cells. This results in cleavage of any nearby C3b to C3bi and binding of the C3bi to lymphocyte CR2 receptors (Ross *et al.*, 1981).

F. Direct Involvement of Complement Components in Immune Induction

Moller and Coutinho (1975) using steroyl dextran which binds to cell membranes and fixes complement, showed that lymphocytes treated with this compound and subsequently incubated with mouse serum were blocked from rosetting with EAC_{mo}. They reported that this treatment had no effect on the number of plaque forming cells (PFC) versus three different antigens (SRBC, FITC and NNP), nor on polyclonal activation of B cells by LPS or PPD. They concluded that surface-bound C3 doesn't activate or prevent activation of B cells by these antigens. In contrast, when LPS or PPD was pre-treated with fresh serum, reduced amounts were needed for optimal stimulation of B cells, suggesting that these polyclonal B cell activators (PBA's), after reacting with serum C3, bound more efficiently to B cells so less was needed to stimulate them.

Mason (1976) labelled rat lymphocytes *in vivo*. He concluded from

his data that the precursors of IgM PFC were complement receptor lymphocytes (CRL), while those of IgG were both CR+ lymphocytes and CR- lymphocytes. This was interpreted to mean that C3 may not be essential in the secondary IgG immune response.

In a series of experiments on the generation of memory B-cells, Klaus and Humphrey (1977) initially found that mice chronically depleted of serum C3 by CVF treatment never developed memory B cells. CVF treatment of thymectomized mice prevented the 10 fold increase in the number of DNP binding B cells in the spleen after injection of DNP-KLH. The CVF treatment blocked proliferation of antigen binding B cell after priming. If mice were given passive antibody to DNP-KLH, followed by labeled DNP-KLH, the antigen was localized 4 times as much as antigen localized in the spleen if no antibody was given. If CVF was administered, this effect of antibody was abolished. In studies using DNP-levan, a compound which activates C3, Klaus found that this antigen rapidly localizes in the lymphoid follicles of spleen and lymph nodes and remains at least 10 days. If CVF was given to mice before DNP-levan, it did not concentrate in follicles indicating that C3 was needed for antigen to concentrate in lymphoid follicles.

Klaus next studied the generation of B memory cells with preformed Ag-Ab complexes (1978). He found that complexes were much more active and acted faster than antigen alone in inducing memory cells. If C3 was depleted, the effect was gone. This data suggested that B memory cells are generated in follicles after C3-dependent localization of Ag-Ab.

Finally, Klaus (1979) studied the antibody class requirement for the generation of B memory cells by Ag-Ab complexes. He found that

IgM was inactive and tht IgG_2 > IgG_1 > IgA, both with respect to the number of PFC and the amount of antigen retained in the spleen. He found that all classes but IgM activate mouse complement. So from all of his studies, Klaus concluded that complement activation is probably essential for follicular localization of Ag-Ab and also for the generation of B memory cells. He believes that Ag-Ab deposits on the surface of follicular dendritic cells where it remains for many months. He doesn't know if these cells have C3 receptors. This could explain why T-independent Ags don't induce much memory, because they induce mainly an IgM response.

Working with fragments derived from the activation of C3 or C5, Weigle and workers (1982) reported that C3a inhibits polyclonal and anti-SRBC responses of mouse and human lymphocytes in vitro. C3a appeared to act on a $Lyt1^+2^-$ suppressor-inducer cell, didn't affect B or T cell proliferative responses, was active only when a carboxypeptidase inhibitor was present, and did not inhibit in the absence of T cells. C5a, on the other hand, enhanced anti-SRBC and MLC (mixed lymphocyte culture) reactions in mice, four-fold. It had to be added within 48 hr of culture initiation or the effect was lost (Goodman et al., 1982).

Hobbs et al. (1982) found that human or rat C3 blocked rat secondary IgG responses by 80-90%. C3 could be added early or late in the culture period, and it worked with both T-dependent and T-independent antigens. The activity appeared to reside in the low molecular weight C3a fragment.

To date, C3 has not been proven to be an essential signal or component for B cell activation or proliferation, although the

presence of C3 receptors on B cells, T cells and antigen-processing cells suggests that it probably plays some important role. The present study will show that 1) the maturation of B cells correlates with their expression of complement receptors; 2) C3 is synthesized by mouse spleen cells in culture; 3) these cultures also synthesize compounds which will inactivate C3; 4) cells which bear the C3b receptor produce only an IgM response to T-independent antigens; 5) addition of semi-purified C3 to cultures enhances the IgM response of cells bearing C3 receptors, and addition of anti-C3 prevents it; 6) neither semi-purified C3 nor anti-C3 has any effect on the secondary (IgG) response to T-dependent antigens.

MATERIALS AND METHODS

I. Buffers

Isotonic buffers at various ionic strengths were made by mixing different proportions of sucrose-veronal buffer (μ= 0.009) and veronal buffer (μ= 0.15) containing added Ca^{++} and Mg^{++}, according to the method of Rapp and Borsos (1963). All buffers contained 0.1% gelatin (Difco Labs., Detroit, MI) or, for immune adherence (I-A), 0.1% bovine serum albumin (Cohn FrV, Armour Pharmaceutical Co., Kankakee, IL). For I-A with mouse C3, 0.1% ovalbumin (Schwartz-Mann, Orangeburg, NY) was substituted. Gelatin-veronal buffer (μ = 0.15) was designated GVB^{++}; that containing 0.02M EDTA was designated EDTA-GVB.

II. Media

A. RPMI-1640 with 25 mM hepes buffer was purchased from the Cell Culture Facility, University of California, San Francisco.

B. Hank's balanced salt solution (HBSS) was purchased at 10X concentration from Gibco Labs (Grand Island, NY). HBSS was used for spleen cell preparation, washing of lymphoid cells and for the rosette assay.

C. Earle's balanced salt solution (EBSS) was purchased at 10X concentration from Gibco Labs (Grand Island, NY). EBSS was used for harvesting cells for the Jerne plaque assay.

D. Mishell-Dutton medium consisted of RPMI-1640 with 25 mM hepes buffer supplemented with 10% fetal calf serum (FCS) (Sterile Systems, Logan, UT), 2 mM glutamine (Gibco Labs, Grand Island, NY) pen-strep (100 units penicillin, 100 μg streptomycin) (Gibco Labs, Grand Island,

NY), and 5 x 10^{-5}M 2-mercaptoethanol (Sigma, St. Louis, MO). This medium was used for both primary and secondary in vitro immune responses to either sheep red blood cells (SRBC) or horse red blood cells (HRBC).

E. Polyclonal culture medium consisted of RPMI-1640 with 25 mM hepes buffer supplemented with 5% FCS, 2 mM glutamine and pen-strep. This medium was used for both the polyclonal antibody response and the polyclonal mitogen response.

F. Cytotoxicity medium. This medium, used for all cytotoxic reactions, was purchased from Cedarlane Labs (Hornby, Ont., Canada).

III. Animals

A. Female C_{57}Bl/6 or BDF1 mice, 6-8 weeks of age, were used for all primary and secondary in vitro immune response studies. Female mice 4-6 weeks of age were used for the polyclonal responses. Retired female breeders of either strain were bled two times per week for serum or plasma pools. All mice were purchased from Simonsen Labs (Gilroy, CA). The animals were housed six to a cage and were fed standard Purina Laboratory Mouse Chow.

B. Outbred Hartley guinea pigs (250 grams) of either sex were purchased from Simonsen Labs (Gilroy, CA). The animals were housed 3 to a cage and fed standard Purina Laboratory Guinea Pig Chow.

C. Four week old New Zealand rabbits of either sex were purchased from Nitabell Rabbit Tree (Hayward, CA). The rabbits were housed 2 to a cage and fed standard Purina Laboratory Rabbit Chow.

IV. Immunizations.

For secondary immune responses in vitro, mice were first primed with HRBC by a single intraperitoneal injection of 0.1 ml of a 10% suspension of thrice washed HRBC in HBSS. The spleen cells were placed in culture 14 days later.

V. Antigens

A. T-dependent antigens

1. Pooled HRBC, preserved in Alsever's solution, were purchased from Colorado Serum Co. (Denver, CO).

2. Unpooled SRBC from sheep number 298, preserved in Alsever's solution, were purchased every 2 weeks from Colorado Serum Co.

B. T-independent antigen: (DNP_{121})-Ficoll was prepared according to Inman (1975). This compound was generously provided by Dr. George Lewis of this department.

C. Haptenated SRBC

1. TNP-SRBC. SRBC were washed 4 times with phosphate buffered saline (PBS); 2,4,6-trinitrobenzene sulfonic acid (TNBS) (Sigma Chemical Co., St. Louis, MO) 30 mg, was dissolved in 7 ml of sodium cacodylate buffer pH 7.2. The TNBS was added dropwise to one ml of packed SRBC. The mixture was stirred for 10 min at room temperature in the dark. The TNP-SRBC conjugate was washed with 1% FCS-PBS until the supernate was clear. These cells were used in the Jerne plaque assay to measure the DNP-specific antibody response to DNP-Ficoll.

2. Fl-SRBC. SRBC were washed 3 times with PBS and 1 time

with carbonate-bicarbonate buffer 0.25 M, pH 9.0. Fluorescein isothiocyanate isomer I (Sigma Chemical Co., St. Louis, MO) was dissolved (10 mg/ml) in carbonate-bicarbonate buffer, and added in the proportion of 2 ml per ml of packed SRBC. The reaction mixture was rotated end over end for 1 hr at room temperature in the dark. The cells were washed extensively with PBS until the supernate was free of hemoglobin color. These cells were used in the Jerne plaque assay to measure the polyclonal antibody response to lipopolysaccharide or Purified Protein Derivative (Coutinho and Moller, 1975).

D. Mitogens

1. Lipopolysaccharide (LPS) from E. coli (serotype 055:B5) was purchased from Sigma Chemical Co., (St. Louis, MO) and used at a concentration of one or 10 μg per culture for the polyclonal mitogen response, and at a concentration of 100 μg per culture for the polyclonal antibody response.

2. Purified Protein Derivative (PPD) (PPD-CT68), preservative-free, was purchased from Connaught Laboratories (Toronto, Canada), and used at a concentration of 100 μg per culture for both the polyclonal mitogen and antibody responses.

3. Concanavalin A (Con A) was purchased from Pharmacia Fine Chemicals (Piscataway, NJ), and used at a concentration of 0.5 μg per culture for the T-cell mitogen response.

VI. Antisera

A. Anti-Thy1.2. This monoclonal IgM antibody was purchased from New England Nuclear (Boston, MA) and was used to deplete T cells from the mouse spleen cell suspensions.

B. Anti-Theta. Polyvalent rabbit anti-Theta (IgG) was purchased from Accurate Biochemicals (Hicksville, NY). This antiserum was used along with fluorescein-Protein A, to enumerate the T cells in the mouse spleen cell suspension.

C. Fl-Anti-Mouse IgM. Rabbit (IgG) anti-mouse IgM coupled with fluoroscein was purchaed from Accurate Biochemicals. This reagent was used for the enumeration of B cells in mouse spleen cell suspensions.

D. Rabbit anti-mouse IgG. This reagent was purchased from Cappel Labs (Cochranville, PA), and used to develop IgG plaques in the Jerne plaque assay.

E. Rabbit anti-mouse IgM. This reagent was purchased from Cappel Labs and used in the Jerne plaque assay to inhibit IgM plaques.

F. Anti-mouse C3. Rabbit anti-mouse C3 was prepared according to the procedure of Mardiney and Muller-Eberhard (1965). The antiserum gave a specific agglutination reaction with mouse C3, C3b and C3d and gave a single precipitation arc vs normal mouse serum in immunoelectrophoresis. For use in Mishell-Dutton cultures, the antiserum was adsorbed 2 times at $0^{o}C$ with mouse spleen cells. $F(ab)_2$ fragment of goat anti-mouse C3 (IgG) was purchased from Cappel Labs.

G. Anti-bovine C3. Rabbit anti-bovine C3 was purchased from Cappel Labs and dialyzed against RPMI-1640 to remove the sodium azide preservative. The antiserum was sometimes absorbed 2 times for 20 min at $0^{o}C$ with mouse spleen cells before addition to cultures.

H. Rabbit anti-SRBC. Rabbit antibody to boiled sheep erythrocyte stroma was prepared as described by Kabat and Mayer (1961). The IgM fraction was separated by passage over a Sephadex G-200 column.

I. Bovine conglutinin was prepared using the method of Lachmann (1967). Forty ml of heated adult bovine serum (Colorado Serum Co.) were made 0.01 M with respect to EDTA, then absorbed once for 30 min at 37°C and 30 min at 0°C with 5 ml of boiled, packed zymosan, to remove antizymosan immunoglobulins but not conglutinin. After centrifugation, the supernatant serum was recalcified with $CaCl_2$ and stirred for one hr at 37°C with 7 ml of packed zymosan particles. The zymosan was then washed 5 times with veronal buffer, pH 7.4. The adsorbed conglutinin was eluted with 15 ml of 0.02 M EDTA-VB for one hr at 37°C, recalcified and stored at -70°C. Conglutinin prepared in this manner contained no detectable C3bINA, C1, C2, C3, C4, C5 or C8 activity, but had small amounts of C6, C7 and C9 (Linscott et al., 1978).

J. Anti-zymosan. This antiserum was produced by immunizing a rabbit with 50 mg of boiled zymosan intravenously and subcutaneously. The rabbit was boosted 2 times at 3 week intervals and then exsanguinated. IgM antibody was separated with a Sephadex G-200 column.

VII. Normal serum

A. Guinea pig serum. Guinea pigs were bled by cardiac puncture. The unpooled blood was allowed to clot for 2 hr at 4°C. The separated serum was pooled, aliquoted, and stored at -70°C. This pool of serum was used for complement component purifications and to make EAC4. Serum heated at 56°C for one hr was used as a source of C3bINA. Pooled commercial guinea pig serum was purchased from Colorado Serum Co. and used in hemolytic reactions as EDTA-C.

Lyophylized guinea pig serum was purchased from Gibco, and usd to develop plaques in the Jerne plaque assay.

B. Rabbit serum. Weanling rabbits were bled by cardiac puncture. The unpooled blood was allowed to clot for 2 hr at 4°C. The separated serum was screened for cytotoxic antibodies, and sera with low cytotoxicity were pooled and stored at -70°C, for use as rabbit complement. Normal rabbit serum was taken from a control rabbit used by the laboratory, and used as a control for rabbit antibody reactions. It was also used to block Fc receptors on lymphocytes. This serum was heat-inactivated for one hr at 56°C.

C. Mouse serum. Adult female mice were bled from the retro-orbital plexus and the blood was pooled, allowed to clot for one hr at 4°C, then centrifuged and stored at -70°C. If plasma was to be collected, 25 µl of 0.2 M EDTA was added to the collecting tubes. The serum was used as a source of mouse complement in making rosette indicator particles, and the plasma was used for C3 purification. Serum heated at 56°C for one hour was used as a source of C3b-INA.

D. Normal goat serum was purchased from Antibodies Inc. (Davis, CA), and used as a control serum for goat antibody reactions. This serum was heat-inactivated for one hr at 56°C.

E. Fetal calf serum was purchased from Sterile Systems (Logan, UT). Lot No. 171 was shown to be a good supportive serum for the Mishell-Dutton in vitro immunization assays. Five ml of this FCS were absorbed for 60 min at 37°C with one ml of packed boiled zymosan particles (Fleishman Labs, Brooklyn, NY). The zymosan was removed by centrifugation and the serum was heat-inactivated for one hr at 56°C and stored at -70°C. Hemolytic bovine C3 was undetectable. This

absorbed FCS was used in all Mishell-Dutton assays.

F. Guinea pig complement components

1. Semi-purified guinea pig complement components were purchsed from Cordis Corp. (Miami, FL).

2. Crude guinea pig C1 was prepared according to Nelson et al. (1966). Two hundred ml of guinea pig serum were adjusted to pH 7.5 and to 0.04 M with water. The serum was stirred for one hr in the cold, the resulting precipitate was rinsed with cold 0.04 M VB^{++}, dissolved in 40 ml of 2X concentrated VB^{++}, recentrifuged and the precipitate discarded. The C1 was reprecipitated with 6.5 volumes of cold water, adjusted to pH 7.5 and collected as before. The final C1 preparation was made isotonic with water, and gelatin was added to a concentration of 20 mg/ml. This preparation was stored at -70°C and contained C5, C6, C7, C8, C9 and C3bINA but not C2, C3 or C4.

3. Crude guinea pig C2 was purified by adsorption to and elution from EAC4 according to Mayer et al. (1970). Briefly, the conductivity of 200 ml of GPS was lowered to 0.04 M to remove C1. The resulting supernate was concentrated to 150 ml, then dialyzed against 4 liters of buffer (0.05 M PO_4, pH 7.6, 0.001M EDTA) for 24 hr to precipitate the C3. The clarified supernate was brought to 0.054 M conductivity, and diluted to 4000 CH_{50} units of C2 per ml. Two hundred ml of this C2 was incubated with EAC4 (2 x 10^8/ml) for 10 min at 30°C and washed 3 times with sucrose buffer (0.018 M, pH 8.5). C2 was eluted from the cells with 0.145 M veronal buffer, pH 6.5, for 10 min at 0°C. The cells were centrifuged and the C2 eluates pooled, concentrated 4-fold by negative pressure dialysis and dialyzed against VB^{++} before being aliquoted and stored at -70°C. This preparation

contained hemoglobin and some C5, C6, C7 and C9.

4. Guinea pig C3 was prepared according to the method of Shin and Mayer (1968). Briefly, the supernate from C1 precipitation of guinea pig serum was dialyzed and concentrated against 0.005 M phosphate buffer pH 5.5. The resulting precipitate was dissolved in 100 ml of 2X concentrated VB^{++} and centrifuged. To the supernate was added 6.5 volumes of cold distilled water so the final ionic strength was 0.04 M, and the pH was adjusted to 7.5. The crude C3 was added to a DE-52 column equilibrated with 0.4M phosphate buffer at pH 7.5. The column was then washed with 0.045 M NaCl and the C3 was eluted with 0.075 M NaCl. C3 fractions were pooled and adjusted to pH 5.0 and ionic strength 0.005 M, and applied to a CM-52 column. The column was washed with 0.094 M NaCl and the C3 was eluted with a linear NaCl gradient. The C3 came off the column at 0.014 M NaCl. Fractions were pooled and gelatin was added at a final concentration of 3%. The fractions were stored at $-70^{o}C$.

G. Mouse C3 was prepared according to the method of Gyongyossy and Assimeh (1977). Fifty ml of mouse plasma in EDTA were dialyzed overnight at 4^{o} C against 2 liters of 5mM phosphate buffer (pH 7.6) containing 1mM EDTA. The precipitate was removed by centrifugation and the supernate was dialyzed overnight at $4^{o}C$ against the starting buffer for SP-Sephadex chromatography. The dialyzed pseudoglobulin fraction was cleared by centrifugation and applied to an SP-Sephadex column. The column was washed extensively with starting buffer (pH 7.6) until the A_{280} of the effluent was less than 0.1. A linear salt gradient was applied and 5 ml fractions were collected, neutralized with one ml of 0.2 M veronal buffer and tested for C3 by I-A

(Nishioka, 1963). The C3 positive fractions were pooled, dialyzed overnight against the next buffer and applied to a column containing QAE Sephadex equilibrated with 50mM phosphate buffer pH 8.25 containing 1mM EDTA and 25mM NaCl. The column was washed and eluted with a linear salt gradient, and one ml fractions were collected and tested for C3. The positive fractions were pooled, gelatin added to a concentration of 2%, then quick-frozen in liquid nitrogen and stored at -70°C.

H. Bovine C3. Semi-purified bovine C3 was generously provided by Richard Triglia of this department.

VIII. Complement Component Assays

A. Guinea pig C1 hemolytic assay. To measure GP C1, $EAC4_{gp}$ (Nelson et al., 1966) (2 x 10^7 cells, made according to the method of Borsos and Rapp, 1967) were incubated with 2 x 10^3 e.m. C1 cell at μ = 0.065 for 15 min at 30°C. Then 3 ml of warm μ = 0.044 buffer were added, the tubes were centrifuged at room temperature and washed twice with μ = 0.044 buffer at room temperature. One hundred effective molecules (e.m.) of guinea pig C2/cell (μ = 0.065) were added and after 10 min at 30°C, 4.5 ml of guinea pig serum 1:50 in EDTA-GVB was placed in each tube. After one hr at 37°C, the tubes were centrifuged and the O.D. of the supernates was read at 413 nM.

B. Guinea pig C2 hemolytic assay. The method of Nelson et al. (1966) was used to make EAC14. The cells at 1 x 10^6/tube were incubated with 0.5 ml of test dilution for 10 min at 30°C at μ=0.044. Then 4.5 ml of guinea pig serum 1:50 in EDTA-GVB were placed in each tube followed by a one hr incubation at 37°C. The tubes were

centrifuged and the O.D. of supernates was read at 413 mM.

C. Guinea pig C3 hemolytic assay. The method of Nelson et al. (1966) was used to make EAC142. The cells at 1 x 10^6/tube were incubated with a C3 dilution at μ=0.065 for 30 min at 30°C. Then 0.25 ml portions of Cordis guinea pig C5 1:10, C6 1:10 and C7 1:20 were added (μ=0.065); after an additional 30 min at 30°C, 0.25 ml portions of C8 1:20 and C9 1:20 (μ = 0.15) were added, and the tubes incubated at 37°C for 90 min.

D. Mouse C3 hemolytic assay. This assay (Ranken and Linscott, 1979) was essentially the same as for measurement of guinea pig C3 with the following exceptions:

1. Extra GP C4 was added to EAC14 by treating them with GP C4 diluted 1:3 with 0.009 SVB^{++} for 12 hr at 0°C.

2. Before addition of C2, the EAC14 were washed with μ = 0.065 buffer.

3. The C2 reaction was carried out at μ = 0.086 and after the incubation, EAC142 cells were washed one time at 0°C with 0.15M VB^{++}.

4. The final ionic strength during the C3 reaction was 0.118, achieved by diluting the C3 in VB^{++} (the EAC142 being at μ = 0.086). The C3 reaction was carried out at 37°C for 10 min, and the cells were then washed one time with μ = 0.044 buffer.

5. Two hundred effective molecules of C2 in μ = 0.065 (0.25 ml) was added with the C5, C6 and C7.

E. Bovine C3 hemolytic assay. The method of Linscott and Triglia (1980) was used. Washed EAC142 were mixed with dilutions of bovine C3 at μ = 0.037 (0.25 ml); after 30 min at 30°C, 0.25 ml

portions of Cordis guinea pig C5 1:10, C6 1:10 and C7 1:20 were added (μ= 0.065). After an additional 30 min at 30°C, 0.25 portions of C8 1:20 and C9 1:20 (μ = 0.15) were added and the tubes held 90 min at 37°C.

F. Test for the presence of C3 and C3-Inactivating Enzymes in Culture

Macro cultures of mouse spleen cell suspensions (2 x 10^7 cells/ml) were incubated with or without antigen or FCS. Twenty-five μl of culture supernate was used as a source of C3 in the mouse C3 hemolytic assay described above. To test for C3-inactivating enzymes, semi-purified mouse, guinea pig or bovine C3 was incubated for varying periods of time with an equal volume of the above cell culture supernate. Dilutions of the mixture were made and tested for C3 in the hemolytic assay. To test for the presence of C3b-INA, $EAC4^{3}b$, prepared with limiting guinea pig or mouse C3, were incubated with spleen cell supernates for 3 hr at 37°C, μ = 0.037. The washed cells were then reacted with Cordis guinea pig C1, C2 and C5 through C9.

G. Guinea pig C3b Immune-Adherence (I-A) Assay (Nishioka, 1963). The cells to be tested for surface C3b were resuspended in distilled water and the lysed cells were then made isotonic with 5 times concentrated veronal buffer. Two-fold dilutions were made of the stroma to be tested, in VB^{++} 0.1% BSA buffer, and one ml aliquots of these dilutions were placed in hemagglutination tubes with 0.1 ml of human erythrocytes (2 x 10^8/ml). The tubes were shaken, incubated 10 min at 37°C, shaken again and allowed to stand undisturbed for 60 min at 37°C, after which the hemagglutination patterns were read. The reciprocal of the highest sample dilution giving a 2+ hemagglutination

pattern was multiplied by 2 to give the titer of the sample in 50% I-A Units ($CI\text{-}A_{50}/ml$).

H. Mouse C3b Immune Adherence Assay. The method was the same as for the guinea pig C3b I-A assay, except that the buffer contained 0.1% ovalbumin instead of BSA.

I. Conglutination Assay for guinea pig or mouse C3 on Cells (Linscott et al., 1978). A semiquantitative test was devised utilizing a Coulter Counter (Vh model) to determine the extent of aggregation of C3 coated cells treated with bovine conglutinin. Exactly 2.5×10^7 of EAC43 were washed in GVB^{++} and centrifuged in 13 x 100 mm test tubes. The cells were vigorously resuspended without adding any fluid to the tube and 0.5 ml of bovine conglutinin diluted 1:100 in GVB^{++} was then added followed by incubation with shaking for 30 min at 37°C. The tubes were then held overnight at 4°C, gently resuspended by hand and then exactly 5 µl were added to 10 ml of $\mu = 0.15$ veronal buffer which had previously been heated for one hr at 56°C to dissolve fine aggregates that register in the Coulter Counter. This suspension was counted 3 times with the following setting on the instrument: 1/amplification = 1/2; 1/aperture current = 1/2; aperture select=high resolution out; exclusion = 7.5; lower threshold = 10; upper threshold = 30; matching switch = 20k; gain = 5; aperture = 100 µm. Under these conditions, 88% of unsensitized erythrocytes (E) were counted between thresholds of 10 and 30 with minimal noise. The mean number of counts, minus the counts with buffer alone, was divided by the number of similarly corrected counts in a control. The resulting fraction was subtracted from one and multiplied by 100, to give the percentage reduction in the number of particles (cells and their aggregates) per

tube as a consequence of conglutination.

IX. Spleen Cell Preparations

A. Whole spleen cell suspension. Mice were sacrificed by cervical dislocation. Their spleens were aseptically removed and gently teased in HBSS. Suspensions were prepared from 6 mice at a time, then allowed to settle by gravity for 10 min at room temperature to remove cellular debris. The cells were transferred to a new tube, centrifuged, washed three times with HBSS and counted. For cell counts, the spleen cells were diluted 1:200 with trypan blue (0.04%) in a white blood cell diluting pipette, and the viable lymphocytes were counted in an AO hemocytometer under phase contrast at 30 X magnification.

B. Macrophages for Enrichment Studies. Three ml of sterile 3% thioglycollate broth was injected intraperitoneally into 6 week old mice. Four days later, the mice were killed by cervical dislocation and their fur dampened with alcohol. Their peritoneal cavities were lavaged with 5 ml of HBSS-heparin (5 units/ml). Peritoneal aspirates from batches of 6 mice were pooled. The red blood cells were lysed with tris buffered ammonium chloride, and the washed spleen cell suspension was given 3300 rads from a Cobalt-60 source (Nuclear Systems). The dose rate was 750 rads/min. The cells were used in culture the same day.

C. T cell enrichment or depletion

1. T cell depletion. Spleen cells were resuspended in Cedarlane cytotoxicity medium and monoclonal anti-Thy1.2 antibody diluted 1:30,000. After 30 min at 0^{o}C, the cells were centrifuged and

resuspended in cytotoxicity medium plus rabbit complement diluted 1:60. After 30 min at 30°C, the cell suspension was centrifuged, washed one time with RPMI-1640 and resuspended in the same medium at a concentration of $5x10^6$ lymphocytes per ml. The cells were then layered over Lympholyte-M (Accurate Biochemicals), and centrifuged at 1850 rpm for 20 min at room temperature. The resulting band of cells was collected, pooled and washed with HBSS. These cells were free of T cells based on their lack of stimulation by Con A.

2. T cell enrichment. Spleen cells suspended in RPMI-1640 were added to a nylon wool column according to procedures of Handwerger and Schwartz (1974). After incubating the column for 45 min at 37°C, cells were eluted by washing the non-adherent cells through with RPMI-1640 warmed to 37°C. The eluted cell population was greater than 90% T cells based on staining with anti T-cell reagents (see below).

D. Assessment of cell purity

1. T Cells. Cell suspensions were incubated for 30 min at 0°C with rabbit (IgG) anti-mouse T cell in RPMI-1640 containing 25 mM hepes buffer and 20 mM sodium azide. The cells were centrifuged and washed 3 times with cold PBS and then resuspended in RPMI-1640 containing 20 mM sodium azide and fluorescein-protein A for 30 min at 0°C. The cells were then centrifuged through FCS 2 times to remove unbound fluorscein, resuspended in RPMI-1640, smeared on slides, allowed to air dry and then fixed with absolute methanol for 20 min at room temperature. The cells were scored for fluorescence under oil immersion using a Zeiss fluorescent microscope.

2. B cells. Spleen cells were incubated in RPMI-1640

containing 20 mM sodium azide and fluorescein-labeled rabbit anti-mouse Ig for 30 min at 0°C. The cells were centrifuged through FCS and washed 2 times with cold PBS containing 20mM sodium azide, then smeared on slides and treated as described above.

3. Macrophages. Spleen cells were incubated for one hr at 37°C with a 1% suspension of latex particles or with 2% neutral red dye. The cells were centrifuged through FCS and washed twice with PBS, then resuspended in RPMI-1640 containing 20% FCS and smeared on microscope slides. The slides were fixed in methanol and the number of phagocytic or dye-stained cells was enumerated.

E. Preparation of T cell replacing factor (TRF). TRF was prepared according to the method of Watson et al. (1979). Briefly, spleen cells were cultured at a density of 5×10^6/ml in RPMI-1640 supplemented with 1% FCS, 5×10^{-5}M 2-mercaptoethanol, 1 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Con A was present at a final concentration of 2 µg/ml. The cells were cultured in Falcon 3024 tissue culture flasks (Falcon Plastics, Oxnard, CA) in a volume of 50 ml at 37°C for 18 hr in a gas mixture of 7% oxygen, 10% carbon dioxide and 83% nitrogen, and then harvested. Cells were removed by centrifugation at 2000 rpm for 10 min, then 10,000 rpm for 20 min. This Con A supernate was concentrated 4 fold by negative pressure dialysis, absorbed for one hr at 0°C with Sephadex G-25 to remove Con A, aliquoted and stored at -70°C.

X. Red Cell Indicator Particles for Complement Receptor Binding.

A. EIgMC4. These SRBC were prepared with the IgM fraction of rabbit anti-Forssman antibody, guinea pig C1 and EDTA-guinea pig serum

according to the method of Borsos and Rapp (1967). In order to put extra C4 on them, the cells were further incubated for 12 hr at 0°C with GP C1 diluted 1:50 in μ = 0.065 buffer and Cordis GP C4 diluted 1:4 with μ = 0.009 buffer.

B. $EIgMC43_{mo}$. EIgMC4 cells with extra C4 attached were incubated with C5-deficient (A/J) mouse serum diluted 1:3 with GVB^{++} (1 ml serum dilution/10^9 EAC4) for 12 min at 37°C. The reaction was stopped by adding 4 volumes of ice cold EDTA-GVB, the cells were centrifuged at 4°C and washed 2 times with EDTA-GVB and 2 times with GVB^{++}. These cells carry both C3b and C3d.

C. $EIgMC43b_{gp}$. Guinea pig C1 (μ = 0.065) was incubated with EIgMC4 cells for 15 min at 30°C (1 ml C1/10^8 cells). Four ml of μ = 0.044 buffer (warmed to 30°C) was added to the cells, and they were then centrifuged and washed 2 times with μ = 0.044 buffer. Cordis GP C2 diluted 1:4 (μ = 0.037) was added at 1 ml C2/10^8 cells, and after 10 min at 30°C, GP C3 diluted 1:3 (μ = 0.065) was added at 1 ml C3/10^8 cells. Following incubation for 30 min at 30°C, the cells were centrifuged, washed twice with EDTA-GVB and 3 times with GVB^{++}.

D. $EIgMC43d_{mo}$. EIgMC4 cells with extra C4 attached were incubated with C5-deficient (A/J) mouse serum diluted 1:3 in GBV^{++} (1 ml/10^8 cells) for 45 min at 37°C. The cells were centrifuged, washed 2 times with EDTA-GVB, and then decayed for 90 min at 37°C in EDTA-GVB to remove C1 and C2. The cells were centrifuged and resuspendedd in μ = 0.037-EDTA-guinea pig serum 1:10 (heat inactivated for 1 hr at 56°C), and incubated at 37°C for 2 hr to convert C3b to C3d. The cells were centrifuged, washed 2 times with EDTA-GVB and 3 times with GVB^{++}.

E. ZIgMC43d$_{mo}$. These were made the same as EIgMC43d$_{mo}$, except that zymosan particles were used instead of sheep erythrocytes and the IgM antibody was against zymosan.

F. Rosette Assay. One ml of lymphocyte suspension (5-7 x 10^6/ml) and an equal volume of the desired C3 bearing indicator particle (5 x 10^7/ml) were placed in small plastic tubes in HBSS, covered with parafilm and rotated end over end for 30 min at room temperature (approximately 10 rpm), then centrifuged for 5 min at 1200 rpm. The cells were then resuspended by rotation for 5 min at room temperature. When feasible, 100 rosetted cells (with 3 or more adherent indicator particles) were counted, along with all accompanying unrosetted cells, and the percentage of rosettes was calculated from those figures. When the percentage of rosettes was very low, at least 500 cells were counted.

G. Double Rosette Assay. This assay was carried out the same as for the single rosette assay except EIgMC43b$_{gp}$ were added first and rotated for 15 min at room temperature, then ZIgMC43d$_{mo}$ particles were added and the total mixture was rotated at room temperature for 30 min. Lymphocytes with only red blood cells attached, those with only zymosan attached, and those with both particle types attached, were counted.

XI. Separation of Spleen Cells into Complement Receptor Subpopulations

The flow chart illustrated in Figure 1 was followed. Briefly, spleen cell suspensions were depleted of T cells by treatment with anti-Thy1.2 plus complement. The suspension was then layered over

Lympholyte M and centrifuged at 1850 rpm for 20 min at room temperature, and the banded cell layers from several tubes were pooled and washed 3 times with HBSS. These lymphocytes were then incubated with red cells bearing the appropriate C3 fragment(s), for 30 min at room temperature. The rosetted suspension was brought up to 5 ml with HBSS and layered over Ficoll-hypaque according to the method of Boyum (1968). After centrifugation at 1350 prm for 30 min at room temperature, the banded cells were harvested, pooled and washed 3 times in HBSS. The pelleted cells were pooled, washed one time in HBSS and then incubated for 15 min at room temperature with Tris-buffered ammonium chloride to lyse the erythrocytes. This suspension was then layered over FCS for 10 min at room temperature, transferred to a new tube, layered over FCS and centrifuged for 10 min at 1000 rpm at room temperature to remove cell debris, and washed 3 times with HBSS.

XII. In Vitro Immune Response Techniques.

A. Polyclonal Responses

1. Mitogen induced proliferation. Mitogens were dissolved in RPMI-1640 containing 25 mM hepes buffer and 0.1 ml was added to each culture. Control cultures received 0.1 ml of RPMI-1640 alone. (The optimal concentration of LPS was found to be 1 µg/culture; PPD 100 µg/culture; and Con A 0.5 µg/culture.) A suspension of 5×10^5 lymphocytes in 0.2 ml of RPMI-1640 supplemented with 5% FCS was cultured with or without mitogen for 2 days for Con A responses and for 3 days for PPD or LPS responses. Six hours before harvest 0.2 µCI tritiated thymidine in 0.05 ml RPMI-1640 was added to each culture.

Proliferation was terminated by washing the culture onto glass fiber filters followed successively by TCA and methanol washes. After drying, incorporated radioactivity was determined by liquid scintillation spectrometry.

2. Polyclonal antibody response. Culture conditions and mitogen concentration were the same as for the proliferative response, except that 10^6 lymphocytes were cultured, and harvested on day 3. The plaque-forming response of the harvested cells was determined against fluorscein-SRBC.

3. Mishell-Dutton primary and secondary immune responses. The microculture primary or secondary immune response of mouse lymphocytes to HRBC was carried out as described by Mishell and Dutton (1967). Cell cultures were set up with spleen cells (1×10^6 cells in 0.05 ml RPMI-1640), 0.15 ml TRF, 0.05 ml macrophage feeder cells (5×10^4 irradiated spleen cells) and 0.05 ml (2×10^4) HRBC/culture. Cultures were incubated in gas boxes at $37^{\circ}C$ with continuous rocking, and harvested on day 5.

4. T-independent response. Cells were cultured as for the polyclonal antibody response above except 1 μg of DNP-Ficoll was used to immunize the cells in culture.

XIII. Jerne Plaque Assay

A modified Jerne plaque assay was used (Jerne et al., 1974). Briefly, cells were harvested in EBSS, centrifuged and washed twice in EBSS. Two cultures were pooled for each point and triplicate points were determined for each culture condition. The cells were then resuspended to an appropriate concentration in EBSS depending on the

expected response. The plaquing mixture included: 0.5 ml of 5% nutrient agar with 3% DEAE-dextran; 50-100 µl of cell suspension; 50 µl of GP C' diluted 1:6 in EBSS; and 50 µl HRBC diluted 1:7 in EBSS. The suspension was mixed and layered on a Falcon #1001 Optilux plastic petri dish. When the agar had solidified, the plates were placed in a humidified chamber and incubated for 4 hr at 37°C, then overnight at 4°C. Results were expressed as the number of plaques per culture. If the IgG response was being investigated, 10 µl of undiluted goat anti-mouse IgM (heavy chain specific) and 50 µl of rabbit anti-mouse IgG (heavy chain specific) diluted 1:800 with EBSS were incorporated in the plaquing mixture. If an anti-hapten response or a polyclonal antibody response was being measured, the reaction mixture was the same as for the primary IgM response except the dilution of GP C' used was 1:10.

RESULTS

I. Sensitive Hemolytic Assay for Mouse C3

Since our research on the role of C3 in the immune response was hindered by the lack of a sensitive, quantitative and functional assay for mouse C3, we set out first to develop an assay system for this complement component. Eventually the following procedure was worked out: $EAC\overline{4}$ were reacted with approximately 200 effective molecules (e.m.) of guinea pig (GP) C1/cell for 15 min at 30°C, μ=0.065 and then washed with warm μ=0.037 buffer. These $EAC\overline{14}$ were treated with approximately 600 e.m. of GP C2/cell for 5 min at 30°C, μ=0.037, then cooled to 0°C and washed once with μ=0.037 buffer. The cells were then resuspended to a concentration of 2.5×10^7/ml in ice cold μ=0.037 buffer, and 0.5 ml was placed into tubes containing 0.25 ml each of: diluted mouse serum, GP C5, GP C6 and GP C7 (all reagents at μ=0.065). After 30 min at 30°C, 0.25 ml each of GP C8 and GP C9 (μ=0.15) were added to each tube, followed by 90 min at 37°C. Three ml of cold EDTA-GVB were then added, the tubes centrifuged, and the optical density at 412 nm was determined.

The results (Table 1) indicate that C3 could be detected in high dilutions of mouse serum; that a strong inhibitory effect was present at lower serum dilutions but not in semi-purified C3; and that mouse C3 was moderately heat labile.

In order to help determine whether the assay was indeed measuring C3, mouse serum was treated with cobra venom factor: 0.5 ml of serum was incubated for 15 min at 37°C with 0.1 ml of undiluted cobra venom factor, and a control serum was similarly incubated with 0.1 ml of

0.15 buffer. Also, since it is known that C3b inactivator (C3b-INA) is blocked by the drug Suramin, this drug was tested to see if it would reverse the strong inhibition of lysis seen at low serum dilutions. Ten mg of Suramin were dissolved in 1 ml of distilled water and then added to a portion of cobra venom factor treated mouse serum and the untreated control serum, to a final concentration of 0.8 mg/ml. After 15 min at 37°C, these preparations were diluted in buffers containing 0.8 mg Suramin/ml, and tested for C3 activity.

The results (Table 2) show that cobra venom factor depleted the serum C3 levels almost to 0 while Suramin greatly reduced the inhibitory effect of high serum concentrations on lysis by C3, suggesting that this inhibition might be due to C3b-INA.

To determine optimal conditions for the mouse C3 assay, the reaction was carried out at 3 different ionic strengths (μ-0.065, 0.099 and 0.134) for different periods of time at 30°C or 37°C. We also tested the effect of a lower concentration of Suramin (0.2 mg/ml). Semi-purified mouse C3 was also titrated at μ=0.134, for comparison. The data obtained for a C3 reaction time of 10 min at 37°C are shown in Fig. I, where the negative natural log of (1-fraction of lysed cells) is plotted against the serum dilution. It can be seen tha the best ionic strength for the mouse C3 reaction was 0.134, while the poorest results were obtained at μ=0.065. The inhibition at low serum dilutions is seen quite clearly and seems to be strongest at the lowest ionic strength. Much but not all of this inhibition was eliminated by Suramin at a concentration of 0.2 mg/ml, but Suramin also reduced the sensitivity of the assay, probably through its effects on C1, C4 and C2 (Fong and Good, 1972). Semi-

purified mouse C3 which contained no C3b-INA gave a nearly linear dose-response curve with a slope of 0.97, very close to that seen with whole serum in the presence of Suramin, at μ=0.134. Other experiments established that μ=0.15 was not preferable to μ=0.134; that reaction times of 5 min or 30 min gave less lysis than 10 min; and that 30°C gave slightly less sensitivity than 37°C.

The optimal conditions for measuring mouse C3 in serum as determined above were applied in a comparison of unpooled sera from several different mouse strains. The negative natural log of (1 minus fraction of lysed cells) was calculated, and the 63% lysis titers were normalized to 1 ml of undiluted serum (Table 3). It can be seen that serum C3 levels of females of 8 different mouse strains varied over a 4 fold range, from a low of 13,000 $C3H_{63}$/ml for C3D2F1/J, to 55,000 for C3HeB/FeJ. Although the serum C3 in a C57B1/6 male was about 30% less than in a female of the same strain, this sex difference was reversed in the CBA/N strain. These strain and sex differences may reflect differences in C3 concentration, differences in the amounts of C3-inactivating enzymes in the serum, or both. Also, since these were unpooled sera from single mice (except A/J), the relative C3 levels reported here should not be taken as representative values for all members of the strain.

II. Generation of C3 Conglutination, Immune-Adherence and Rosetting Activity on Cells by Mouse Serum

Most studies of mouse lymphocyte C3 receptors have been carried out using a rosette assay with IgM-sensitivized sheep erythrocytes treated for 30 min at 37°C with C5-deficient (A/J) mouse serum (EIgMC)

(Ross and Polley, 1976). Since two populations of complement receptor lymphocytes had recently been reportd, one bearing a receptor for C3b and the other a receptor for C3d (Ross and Polley, 1976), it seemed likely that the length of time EIgMC were exposed to serum would affect their specificity as C3 indicator cells, by altering the surface C3b:C3d ratio. The assays chosen were immune-adherence (I-A), which detects C3b (Linscott and Ranken, 1978); the conglutination assay, which was believed at that time to detect C3d (Lachmann, 1967); and the lymphocyte rosette assay which detects both forms of C3. IgM-sensitized SRBC (EIgM) were treated with A/J mouse serum at 37°C, μ=0.15, in the ratio of 1 ml of serum diluted 1:3/10^9 packed cells. Samples containing the EIgMCmo were removed at intervals into 60 volumes of ice cold EDTA-GVB, centrifuged and washed. The cells were tested for their ability to support I-A, conglutination and mouse spleen cell rosettes. As shown in Table 4, I-A activity appeared within 30 sec, peaked by 1 min., declined gradually and was absent at 1 hr. Spleen cell rosetting activity also appeared very rapidly (30 sec), peaked at about 15 min., then declined slowly but not to 0. Conglutinability was absent at 30 sec, strong by 1 min, maximal by 10 min, and declined gradually until it was no longer detectable on cells sampled after 2 hr at 37°C. This loss of conglutinability at 2 hr could not have resulted from complete loss of c3d from the cells, because cells sampled then still formed rosettes with mouse spleen cells. It was a strong indication that conglutinin does <u>not</u> react with C3d, but with an earlier C3 degradation product (Linscott, <u>et al.</u>, 1978). Loss of I-A (i.e., C3b) reactivity by 1 hr at 37°C suggests that the rosettes with 2 hr cells were due primarily to C3d,

not C3b, although at 30 sec they were probably due mainly to C3b. It is not clear why rosetting activity peaked at 15 min, but this was observed on numerous occasions. It may be that binding of $EIgMC_{mo}$ to lymphocytes is strongest at that time because both C3b and C3d are acting as ligands; also, there are lymphocytes bearing only C3b or only C3d receptors, which may contribute to the total number of rosettes only in the presence of cells bearing relatively large amounts of both C3b and C3d. It was concluded that a 15 min incubation at 37°C would yield indicator particles reacting with the maximum number of complement receptor lymphocytes.

III. A Specific Indicator Particle for Mouse C3b Receptors

The study of mouse lymphocyte C3b and C3d receptor function has been difficult because of the problem of separating cell populations bearing only one receptor type or the other, due to the difficulty of preparing indicator particles bearing only C3b, or only C3d. A fortuitous observation pointed the way out of this dilemma: mouse lymphocytes will not react with guinea pig C3d. $EIgMC43b_{gp}$ were prepared with 350 e.m./cell of Cordis guinea pig C3. Some of these cells were then treated with heated serum in low ionic strength EDTA buffer to convert C3b to C3d (Linscott and Ranken, 1978), under the conditions indicated in Table 5. All preparations were then washed and tested for I-A and rosetting activity. By comparing Table 5 with Table 4, it can be seen that unmodified $EIgMC43b_{gp}$ rosetted as well with mouse spleen lymphocytes as did the best EAC_{mo} preparations. Yet when the C3b was converted to C3d, rosetting activity with mouse spleen cells was completely lost, whereas that with guinea pig cells

decreased only moderately. It was also found (data not shown) that human C3 does not bind well to mouse cells, although mouse C3 will bind to receptors on human cultured cell lines.

The data presented in Table 6 illustrate the kinetics of cell-bound C3b inactivation by serum. EIgMC43gp (1×10^8) were treated at 0° or 37°C with 0.5 ml of heat-inactivated guinea pig or mouse serum in EDTA. Some of the cells were then washed, treated with C1, C2 and C5-C9, and incubated at 37°C to check for lysis. Other portions were tested for residual C3b activity in I-A, or for rosetting activity with mouse or guinea pig spleen cells. Heated serum in EDTA rapidly inactivated guinea pig C3b, the most sensitive indicator of the loss of activity being loss of the ability to rosette with mouse spleen cells. The loss of C3b hemolytic activity was slightly less sensitive, as was expected since about 300 e.m. of C3 were offered per cell during their preparation. If 10% of this was taken up, then more than 90% of the C3b on the cell surface would have to be blocked or degraded before a drop in lysis below 100% could be detected. Yet this occurred within 1 min in heated guinea pig serum, even at 0°C, and within 10 min in mouse serum. I-A was rather insensitive to alterations in C3b activity and did not approach negative until lysis had been reduced to less than 10%. Other tests (data not shown) have indicated that under normal conditions it takes three to five times as much guinea pig C3 per cell to generate detectable I-A as it takes to produce lysis. Thus it would appear that guinea pig C3b can be very rapidly degraded or blocked from participating in hemolysis or rosetting with mouse spleen cells, without affecting its ability to participate in I-A or to rosette with guinea pig spleen cells.

There seems to be a real difference between the C3b receptors on human E and those on mouse lymphocytes, with the latter being much more sensitive to alterations in the C3b fragment than the former. It is possible that the rapid loss at $0^{o}C$ of the ability to rosette with mouse spleen cells reflects uptake of B1H from heated serum by the C3b sites, producing inhibition of binding to mouse lymphocytes without any alteration of the C3b itself. This seems unlikely in the absence of any decrease in guinea pig spleen cell rosettes with $0^{o}C$ treatment, even after 3 hr, but perhaps some B1H uptake plus lower-affinity binding to heterologous C3b (see below) could account for the observations.

The gradual decrease in mouse spleen rosetting with prolonged $37^{o}C$ incubation of $EIgMC43b_{gp}$ in the absence of heated serum may reflect elution of some C3b from the cells; a similar phenomenon has sometimes been observed with I-A activity. The absence of a parallel in guinea pig spleen rosettes probably reflects the fact that it takes 5 times more guinea pig C3b per cell to produce strong rosetting with mouse spleen cells than with homologous spleen cells (data not shown).

The data presented in Tables 5 and 6 led us to conclude that guinea pig C3b binds to the C3b receptors of both guinea pig and mouse lymphoid cells, whereas guinea pig C3d will bind to receptors of guinea pig cells, but not to those of the mouse. Thus cell-bound guinea pig C3b provides a new and useful tool: a specific indicator for the C3b receptors on mouse lymphoid cells. A major advantage of using guinea pig C3 for this purpose is that, should some of the C3b be converted to C3d by enzymes or other factors in the media or cell preparation used, misleading binding to mouse C3d receptors will not

occur, as could happen with mouse C3.

IV. Separation of C3b-Receptor-Bearing Lymphocytes ($C3bR^+$) and C3d-Receptor-Bearing ($C3dR^+$) Lymphocytes from a Mouse Spleen Cell Population

In order to study the immune response of each of the various complement receptor-bearing B lymphocytes, we next sought to enrich for each cell type using the rosette technique with cell separation on a Ficoll-Hypaque gradient. The procedure is outlined in Fig. II. Briefly, a single cell suspension was prepared from a pool of 50 mouse spleens. This suspension was then treated sequentially with Thy1.1 antiserum and low-tox rabbit complement followed by Ficoll-Hypaque centrifugation to remove the dead cells. [The Con A response of the spleen cell suspension was obliterated by this treatment (120,000 cpm vs 2500 cpm).] These T cell-depleted spleen cells were then divided into 3 aliquots. One was rosetted with $EIgMC43b_{gp}$, one with $EIgMC43d_{mo}$ and the last with $EIgMC_{mo}$ (EIgM incubated with mouse serum for 15 min at $37^{o}C$). The rosetted cells were then layered on top of Ficoll-Hypaque gradients and centrifuged. The cells separated into a band at the interface with the Ficoll-Hypaque, and a pellet at the bottom of the tube. The banded cells and the pellet were then washed 3 times with medium, the SRBC lysed with Tris-buffered NH_4Cl, and samples were then re-rosetted with the same or different indicator particles. Table 7 lists the types of complement receptor bearing lymphocytes and the number of macrophages found in each fraction, with each type of indicator particle in turn. The data show that use of $EIgMC43b_{gp}$ for fractionation enriches the pellet and completely

depletes ths supernate of $C3bR^+$ cells, leaving $C3dR^+$ and CR^- cells in the supernate. $EIgMC43d_{mo}$ enriches for $C3dR^+$ cells and 15 min $EIgMC_{mo}$ enriches for both types in the pellet, again virtually completely removing that cell type from the supernate. By fractionating with $EIgMC_{mo}$ a true CR^- population is left in the supernate by negative selection. The data also suggest that there is a population of lymphocytes bearing both receptor types, since the pellet may contain more CR^- cells than would be expected by subtracting the $C3bR^+$ and $C3dR^+$ cells from 100. Macrophage contamination is not high. (It should be pointed out that while enrichment for each cell type is good, final cell yields are poor as there is approximately a 50% cell loss at each enrichment step.)

This separation scheme allowed us to define the various cell populations used in subsequent culture experiments as follows: $EIgMC43b_{gp}$ supernate cells consist of negatively selected $C3dR^+$ and CR^- cells ($C3dR^+ + CR^-$); the corresponding pellet contains $C3bR^+$ as well as $(C3b + C3d)R^+$ cells and some CR^- cells. $EIgMC43d_{mo}$ supernates consist of negatively selected $C3bR^+$ and CR^- cells, while the pellet contains $C3dR^+$, $(C3d+C3b)R^+$ and a few CR^- cells. $EIgMC_{mo}$ supernates consist solely of CR^- cells, and the pellet contains CR^-, $C3bR^+$, $C3dR^+$ and $(C3b + C3d)R^+$ cells. All supernate populations are referred to as negatively selected, as their receptors have not reacted strongly with C3. It may be that some of the "CR^-" cells in the pellets actually have C3 receptors which are blocked by C3-membrane fragments left from NH_4Cl lysis of the attached RBCs.

V. Modulation of Complement Receptors on Lymphocytes Cultured Under Various Conditions

We next studied the behavior of cell surface complement receptors under various culture conditions. The results listed in Table 8 are for mouse spleen cells cultured in RPMI-1640 plus supplements for varying periods of time without antigen. The data indicate that when unfractionated cells are cultured, both C3b and C3d receptor cells are present throughout the culture period, although they tend to decrease with time, especially $C3dR^+$ cells. The CR^- population remains essentially negative for both types of C3 receptors throughout the culture period. The positively selected CR^+ population is low in $C3dR^+$ cells throughout the culture period, while cells bearing C3b receptor increase rapidly in number during the second day and remain at nearly 50% during the rest of the culture period.

Table 9 shows the effect on complement receptors of culturing whole spleen cells with HRBC antigen or LPS. In the HRBC stimulated culture, $C3bR^+$ cells remained constant throughout the culture period, while $C3dR^+$ cells declined markedly after 48 hr. On the other hand, in the LPS stimulated culture system, $C3bR^+$ cells decreased rapidly in number during the first day and remained low throughout, while $C3dR^+$ lymphocytes remained numerous and relatively stable for 96 hr. Comparing Table 8 with Table 9, it appears that whole spleen cell suspensions lose $C3dR^+$ cells after 3-4 days of culture, with or without antigen, but that LPS prevents this loss entirely. Conversely, $C3bR^+$ cells remain relatively constant during culture, with or without antigen, but are rapidly lost in the presence of LPS.

Table 10 shows the effects of culture, with and without antigen, on cells previously fractionated on the basis of their complement receptors. CR^- cells did not develop C3 receptors under any

conditions. C3b receptors decreased rapidly from zero-time levels, and remained low throughout the culture period with or without stimulation. C3d receptors, on the other hand, were quite variable depending on the cell type and the culture conditions. With $C3bR^+$ cells as the starting population, culture without antigen resulted in a drop in C3d receptors from 23% at zero time to 8% at day 1, a sharp rise to 39% at day 2, and a fall-off again at day 3. In the presence of HRBC or LPS the sharp rise in C3d receptors was accelerated, being maximal by day 1, and falling off more slowly with LPS than with HRBC. Under the same conditions, $C3dR^+$ cells showed a somewhat different response. Again C3b receptors dropped rapidly and stayed low, but the C3d receptors declined only moderately in the presence of LPS, remaining relatively high and constant throughout the culture period. In the absence of LPS, the C3d receptor loss was much greater, and quite rapid.

One of the problems in interpreting the results shown in Tables 8-10 is that the C3 receptors of positively selected cell populations have already been in contact with C3. Some of these receptors may even be blocked by C3-bearing membrane fragments left after lysis of the EIgMC3 cells used in the fractionation process. In addition, positively selected $C3bR^+$ and $C3dR^+$ cell populations would also be expected to contain cells bearing <u>both</u> receptor types. These problems can be eliminated by using negatively selected cell fractions. Although negatively selected cell populations all contain CR^- cells as well, the responsiveness of CR^- cells is quite low (see below), and we have already shown that these cells do not acquire C3 receptors under any of the culture conditions employed. Table 11 compares the C3

receptors on both positively and negatively selected cell fractions after 1 or 2 days of culture with HRBC. Once again the positively-selected cells showed a rapid loss of C3b receptors, while the C3d receptors were less rapidly affected. The negatively selected preparations behaved quite differently, both from the positively selected cells and from each other. Although both showed high and increasing levels of C3d receptors, only the ($C3bR^+$ + CR^-) population contained C3b receptors, and the fraction of cells bearing these receptors did not decrease rapidly with time, but actually increased.

It was reported in the literature (Danielle and Rowlands, 1974) that if lymphocytes are incubated with 2-mercaptoethanol, there is an increase in the number of complement receptor lymphocytes. We looked at this phenomenon to see which C3 receptors were affected. Negatively selected cell populations were incubated at 37°C for 1 hr with or without 5×10^{-5}M 2-mercaptoethanol in the culture medium, then washed and rosetted. The data in Table 12 show that the ($C3bR^+$ + CR^-) cells, after exposure to 2-mercaptoethanol, rapidly expressed C3d receptors on their surface, while the C3 receptors of the ($C3dR^+$ + CR^-) cells were unaffected. All cultures in the present study contain 5×10^{-5}M 2-mercaptoethanol unless otherwise indicated.

VI. Measurement of C3, C3 Inactivating Activity and C3b-Inactivator Activity in Spleen Cell Culture Supernates

Since C3 is secreted by macrophages (Colten, et al., 1979; Sundsmo, 1980), we wanted to assess the amounts of C3 produced by mouse cells in culture, as well as any C3 inactivating activity which might also be present. Preliminary results indicated that in order to

detect hemolytically active mouse C3 in culture supernates, macro cultures (containing 20 times as many spleen cells as the standard micro culture) had to be used. It made no difference whether the cells were being stimulated by antigen or not. Adult bovine serum contains a high level of hemolytically active C3 (Triglia and Linscott, 1980). Therefore, when fetal calf serum was used, it was first adsorbed with zymosan and then heat-inactivated for 1 hr at 56°C. The hemolytic C3 activity of fetal calf serum decreased from 30% lysis to less than 1% lysis at a 1:10 dilution of the serum, following this treatment.

Spleen cells at a concentration of 3×10^7 cells in 1 ml of medium were used except for cultures in which fractionated cell populations were used; then the concentration was decreased to 2×10^7 cells per culture. Cultures were stimulated with HRBC for a 5 day period, or with LPS for a 3 day period. Two-tenths ml of culture supernate was tested for C3 hemolytic activity. To look for C3 inactivating substances, 0.2 ml of culture supernate was incubated with an equal volume of semi-purified mouse C3 for 3 hr at 37°C, then tested for residual C3 activity. Culture fluids were tested for C3b-INA by incubating them with EIgMC43b$_{gp}$ (bearing 1-2 e.m. of C3/cell) for 3 hr at 37°C, then testing these cells for loss of C3 hemolytic activity by addition of C1, C2, and C5-9.

Table 13 shows that semi-purified mouse C3 gradually lost hemolytic activity during prolonged incubation in RPMI-1640 medium at 37°C, and that this breakdown was greatly accelerated by 10% heat-inactivated zymosan-adsorbed fetal calf serum. Mouse C3 was easily detected in spleen cell culture fluids within 24 hr. By 48 hr, the C3

levels were decreasing and a C3-destroying activity had appeared in the supernate. The rapid drop in C3 levels at 48 hr in cultures containing FCS is due mainly to the FCS (see line 2 of Table), but partly to this other C3- destroying factor, generated by the cultured cells (line 3). In the HRBC + FCS system, C3 dropped to 0 at 48 hr but began to reappear by 72 hr. With LPS the results were very similar.

We next examined which cell types within the spleen cell preparation were responsible for C3 synthesis and C3 destroying activity. Cells were harvested from a 4 day thioglycolate-induced peritoneal exudate in mice. The adherent layer of cells on plastic tissue culture dishes after incubation at 37°C for 3 hr was used as the source of macrophages. T cells were obtained from a whole spleen cell population by passage through a nylon wool column (Handwerger and Schwartz, 1974). B cells were eluted from the nylon wool column. All cells were cultured for 48 hr in the presence of Con A, and the supernates were then tested for C3 and for C3 destroying activity. Table 14 shows that only the macrophages produced C3 in culture, as was expected. Both the macrophages and the T cells produced some C3 destroying activity, but the B cells did not.

In order to determine whether the C3 destroying activity of mouse cell culture supernates might be related to the C3b-INA system present in serum, EIgMC43b$_{gp}$ were prepared with limiting C3b. These cells were incubated for 3 hr at 37°C with various mouse cell culture supernates prepared in the absence of FCS. The EIgMC43b$_{gp}$ were then washed and exposed to C1, C2 and C5-9, to test for residual C3b. The data in Table 15 indicate that the C3 destroying activity of mouse

cell culture supernates is not due to the C3b-INA system. (Line 3 of the Table shows that mouse C3b-INA can destroy guinea pig C3b.) Other work showed that the C3 destroying factor was not inactivated in 1 hr at 56°C, and that it remained active in the presence of 0.1M EDTA.

The species specificity of C3 inactivation by culture supernates was checked. Supernates from Con A stimulated BDF1 cultures were incubated with either semi-purified mouse C3, semi-purified guinea pig C3 or semi-purified bovine C3 for 3 hr at 37°C, and the residual C3 hemolytic activity was measured. As can be seen from the data in Table 16, only mouse C3 was affected by the mouse spleen cell culture supernate.

We also looked at synthesis of C3 and C3 destroying factor by the CBA/N mouse strain. CBA/N males have a B cell defect which is manifested by inability to respond to LPS stimulation, while females give a normal LPS response. Table 3 showed that serum levels of C3 were the same for both the male and female. In Table 17 we see that CBA/N male spleen cells synthesize much more C3 than do female cells, but don't produce much C3 destroying substance. However, these higher C3 levels could simply be the result of less C3 destruction in vitro.

VII. Culture Conditions Necessary for Optimal In Vitro Antibody Production By Mouse Spleen Cells and Fractions Thereof

Since the spleen cell population was depleted of T cells by anti-Thy1.1 treatment plus complement, and macrophages were lost during the cell separation and purification process, it was necessary to replace these elements in order to study antibody synthesis in vitro by the resulting cell fractions. T cells were replaced by T cell

replacing factor (TRF), prepared as described in Materials and Methods. From the data in Table 18, it can be seen that the response to horse RBCs is lost after anti-Thy 1.1 treatment as would be expected and that this loss is reconstituted to levels above the whole spleen cell response by adding TRF to the culture system. The next step in cell purification, passage over a Ficoll-Hypaque gradient, removes macrophages as well as dead cells and erythrocytes. Table 19 shows that these losses can be compensated for by adding back irradiated whole mouse spleen cells, while by themselves are completely unable to respond to antigen, even in the presence of TRF. Further fractionation, into CR^+ and CR^- populations, also requires addition of macrophages and TRF for good in vitro antibody synthesis as shown by Table 19. On the basis of these and other experiments, a dose of 6×10^4 irradiated spleen cells and 0.15 l of TRF were selected for use in all subsequent T-dependent responses, unless otherwise indicated. This resulted in plaque responses which often greatly exceeded those of the whole spleen suspension from which the responding cells were derived, probably as a result of T suppressor elimination by the anti-Thy 1.1 treatment.

Lastly, a relatively low antigen concentration ($3\text{-}6 \times 10^5$ RBC/culture) was selected so as to give an adequate but not maximal plaque response, thus increasing the chances of detecting an enhancing effect due to complement.

VIII. The Primary In Vitro Response of Separated Cell Fractions Against Horse RBCs

With the culture conditions established, a pool of mouse spleen

cells was separated into various B cell fractions and cultured in the established Mishell-Dutton in vitro system. The cells were harvested on days 4 through 7 of culture and both IgM and IgG plaques were enumerated. Table 20 shows that the peak IgM response was usually on day 5 and that a very weak IgG response could be detected by day 7. Positively selected cells (CR^+, $C3bR^+$ and $C3dR^+$) gave the largest responses, with roughly twice as many plaques as the whole spleen control. The CR^- cells gave the poorest response, with fewer than 100 plaques. The ($C3bR^+$ + CR^-) cells were only slightly better, but the ($C3dR^+$ + CR^-) population gave about half the response of $C3dR^+$ cells alone. Since in these last two fractions up to 50% of the cells may be poorly-responding CR^- cells, simple dilution probably accounts for the lower ($C3dR^+$ + CR^-) response. It seems doubtful that the very low ($C3bR^+$ + CR^-) response could be explained in the same way, however.

IX. The Secondary In Vitro Response of Separated Cell Fractions Against Horse RBCs

Mice were primed with HRBC. Two weeks later, a pool of their spleens was separated into various B cell subpopulations and cultured for up to 7 days with HRBC. Cultures were harvested from day 2 through day 7, and both IgM and IgG plaques were measured. Table 21 indicates that the IgM antibody response peaked on day 4 of culture, and that cells bearing C3 receptors made a good response, that of the $C3dR^+$ cells being clearly the best. The CR^- cell response was very poor, as previously seen in Table 20. Thus the primary and secondary IgM responses were in general similar among the various cell fractions, although the $C3bR^+$ cell response was weaker in the

secondary reaction.

The various cell fractions differed markedly with respect to IgM vs IgG production. Although CR^- cells made very little IgM antibody, they made a good solid IgG response, while the $C3bR^+$ cells made no IgG although they produced a fairly good IgM response. $C3dR^+$ and ($C3bR^+$ + $C3dR^+$)cells made good IgM and IgG responses. Part of the experiment was repeated, this time using negatively selected $C3bR^+$ and $C3dR^+$ cells (Experiment II). Both fractions made a good IgM response, but again the $C3dR^+$ cells made much more IgG than the $C3bR^+$ cells, and most of the latter was likely due to the CR^- component of that fraction.

X. The Effect of Cell-Attached C3 Fragments on the Primary Immune Response

Since all positively selected cell fractions have had their C3 receptors cross-linked by C3 fragments on the rosetting particles used to fractionate the cells, this contact may have affected their subsequent response. This response might also be affected by persisting RBC membrane-C3 fragments left after rosetting. This was examined by sham-rosetting T cell-depleted spleen cells with either C3b, C3d or C3b+d indicator particles. Contrary to usual practice, the cells were not fractionated after rosetting, but the indicator particles were lysed as usual with Tris-buffered NH_4Cl. Then some cell preparations were merely washed with medium, and cultured with antigen. Others, however, were first centrifuged through fetal calf serum, our standard procedure for separating RBC stroma and fragments from the viable nucleated spleen cells. Table 22 shows that this

separation step is critical, because when the rosetting-particle stroma were not removed the subsequent antibody response was markedly inhibited. But note that if the stroma carried antibody and C4, but no C3, they did not inhibit.

These observations led us to check the effect of C3-bearing antigen on the immune response. T cell-depleted spleen cells were cultured with SRBC with or without attached IgM antibody and C4, C3b, C3d or C3b+d ($EIgMC_{mo}$). Table 23 shows that unrosetted spleen cells gave a better response to SRBC coated with C3b and/or C3d, than to SRBC alone or SRBC coated with C4. When the experiment was repeated using positively and negatively selected C3 receptor-bearing cells (Table 24), it was found that the positively selected cells showed at best only a slightly enchanced response to SRBC coated with C3, while negatively selected $C3bR^+$ cells showed a clear-cut 2-3 fold increase in plaques. Negatively selected $C3dR^+$ cells showed an enhanced response when the antigen was coated with C3d, in one experiment out of two.

XI. Test for CR^- Suppressor Cells

Since the CR^- cell fraction gave a poor primary IgM response, we checked to see whether this fraction contained suppressor cells. CR^+ and CR^- cells were cultured together or separately in the presence of antigen, and IgM plaques were enumerated on day 5. Table 25 shows that culturing twice the normal number of CR^+ cells together strongly inhibited their response. The data also show that neither 5 nor 10 x 10^5 CR^- cells suppressed the response of 10^6 CR^+ cells, thus making it unlikely that the low IgM primary response of CR^- cell fractions is

due to the presence of suppressors. The same type of mixing experiment was also performed using the mitogenic response to LPS, and again no suppressor activity was found in the CR^- fraction (data not shown).

XII. Effect of Semi-Purified Mouse C3 on the Primary In Vitro HRBC Response of Subpopulations on B cells

With the kinetics of the response worked out, we next examined the effect of adding mouse C3 to the cultures. Subpopulations of normal B cells were cultured for 4 days with HRBC and varying concentrations of semi-purified mouse C3 (or in some cases bovine C3 since fetal calf serum may contain bovine C3 (Triglia and Linscott, 1980) and IgM plaques were measured. Table 26 shows that C3 stimulated a 2-3 fold increase in the number of IgM plaques produced by cells with C3 receptors, while having no effect whatever on the low IgM mouse response of CR^- cells. Mouse C3 was slightly more active than bovine C3 with $C3dR^+$ cells, while $C3bR^+$ cells responded only to mouse and not to bovine C3. C3 was not a polyclonal activator, as its effect was only seen in the presence of HRBC.

Since $C3dR^+$ cells responded to both mouse and bovine C3, the effect of depleting bovine C3 by zymosan adsorption of fetal calf serum (FCS) was checked (see Methods and Materials). Either CR^+ ($C3bR^+$ + $C3dR^+$) or CR^- cells were cultured for 4 days in medium containing FCS adsorbed with zymosan or not. Semi-purified mouse C3 was added to some cultures. From Table 27 it is evident that IgM responses were about half as great in zymosan-adsorbed FCS as in normal FCS. (In all other experiments, zymosan-treated FCS was used.)

Adding mouse C3 about doubled the response of the CR^+ cells in either medium, whereas once again it failed to affect CR^- cells. This is important because it shows tht the stimulatory effect of C3 is restricted to cells bearing C3 receptors, and thus is unlikely to be non-specific or artifactual.

We next looked for a C3 effect on negatively-selected cells (cells which had not had their C3 receptors stimulated as part of the fractionation process). The subpopulations ($C3bR^+ + CR^-$) (exposed to C3d during fractionation) and ($C3dR^+ + CR^-$) (exposed to C3b) were cultured with HRBC in zymosan-adsorbed FCS with and without supplemental mouse C3, and harvested on day 5. Table 28 shows a 3-fold or greater C3-dependent increase in the number of IgM plaques produced by both negatively selected cell populations, comparable to the results previously obtained with positively selected cells (Tables 26 and 27).

We wanted to verify this C3 effect on cruder cell fractions and to investigate the kinetics of the reaction. T cell-depleted spleen cells were placed in culture and horse RBCs were added with or without C3 at 0 time, 24 hr or 48 hr into the culture period. From the data in Table 29, it can be seen that both antigen and C3 could be added up to 24 hr after the cells were cultured with no loss of effect. By 48 hr a reduced antibody response was found and the C3 stimulatory effect was lost. Note that C3 did not substitute for TRF.

We next varied only the time of C3 addition, as shown in Table 30. T cell-depleted spleen cells were cultured with TRF, macrophages and antigen, and mouse C3 was added at time 0, 24 hr, 48 hr, 72 hr, or 96 hr, all cells being harvested at 120 hr. Surprisingly, C3 added as

late as 24 hr before harvest was as effective as C3 added at the start of the culture period. The experiment was repeated and C3 was added either 12 hr or 5 min before harvest as well as at time 0 and 96 hr into the culture period (Experiment II). C3 did not stimulate when added later than 96 hr after culture initiation.

XIII. Effect of Semi-Purified Mouse C3 on the Secondary In vitro HRBC Response of Subpopulations of B Cells

The effect of mouse C3 on the secondary antibody response to HRBC was also studied. Mice were primed with HRBC two weeks prior to being placed in culture. The B cells were then separated into various fractions and cultured as described under Materials and Methods. HRBC and C3 were added at time 0. Table 31 shows that the positively selected $C3bR^+$ population gave a moderate IgM response which was boosted 3-fold by the addition of mouse C3, much the same as occurred in the primary response. The minor IgG response of these cells, probably attributable to cells bearing both C3 receptors, was not augmented by C3. The negatively selected ($C3bR^+ + CR^-$) population similarly gave a moderate IgM response which was strongly enhanced by C3, and again C3 had no effect on the weak IgG response of this fraction.

The positively selected $C3dR^+$ fraction gave a moderate IgM response and a strong IgG response and C3 did not affect either one. Results were essentially the same with the negatively selected ($C3dR^+ + CR^-$) population. The CR^- fraction gave a very poor IgM response and a good IgG response, and again mouse C3 was without effect.

XIV. Effects of Anti-C3 on Primary and Secondary In Vitro HRBC Responses

In view of the stimulatory effect of C3 on the IgM antibody response of cells bearing C3 receptors, we decided to examine the effect of antibodies against mouse C3. Both the IgG fraction of rabbit anti-mouse C3, and $F(ab')_2$ fragments of goat anti-mouse C3 were tested. T cell-depleted spleen cells were cultured with antigen, and at varying times 25 µl of anti-mouse C3 was added to the culture. As can be seen from Table 32, approximately 75% of the response was eliminated by the addition of anti-C3, even as late as 1 hr before harvesting the cells.

We checked the sepcificity of the anti-C3 effect, by comparing the response of CR^+ and CR^- cell populations. Table 33 shows that rabbit anti-bovine C3, rabbit anti-mouse C3 and goat anti-mouse C3 F(ab')2 all decreases the response of CR^+ cells by about 75%, while having no effect on the response of the CR^- population. (Antisera were free of preservatives when tested.) This would seem to rule out nonspecific cytotoxicity as a cause for the anti-C3 results. As an additional precaution we adsorbed the normal rabbit serum and the rabbit antiserum against mouse C3 for 2 hr at 37° and then for 4 hr at $0^\circ C$ with mouse spleen cells. This procedure was carried out twice. Table 34 shows that the adsorption had no effect on the activity of the antiserum or the control serum. We also tested the anti-C3 preparations for toxicity to mouse spleen cell fractions in the presence of rabbit complement. Table 35 shows that the anti-mouse C3 preparations were not cytotoxic for CR^+ or CR^- cells. Thus the effect of anti-C3 on the IgM antibody response does not appear to be due to

cell destruction by antibody and complement.

The anti-C3 effect was also examined in the secondary response. Table 36 shows that the IgM respone of CR^+ cells to HRBC was inhibited approximately 80% by the addition of anti-C3, while the IgG response was unaffected. The CR^- cells did not give an appreciable IgM secondary response, but gave a good IgG response which was not affected by anti-C3.

We tried to reverse the effect of anti-C3 by washing the cells after anti-C3 treatment and re-supplying additional C3. T cell-depleted spleen cells were cultured with HRBC and either rabbit or goat anti-mouse C3 from time 0. AT 24 hr intervals selected cultures were washed two times with medium and resuspended in fresh medium plus antigen. As can be seen in Table 37, the washing had no effect on the control responses, and failed to reverse the inhibitory effect of anti-C3. Adding mouse C3 to the cultures washed at 96 hr doubled the number of plaques in the control response as observed previously (see Table 30), but did not reverse the effect of anti-C3.

Since exposure to anti-C3 during the first 24 hr of culture was sufficient to turn off the IgM response, we repeated the experiment with even shorter exposures to anti-C3. T cell-depleted spleen cells were cultured with HRBC and anti-C3 from time 0. At 30 in, 3 hr, 12 hr or 24 hr, cells were washed two times with medium and then re-cultured with antigen or antigen plus semi-purified mouse C3. Cultures were harvested as usual on day 5, and IgM plaques were measured. Table 38 indicates that after treatment with anti-C3 for 30 min or 3 hr, the response can be rescued to the control values by washing. The usual enhancing effect of mouse C3 can also be seen.

Washing after 12 hr resulted in recovery of only half the control response, which still was enhanced by mouse C3. By 24 hr, neither washing nor addition of C3 had any effect, as had been found previously (Table 37).

Finally, we attempted to see if the anti-C3 effect on IgM secretion was in anyway related to C3 secretion by the cells in vitro. To measure C3 secretion over time, macro cultures of T cell-depleted spleen cells were set up with HRBC, and samples of supernate were taken for C3 analysis at 1 hr, 3 hr, 6 hr, 12 hr, 18 hr and 24 hr. Micro cultures of the same cell suspension were set up with HRBC and anti-C3 for IgM plaque assay. At appropriate time intervals, selected micro cultures were washed 2 times to remove anti-C3, then recultured with fresh medium + HRBC. To one culture, mouse C3 was added at time 0; after 1 hr, anti-C3 was added to the culture for an additional 2 hr before washing. A final culture was set up in spent medium from a 24 hr culture of spleen cells; after 1 hr, anti-C3 was added to this culture for an additional 2 hr incubation before washing as above. All cultures were then harvested on day 5, and primary IgM plaques were enumerated.

Table 39, Part A, shows that within 1 hr cells in culture were producing detectable C3, the levels increasing steadily over the 24 hr period. In Part B of the Table, the effect of anti-C3 became irreversible after 12 hr of exposure and was even stronger after 24 hr, confirming the results shown in Table 33. Adding exogenous C3 (25 µl) at time 0 did not alter these results. However, incubation with a 24-hr culture supernate followed by anti-C3 for 2 hr did shut down the IgM response. Thus something generated during the first 24 hr of

culture, but not C3 (C3 fragment?) interacted with anti-C3 to turn off IgM secretion by CR^+ but not by CR^- cells.

XV. Effect of Antigen Concentration

Since C3 might enhance the IgM response by making antigen presentation more effective, we checked to see whether an increase in the amount of antigen normally used could mimic the C3 effect. T cell-depleted spleen cells, CR^+ and CR^- cells were cultured with 1/10, 1, 10 and 50 times the normal antigen concentration, in some cases with or without exogenous mouse C3. Table 40 shows that more antigen did not enhance the response, and that too much antigen was inhibitory especially with CR^- cells. Exogenous mouse C3 enhanced the response of CR^+ cells about equally over a 100-fold range of antigen concentrations.

XVI. Comparison of C3 Effect and TRF Requirement

We checked to see if mouse C3 could replace the T cell requirement in the primary immune response. T cell-depleted spleen cells were cultured with and without TRF and with and without C3. From the data in Table 41, it can be seen that mouse C3 could not substitute for TRF.

XVII. The role of Complement Receptors in Polyclonal B Cell Activation

We next studied the polyclonal B cell response to the mitogen lipopolysaccharide (LPS). Various spleen cell fractions were cultured with 10 μg of LPS/culture. At intervals selected, cultures were

pulsed for 12 hr with tritiated thymidine and harvested. As can be seen in Table 42, the peak response of whole spleen cells , T cell-depleted spleen cells, CR^+ cells and positively selected $C3dR^+$ cells was on day 3. CR^- cells and negatively selected $C3bR^+$ and $C3dR^+$ cells peaked on day 2, as did positively selected $C3bR^+$ cells. The strongest responses to LPS were made by T cell-depleted spleen, CR^+ and positively selected $C3dR^+$ fractions, which are all fractions peaking on day 3. Thus the speed of response does not correlate with magnitude. The CR^- fraction and the negatively selected fractions of $C3bR^+$ + $C3dR^+$ cells (which contain many CR^- cells) gave relatively weak responses, as did the positively selected $C3bR^+$ fraction. $C3dR^+$ cells seemed to respond more strongly than corresponding $C3bR^+$ fractions.

Next the polyclonal antibody response of each cell fraction was studied. Cells were cultured with 10 μg of LPS and harvested on day 3 or day 4. Plaques against fluorescein-SRBC were determined (Moller, 1975), and are presented in Table 43. Whole spleen cells gave a poor LPS response and peaked on day 3, while the T cell-depleted fraction gave a good response and peaked on day 4; the CR^+ fraction had a peak LPS response on day 4; and the CR^- cells did not respond well to LPS. The $C3bR^+$ fraction peaked on day 4 for LPS and gave about half the response of the $C3dR^+$ fraction, which also peaked on day 4; and the ($C3dR^+$ + CR^-) fraction gave a good LPS response which was three-fold higher than the ($C3bR^+$ + CR^-) population. Thus, in general, the polyclonal antibody responses were similar to the mitogenic responses of the same cell fractions.

Similar experiments were done utilizing semi-purified mouse C3 to

check for an enhancing effect. Tables 44 and 45 show that mouse C3 had no enhancing effect on either the mitogenic or the polyclonal antibody response to LPS. If anything, it sometimes seemed to be inhibitory.

The effect of anti-mouse C3 was checked with the polyclonal mitogen response. As can be seen from the data in Table 46, anti-C3 did not inhibit the response of either the CR^+ or CR^- population more than normal serum, and thus was not more cytotoxic than normal serum.

XVIII. T-Independent Responses

B cell fractions were also studied for their primary antibody response to a T-independent antigen. Cells were cultured with DNP-Ficoll for 4 days. Mouse C3 was also added to some cultures. Cells were harvested on day 4, and the IgM plaques against DNP-SRBC were counted. Table 47 shows that only $C3bR^+$ cells responded to this antigen, and that exogenous mouse C3 was without effect.

DISCUSSION

Investigation of the role of C3 in the humoral immune response can be approached in several ways. Early workers depleted C3 to 5-10% of normal circulating levels by injecting cobra venom factor, and then studied the response to antigen before the animals became refractory to venom factor through immunization. Pepys et al. (1976) showed that the T-dependent IgA and IgG responses of mice to SRBC were suppressed by cobra venom factor treatment while the IgM response was only slightly impaired. They later showed (1977) that both the primary and secondary IgE responses to ovalbumin could be suppressed if venom factor was given at the time of antigen priming, but if given with the antigen boost, it delayed but did not suppress the secondary response. Lewis et al. (1976, 1977) found that venom factor treatment reduced C3 to 20% of control levels but did not change the number of complement receptor positive lymphocytes in the spleen. Venom factor treatment given before SRBC reduced the IgM response by 60%.

Another approach is to deplete C3 in vitro by the use of anti-C3 antibodies. This treatment decreased the IgM response by 80% while it had no effect on the response of CR^- B-cells in vitro (Lewis et al., 1977). Martinelli et al. (1978) confirmed that C3 depletion suppressed the immune response to a T-dependent antigen but had no effect on T-independent antigens.

Absolute elimination of C3 cannot be obtained by cobra venom factor treatment or any other approach, except the use of genetically deficient subjects. The studies cited above imply a possible role for C3 in the switch from IgM to other Ig classes and in the generation of memory B cells (Romball et al., 1980). Further evidence for such a

role was found in studies in C4-deficient guinea pigs and a C4-deficient patient, in whom C3 activation by the classical pathway could not occur. Identical results were found in each: a very reduced primary IgM response; and a secondary response no higher than the primary, and consisting only of IgM, no IgG (Ochs et al., 1977). On the other hand, Alper et al. (1976) reported that a C3-deficient human patient made a normal immune response to a protein and a carbohydrate antigen. Unfortunately, they neglected to determine whether the antibodies they measured were IgM or IgG.

Another approach to the study of C3 in humoral immunity is to focus on its role in the localization of antigen in the lymph nodes and spleen. Klaus and coworkers (1977, 1978, 1979) showed clearly that C3 played a critical role in the follicular localization of antigen, which was essential for the generation of memory B cells. While this function of the complement system is important in the immune response, we will regard it as a separate issue which can only be studied in vivo, and which probably has no counterpart in in vitro systems where cell suspensions are intimately mixed with antigen before plating. Studies with cobra venom factor in vivo, or in complement-deficient subjects, cannot separate this aspect of immunization from the later interaction between antigen, T cells, B cells and macrophages.

Others have attempted to address the role of C3 in the immune response by fractionating cells into populations enriched in or depleted of C3 receptor-bearing (CR^+) cells, and then studying their response to antigen in vivo (by cell transfer experiments) or in vitro. In vitro studies do not permit the elimination of background C3 any more than did cobra venom factor in vivo, because macrophages

secrete C3 in the culture (Colten et al., 1979), and C3 may be present in the serum supplements needed to support good antibody synthesis in vitro (Triglia & Linscott, 1980). Nevertheless such studies permit one to single out different cell fractions, based on their surface receptors for C3, and to study their responses to antigen under relatively controlled conditions. One can also supplement the cultures with exogenous C3, or add anti-C3, in an effort to manipulate cell responses. We chose this latter approach, as offering the best chance to determine the role of C3 and of C3 receptors in the humoral antibody response.

We began these studies by trying to define our in vitro antibody culture system with respect to its bovine and mouse C3 content, and its ability to degrade mouse C3 and C3b. Previous investigators have been unable to measure mouse C3 functionally, except for the rather non-quantitative immune-adherence (I-A) reaction (Nishioka, 1963). By modifying the ionic strength and other parameters of a previously developed hemolytic assay for bovine C3 (Linscott and Triglia, 1980), we were able to measure functional C3 activity in mouse serum with a high degree of sensitivity (Ranken and Linscott, 1979) (Figures I and II; Table 3). Sera from individual mice were found to vary in C3 activity from 13,000 hemolytic units/ml for C3D2 F_1 to 55,000 for C3HeB/FeJ, with most falling in the 20,000 unit range. (Since only single animals were tested, these levels should not be considered as representative of each strain as a whole.)

With such a sensitive assay it became possible to measure directly the C3 content of mouse spleen cell culture supernates. Although this could not be done in the micro cultures used to assay

for plaque forming cells, hemolytic C3 was detectable in the supernates of macro cultures containing 20 times as many cells in a larger volume (Table 13). Since this assay also detected a small amount of bovine C3 in a few lots of FCS, all FCS used in our investigations were first absorbed with zymosan at 37°C, and then heat-inactivated at 56°C for 60 min. With this treatment, bovine C3 activity dropped from 30% lysis to 0 at a 1:10 dilution of FCS. Thus any C3 detected in culture supernates was of mouse origin.

Mouse spleen culture supernates contained some substance(s) which destroyed the hemolytic activity of mouse C3. In addition, FCS contributed to mouse C3 inactivation, and there was a slow spontaneous loss of activity from semi-purified mouse C3 simply held for several days at 37°C (Table 13). Thus the amount of hemolytic C3 detected in a culture supernate at a given time reflects the balance between C3 secretion, destruction and inactivation. C3 secretion was rapid at first, the highest levels usually being detected after 24 hr of culture. Then there was a decrease (much more rapid in th presence of FCS) as C3-destroying activity appeared, followed by an increase toward earlier C3 levels by days 3-5. Later experiments showed that detectable amounts of mouse C3 were present after as little as 1 hr of culture, and rose steadily during the first 24 hr (Table 39). Macrophages are known to secrete C3 in other species (Colten et al., 1979, and Sundsmo, 1980) and we confirmed that mouse C3 was secreted by macrophages, but not by B or T cells (Table 14).

The nature of the C3-destroying activity secreted by spleen cell cultures is not clear. It was specific for mouse C3 (Table 16), and was not attributable to the C3b-INA system (Table 15). It may have

been a nonspecific protease released by the cells in culture.

These early data indicated that future experiments would have to take into account both the endogenous levels of C3 in our culture system, and the C3-destroying activities of FCS and the culture supernate. The presence of factors that destroy mouse C3 *in vitro* provides a mechanism for generating mouse C3 fragments in the culture system, which may have significance for some of our later observations on the effects of exogenous C3 and anti-C3 on antibody secretion *in vitro*.

In 16 years of research on lymphocyte complement receptors, two major C3 receptors have been identified, for C3b and C3d (Ross and Polley, 1976). They are sometimes referred to as CR1 and CR2, respectively. A third, more recently discovered receptor for C3bi (CR3), is probably not present on B cells (Ross *et al.*, 1983), and will not further be discussed.

Most studies on lymphocyte C3 receptors have lumped all C3 receptor cells together as CR^+, and compared them with putatively CR^- cells, because of the difficulty of cleanly separating $C3bR^+$ from $C3dR^+$ cells. Some have attempted this latter type of separation, but in all cases the methods used have been unable to exclude cross-contamination of lymphocyte fractions as a result of uncontrollable changes in the C3 fragments present on the "indicator particles" used to fractionate the cells. We have been able to circumvent this problem, and thus to prepare relatively clean B cell fractions which have markedly different immune functions, depending on the type of C3 receptor on their surface membranes.

Most people who study the function of lymphocyte C3 receptors,

including ourselves, have done so by separating the cells into CR^+ and CR^- fractions by rosetting techniques. Thus the C3 fragments present on the "indicator particles" used for rosetting are critical to the purity of the cell fractions obtained. Yet most studies have utilized "$EIgMC_{mo}$" made by incubating IgM-sensitized SRBC with C5-deficient mouse serum for 30 min at 37°C, followed by washing. Table 4 makes clear that the duration of this treatment radically affects the proportions of different C3 fragments present on the indicator cells, and that 30 min is a relatively poor treatment time. C3b (assayed by I-A hemagglutination) appeared within 30 sec, peaked at 1 min, and was undetectable (due to conversion of C3b to C3bi by the serum C3b-INA system) by 1 hr. C3bi activity (assayed by conglutination) appeared within 1 min, peaked at 5-10 min, and was undetectable (due to C3bi degradation to C3d,g and C3d by serum proteases) by 2 hr. C3d persisted at 1 and 2 hr as shown by spleen cell rosetting.

If one adds together the proportion of $C3bR^+$ cells (23% with 30 sec indicator cells) and $C3dR^+$ cells (16% with 2 hr indicator cells), one obtains a total (39%) which is strikingly close to the maximum of 35% rosettes obtained with 15 min indicator cells. This suggests that relatively few lymphocytes carry both C3b and C3d receptors, and that optimally reactive indicators for <u>both</u> types of receptors (to isolate CR^+ cells) should be harvested after 15 min exposure to C5-deficient serum. (Of course, the quality and dilution of this serum are also very important and likely to vary in different labs.) Cells exposed for too long a period are likely to be deficient in C3b, and thus to leave $C3bR^+$ cells in the "CR^-" fraction after rosetting. We used 15 min indicator cells for all CR^+ vs CR^- fractionations.

To fractionate $C3bR^+$ from $C3dR^+$ cells, one must have indicator particles coated only with C3b, and others bearing only C3d. To make indicator cells bearing only C3d, we took 15 min $EIgMC_{mo}$, treated them with EDTA at 37°C to remove C1 and to decay C2 (Linscott et al., 1978), and then further treated them for 2 hr at 37°C with heat-inactivated mouse serum in EDTA at low ionic strength, to allow complete degradation of C3b and C3bi to C3d (Linscott and Triglia, 1977; Ross et al., 1982) (See also Tables 5 and 6).

Preparing indicator cells bearing C3b, but not C3d or C3bi which could bind to C3d receptors, has been a major problem for all workers in this field. It can't be done without the use of purified C3, because in whole serum C3bi activity is generated within 60 sec (Table 4). Mouse C3 is quite difficult to purify in any quantity, and even if one could use it to produce $EIgMC43b_{mo}$ indicator cells, there is the likelihood that tissue proteases in a spleen cell preparation could convert some of this C3b to C3bi or C3d, which would cause cross-contamination of any cell fractions separated by those indicator cells. We solved this problem by showing that guinea pig C3bi and C3d do not react with mouse spleen cell receptors (Linscott and Ranken, 1978). Guinea pig C3b bound equally well to mouse and guinea pig spleen cells, but was readily degraded by heated guinea pig or mouse serum to a form which still bound well to guinea pig spleen cells but was unreactive in I-A; could no longer participate in immune hemolysis; and, most importantly, could not bind to mouse spleen cells (Tables 5 and 6). Thus, any degradation of the C3b on $EIgMC43_{gp}$ could not lead to cross-contamination of rosette-separated $C3bR^+$ and $C3dR^+$ cells.

With the above reagents in hand, it was possible to devise the mouse B cell fractionation procedure presented in Figure II and Table 7. BDF1 spleen cells, pooled from 30 to 50 mice, were treated with anti-Thy1.1 and rabbit complement to deplete T cells, then centrifuged through Ficoll-Hypaque to eliminate erythrocytes, dead cells and most macrophages and neutrophils. Depletion of T cells and macrophages was complete enough so that the remaining cells made no response to the T cell mitogen Con A (180,000 cpm nondepleted population vs 4000 cpm for depleted population), and no antibody response to T-dependent RBC antigens such as HRBC and SRBC unless supplemented with TRF and macrophages.

To separate CR^+ and CR^- cells, this T cell-depleted population was rosetted with $EIgMC_{mo}$ (15 min cells bearing optimal amounts of C3b and C3d). None of the negatively selected CR^- cells in the supernate reacted with C3 fragments; 80% carried surface Ig; 7% were Thy1.1-reactive; 3% were macrophages; and CR^- cells made no response to Con A (Fig. II and Table 7). Most (70%) of the positively selected CR^+ lymphocytes in the pellet re-rosetted with $EIgMC_{mo}$; thus the pellet consisted of a mixture of $C3bR^+$, $C3dR^+$ and CR^- cells. What proportion of the C3-nonreactive pellet cells were CR^- and what proportion had blocked C3 receptors could not be determined, but some culture experiments were carried out in an effort to resolve this question (see below).

Positively selected $C3bR^+$ cells were rosetted with $EIgMC43b_{gp}$ and recovered from the pellet. Seventy percent re-rosetted with $EIgMC43b_{gp}$, 23% reacted with C3d, and 20% did not react with C3 fragments, for a $C3bR^+$:$C3dR^+$:CR^- ratio of approximately 7:2:2. It is

apparent that a moderate number of these cells probably carried both C3b and C3d receptors. Negatively selected $C3bR^+$ cells were rosetted with $EIgMC43d_{mo}$, and recovered from the supernate. Approximately half of these cells rosetted with $EIgMC43b_{gp}$; and half did not react with any C3 fragment. Only 2% reacted with C3d (Table 7), for a $C3bR^+:C3dR^+:CR^-$ ratio of 1:0:1, so essentially none of these cells carried both types of C3 receptors.

Positively selected $C3dR^+$ cells were rosetted with $EIgMC43d_{mo}$ and recovered from the pellet. Eighty percent re-rosetted with $EIgMC43d_{mo}$, 8% reacted with C3b and 15% did not react with any C3 fragment. Thus the $C3bR^+:C3dR^+:CR^-$ ratio here was approximately 1:10:2, and at most a few percent of cells carried both C3 receptors. Negatively selected $C3dR^+$ cells were rosetted with $EIgMC43b_{gp}$, and recovered from the supernate. About 70% rosetted with $EIgMC43d_{mo}$, and 30% did not react with any C3 fragment, for a $C3bR^+:C3dR^+:CR^-$ ratio of 0:7:3. None carried both types of C3 receptors.

Thus we were able to separate mouse B cells into fractions with widely differing C3 receptor properties, and an absolute dependence on TRF and macrophage supplementation for an antibody response to RBC antigens. Before we studied these responses in vitro, we investigated the stability of C3 receptor expression shown by these cells during culture. One reason for doing so was to see whether culturing positively selected cells would result in an increase in the proportion of cells capable of rosetting, as occupied (blocked) C3 receptors were replaced or freed.

C3 receptors changed markedly during culture, depending on the cell fraction and the conditions. With whole spleen cell cultures,

$C3bR^+$ cells remained at around 15-25% over a period of 4 days in the absence of stimulation; dropped somewhat lower (10-15%) when cultured with HRBC; and fell rapidly to near zero when cultured with LPS (Tables 8 and 9). This contrasted with $C3dR^+$ cells which fell off gradually in the absence of stimulation; decreased more rapidly and to a lower level with HRBC; but remained at a nearly constant level of 20-30% in the presence of LPS. These data suggest that LPS may stimulate the conversion of $C3bR^+$ and $C3b+dR^+$ to $C3dR^+$ cells (also see Table 10).

Isolated CR^- cells remained relatively invariant under all conditions: only a few percent ever rosetted with C3b or C3d, whether cultured alone or with HRBC or LPS. Thus it seems unlikely that this fraction contains CR^+ precursors, although we can't rule out precursors which are only expressed under synergism with other cell fractions.

The CR^+ fraction showed very low proportions of $C3bR^+$ and $C3dR^+$ cells after culture for 1 day with no stimulation, but by day 2 $C3bR^+$ cells were 45% of the total and remained at this level. $C3dR^+$ cells never exceeded 5% (Table 8).

When the positively selected $C3bR^+$ fraction (containing 23% C3d-reactive cells) was cultured, C3b-reactive cells dropped to about 5% in 1 day and stayed there, with or without antigenic stimulation. In contrast, C3d-reactive cells dropped to 8% at day 1, rose to about 40% at day 2, then fell off again, with no stimulation. In the presence of HRBC, C3d-reactive cells were at 40% by day 1, fell rapidly on day 2 and reached 5% by day 3. With LPS, C3d-reactive cells stayed at the 40% level for 2 days, then declined sharply (Table 10). In another

experiment similar results were obtained, but with slightly slower kinetics. In contrast, when the negatively selected $C3bR^+$ fraction (which initially contained equal numbers of $C3bR^+$ and CR^- cells but almost no C3d-reactive cells) was cultured with HRBC, both C3b-reactive cells and C3d-reactive cells stood at 25% by day 1 and 40% by day 2 (Table 11). Since we have shown that CR^- cells do not give rise to significant numbers of CR^+ cells, and since the initial $C3bR^+$ fraction contained almost no C3d-reactive cells, these cells must have evolved from the C3b-reactive half of this fraction, not the CR^- half. All of our data are compatible with a maturational sequence in which the initial CR^+ cell carries C3b receptors, then both C3b and C3d receptors, then only C3d receptors and finally no C3 receptors. (Some of the data in Table 10 suggest that in the absence of stimulation, most C3b receptors can be lost before C3d receptors appear.) This sequential change in C3 receptors appears to proceed in the absence of stimulation, to be accelerated moderately by HRBC, and more strongly by LPS. In addition, LPS seems to retard the loss of C3d receptors at the final stage. Negatively selected $C3bR^+$ fractions, which lack $C3b+dR^+$ and $C3dR^+$ cells, are thus less mature than positively selected $C3bR^+$ fractions, and would be expected to retain C3b-reactive cells longer, as seen in Table 11.

$C3dR^+$ fractions which were positively selected lost their C3 receptors very rapidly under normal culture conditions or in the presence of HRBC, but retained them moderately well when LPS was present. Interestingly, negatively selected $C3dR^+$ cells seemed to retain their C3d receptors well in the presence of HRBC, though these cultures were only followed for 2 days (Table 11). In no case did

$C3dR^+$ cell fractions later express a significant number of C3b receptors.

Danielle and Rowlands reported (1974) that 2-mercaptoethanol promoted an increase in the number of CR^+ lymphocytes when spleen cells were incubated with this compound for 1 hr at 37°C. We re-investigated this problem utilizing our ability to distinguish between $C3bR^+$ and $C3dR^+$ cells, and found that while 2-mercaptoethanol had no effect on $C3dR^+$ cells, it rapidly converted $C3bR^+$ to $C3b+dR^+$ cells (Table 12). Thus 2-mercaptoethanol seems to accelerate a normal B cell maturational event, the acquisition of C3d receptors by $C3bR^+$ cells.

Having defined our culture system with respect to endogenous C3 production and destruction, and having separated mouse B cells into several fractions with distinctly different C3 receptors, we then explored the immune functions of these different cell fractions in vitro.

Most of these studies were done on the primary and secondary antibody responses to HRBC, which were chosen over SRBC because the latter were utilized to prepare the C3-coated indicator particles used to fractionate spleen cells, and could have affected antigen binding cells as well as CR^+ cells. First we determined that our method for T cell depletion of spleen cells was adequate to obliterate the primary response to HRBC, and that TRF could replace the requirement for T cells with this antigen. Table 18 shows that this goal was achieved, and that the response to HRBC by T cell-depleted, TRF-supplemented spleen cells was several times as high as that by whole spleen cells. We also showed that the Con A mitogen response was absent from our T

cell-depleted preparations, as would be expected.

Since most of our B cell fractions were quite low in macrophage content, we needed to show that we could supplement the cultures with an adequate source of macrophages that would not also supply B or T cell functions. We found that 6-8 x 10^4 irradiated (3300 rads) mouse spleen cells per culture met this goal (Table 19), and all cultures with T-dependent antigens were supplemented with macrophages and TRF unless otherwise indicated. Again, responses by supplemented cultures usually exceeded those of whole spleen cells, probably because of the absence of T suppressor cells.

The intensity of the antibody response varied greatly from one cell fraction to another, but differences in kinetics were much smaller. The primary IgM response to HRBC peaked in 5 days for high responding fractions (T cell-depleted spleen, CR^+, $C3dR^+$ and positively selected $C3bR^+$ cells), and in 6 days for low responders (CR^- and negatively selected $C3bR^+$ cells). The former produced 900-2000 plaques per culture with a sharp peak, and the latter 80-240 plaques, with a rather flat profile. It is quite clear that the low responses were not due to missing an unanticipated peak response time (Table 20). The beginning of IgG secretion was evident by day 6-7.

The secondary IgM response was accelerated by about 1 day, with a peak on day 4 for all including the CR^- fraction, but the relative responses of each fraction were similar to those seen in the primary response: CR^- cells were very low in activity, CR^+ and $C3dR^+$ were high, and others were intermediate. Thus the major difference between the primary and secondary IgM responses to HRBC was in tempo, and no important shifts in reactivity were seen except with negatively

selected $C3bR^+$ cells (see below) (Table 21). To summarize, CR^- cells produced a real but extremely limited IgM response, which was not due to contamination by a few CR^+ cells, as will be discussed later. Nor could we demonstrate suppressor cells as a cause of low reactivity in the CR^- fraction (Table 25). $C3dR^+$ cells responded very strongly, although the negatively selected fraction produced fewer plaques than the positively selected fraction, probably because it was diluted with many more CR^- cells. Positively selected $C3bR^+$ cells made good primary and secondary IgM responses, while the cleaner and less mature (see above) negatively selected fraction made a very poor primary response but a good secondary one. Thus we found a dramatic difference between CR^- and CR^+ cells, but not a very great difference between $C3bR^+$ and $C3dR^+$ cells, in the IgM response to a T-dependent antigen.

Results with the (secondary) IgG response were quite different. It peaked on day 5 for all fractions; was very high with CR^+ and $C3dR^+$ cells; was also good with CR^- cells; and was very low with $C3bR^+$ cells, especially positively selected ones with low CR^- contamination (Table 21). So here we have a big difference between the IgG and IgM responses of CR^- cells, as well as a striking difference between the IgG responses of $C3bR^+$ and $C3dR^+$ cells. Positively selected $C3bR^+$ cells made essentially no IgG response, and the response of the negatively selected fraction was far lower than that of negatively selected $C3dR^+$ cells.

These results correlate rather well with our proposed B cell maturational sequence, in which the most immature CR^+ cell carries only C3b receptors, makes a poor IgM response (which improves after

sensitization), and makes essentially no IgG. As C3d receptors appear and C3b receptors dwindle, a strong IgM responsiveness develops, as well as the ability to produce IgG. When all C3 receptors have been lost, IgM responsiveness too is no longer evident, while the ability to secrete IgG remains. Obviously, there must be a C3 receptor precursor cell which is also CR^-, but either it is uncommon in the spleen or is screened out by our fractionation procedures, because our CR^- fractions never gave rise in culture to significant numbers of CR^+ cells. The development of C3d receptors must be fairly closely coupled to the loss of C3b receptors, because our data do not indicate a very large population of cells bearing both receptor types simultaneously. This sequence of C3 receptor development seems to occur without antigenic stimulation, and to be accelerated moderately by HRBC and more strongly by LPS. C3d receptors were induced within 1 hr at 37°C by 2-mercaptoethanol.

It seems clear that loss of C3d receptors does not equate with cell death, or we would not see such a vigorous IgG response by CR^- fractions. And we have shown that this CR^- response can't be explained on the basis of CR^+ contamination of CR^- fractions, which is extremely low, or "maturation" of CR^- cells to CR^+, which cannot be demonstrated. Furthermore, other data indicating strong stimulation of CR^+ cells by exogenous mouse C3, and strong inhibition by anti-C3, were accompanied by observations showing that the responses of CR^- cells were completely unaffected by these reagents (Tables 27 & 33), which could not be the case if CR^- responses were due to contamination by CR^+ cells.

Our results are compatible with those of Feldbush (1980), who

found that a high proportion of the secondary IgG response to DNP-BGG *in vivo* was due to CR^- cells, especially at 2 weeks after priming, while at 6 months CR^+ cells were relatively more important. He also found that CR^- lymphoyctes were small, while CR^+ cells were medium to large in size. Mason (1976) reported that primed CR^- rat thoracic duct cells transferred a good IgG response, but a poor IgM one. Perhaps CR^- cells are end-stage secretors, while long-term memory is maintained by $C3dR^+$ cells.

In addition to the T-dependent antigen, HRBC, we tested the polyclonal and mitogenic responses of B cell fractions to LPS, and the IgM response to DNP-Ficoll, a T-independent antigen. The strongest mitogenic response to LPS was made by CR^+ and $C3dR^+$ cells, with a moderate response by $C3bR^+$ fractions and the weakest response by CR^- cells. (This is similar to the results seen in the IgM response to HRBC.) The polyclonal antibody response to LPS was quite similar (Tables 42 and 43). Moller's suggestion (1975) that LPS activates more mature cells would be consistent with our finding a stronger response by $C3dR^+$ than by $C3bR^+$ fractions, but cells which have differentiated further and lost their C3 receptors responded much less strongly, in our hands. Thus we would suggest that cells that have switched from IgM to the production of other Ig classes have lost some of their sensitivity to LPS.

Lewis *et al.* (1976) reported that CR^- cells make the major antibody response to the T-independent antigen, DNP-Ficoll. This is contrary to our data (Table 47) and those of Nariuchi and Kakiuchi (1982), and is most easily explained by the use by Lewis *et al.* of $EIgMC_{mo}$ treated with mouse C' for 30 min at 37°C, whereas we used 15

min and Nariuchi and Kakuichi used a 20 min incubation. From the results presented earlier it is likely that the 30 min cells were deficient in C3b (Table 4), and this would have the effect of causing $C3bR^+$ cells to contaminate the CR^- fraction. Since in our hands the major response to DNP-Ficoll was produced by $C3bR^+$ cells (Table 47), while CR^- cells responded quite weakly, the presence of $C3bR^+$ cells in the CR^- fraction would be expected to give the results reported by Lewis et al., (1976) and those reported by Dukor et al. (1974) with DNP-dextran. To make a clean separation of CR^+ from CR^- cells by rosetting, it is essential to use particles bearing large amounts of C3b as well as C3bi and C3d.

In studies of the CBA/N mouse, which carries an X-linked immune defective gene (Xid), Ahmed et al (1977) found that male mice could not make an antibody response to Type 2 T-independent antigens such as DNP-Ficoll. This correlated with the absence from B cells in these animals of the Ly5b surface marker, which is normally acquired at a fairly early stage in B cell maturation. On the other hand, we have observed that B cells carrying C3d but not C3b receptors, which we regard as a relatively late maturational stage, do not respond to DNP-Ficoll. This apparent discrepancy is most readily explained by assuming that the ability to respond to Type 2 antigens is acquired at about the time B cells acquire C3b receptors, continues through the stage when both C3b and C3d receptors are present and is lost by the time the C3b receptors have disappeared and only C3d receptors remain, at which time responsiveness to T-dependent antigens is still present. It would be interesting to determine the exact type(s) of C3 receptors on male CBA/N B cells. Kung and Paul's observation (1982) that the

response to Type 2 antigens exhibits little if any memory is consistent with our data showing that the relatively immature $C3bR^+$ cell makes only an IgM response.

Having in hand good evidence that B cell function correlated with the type of cell surface C3 receptor, we sought to modify antibody secretion by adding exogenous C3 to the cultures. We had found earlier that small amounts of murine C3, secreted by macrophages, were detectable in culture supernates within 1 hr of initiation. However, it seemed likely that higher local concentrations of C3 would be needed to affect processes at the cell surface in tissues, where the main C3 source may be local secretion by macrophages. Therefore we added to the cultures 25 µl of undiluted semi-purified murine C3, representing about 100-500 times as much C3 as was found in our culture supernates. This approximated 2×10^9 effective molecules of C3/culture, or about 2000 molecules/cell. C3 additions were controlled by substituting the gelatin-veronal buffer in which C3 was normally stored.

Repeated observations confirmed that the weak IgM responses made by CR^- cells were completely unaffected by exogenous mouse C3, while cells bearing C3b or C3d receptors produced 2 to 3 times as many IgM plaques against HRBC when C3 was added. Interestingly, bovine C3 was stimulatory to $C3dR^+$ cells, although not to $C3bR^+$ cells (Table 26), indicating that C3 in serum supplements may also modify immune reactions <u>in vitro</u>. This led us to compare zymosan-adsorbed FCS (which we used routinely) with unadsorbed FCS from the same lot, and we found that the latter supported twice as many IgM plaques as the former. Cultures in adsorbed and unadsorbed FCS were both stimulated

by exogenous mouse C3 (Table 27).

Further work showed that C3 was equally stimulatory to positively and negatively selected CR^+ fractions, and to unfractionated T cell-depleted spleen cells. It was stimulatory in the secondary IgM response as well as the primary one, but surprisingly here it stimulated only the $C3bR^+$ cells, and actually seemed inhibitory to $C3dR^+$ cells (Table 31). C3 did not enhance IgG secretion by any cell fraction, did not stimulate the mitogenic or polyclonal antibody response to LPS, and did not stimulate the IgM response to a T-independent antigen, DNP-Ficoll (Tables 45, 46 and 47). Lewis et al. found that cobra venom factor depletion of C3 did not affect the in vivo response to DNP-Ficoll, although it greatly reduced the IgM response to SRBC (1976). C3 did not stimulate CR^+ cells in the absence of antigen; did not substitute for TRF; and its effect was equally great over a 100-fold range of antigen concentration (Table 40).

In the above studies, exogenous C3 was added at the initiation of culture. When we added it progressively later after the start of culture, we found that the full stimulatory effect was still seen when C3 was added as late as day 4 (24 hr before harvest), but not when it was added 12 hr before harvest (Table 30). This suggested that C3 stimulation may be directed at antibody synthesis or secretion, rather than at earlier stages of immune induction.

It is important to keep in mind the context of these experiments, when small amounts of secreted C3 are present throughout the culture period, and may have effects on the IgG response or other events which we cannot detect, because unless the amount of C3 secreted by the

culture is sub-optimal, adding more will produce no observable response. This same problem occurs in in vivo experiments utilizing cobra venom factor, which depletes C3 levels by about 90-95% but never to zero. In addition, our experiments utilize mouse spleen cell fractions in culture, and such a system is far-removed from the structural organization of the immune system in a live mouse. The importance of C3 in "focusing" antigen into the lymphoid germinal centers in vivo seems quite clear (Klaus and Humphrey, 1977), yet there probably is no in vitro equivalent of this important early function.

We expected that anti-C3 antibody might modify the activities of CR^+ cells in vitro, either by removing secreted C3 from the cultures, or by binding to C3 fragments which had already attached to B cell C3 receptors. Nonspecific toxicity was eliminated by dialyzing anti-mouse C3 preparations which contained preservatives, and some preparations were absorbed with pooled mouse spleen cells. In the amounts used in culture, anti-C3 preparations were not cytotoxic for mouse spleen cells in the presence of complement, and as will be seen their effects were so specific as to rule out general cytotoxicity. $F(ab')_2$ preparations were just as active as whole antiserum and rabbit and goat antibodies were about equally effective.

Anti-mouse C3, added at any time after initiation of culture, up to and including 1 hr before harvest on day 5, reduced the primary IgM plaque response against HRBC to 20-30% of control levels. When applied to CR^+ and CR^- fractions, anti-C3 had no effect whatever on the low IgM response of CR^- cells, but drastically reduced that of cells bearing C3 receptors (Tables 32 and 33). When the secondary

response was studied, anti-C3 was found to reduce only the IgM response of CR^+ cells and to have no effect on the IgM or IgG response of CR^- cells (Table 36).

We next investigated the effect of removing anti-C3 after different intervals of exposure. Anti-C3 was added at initiation of culture, and at different times selected cultures were washed twice with medium, resuspended in fresh medium plus HRBC, and continued in culture. All were harvested at day 5. It was found that if anti-C3 was removed from the culture at any time after the first 24 hr, its full inhibitory effect persisted. When shorter intervals were studied, we found that removal of anti-C3 within 3 hr left the culture unaffected; removal after 12 hr partially suppressed the response; and removal after 24 hr suppressed the response by 80%. The effect of anti-C3 was not cancelled by restoring exogenous mouse C3 after the wash step (Tables 37, 38 and 39).

Since anti-C3 would not be expected to affect cultures in the absence of C3 (and probably C3 fragments), it seemed possible tht the reason anti-C3 failed to suppress IgM secretion when removed before 12 hr of culture was because it took 12 hr for enough C3 fragments to form and bind to cell receptors, so that they could be cross-linked by anti-C3. We tried to bypass this delay by adding exogenous C3 and mature culture supernates at 0 time, followed by anti-C3 and later a wash. We added mouse C3 or a 24 hr mouse spleen cell culture supernate at 0 time, followed by anti-C3 at 1 hr. The cultures were washed 2 hr later, followed by restoration of fresh medium and HRBC. For comparison, anti-C3 was added to other cultures at time 0 and washed out after varying intervals. At the same time we monitored the

appearance of functional C3 in the supernate of additional paralled cultures. The results of this complex experiment were quite decisive (Table 39). Providing exogenous C3 for the anti-C3 to interact with during the first few hr of culture did not inhibit subsequent IgM secretion, whereas providing a 24 hr culture supernate did. The critical time for anti-C3 to act was confirmed to be between 6 and 12 hr after culture initiation, and monitoring of C3 levels in paralled cultures indicated no sudden appearance of C3 at this time but rather a slow accumulation over 24 hr. It was not necessary for anti-C3 to be present for 6-12 hr; its full effect was manifested within 2 hr when an appropriate substrate (24 hr culture supernate) was present. We surmise that this supernate provided C3 fragments capable of binding to the C3 receptors on B cells, which could then react with anti-C3. (Table 13 showed that 24 hr culture supernates contain C3 and a C3-destroying substance, presumably capable of generating C3 fragments.)

The ability of anti-C3 specifically to inhibit IgM secretion by CR^+ cells, when added as late as 1 hr before cell harvest, was somewhat unexpected, as was the finding that removal of anti-C3 after a few hr of contact early in the culture period leaves the cells permanently impaired for IgM secretion. The high degree of specificity of this phenomenon appears to rule out nonspecific cytotoxicity, since only CR^+ cells are affected, and only their ability to secrete IgM, not IgG. There may be some relationship between these observations and the finding that lysed SRBC stroma bearing IgM, C4 and C3, but not SRBC bearing IgM and C4, strongly inhibited the IgM response of mouse spleen cells to HRBC (Table 22). This inhibition was only seen when

the spleen cells were left in prolonged contact with lysed SRBC-C3 fragments, and not when these fragments were removed promptly by centrifugation through FCS. C3-bearing membrane fragments may be capable of cross-linking B cell C3 receptors in the same way as C3 fragments plus anti-C3, leading to a prolonged shut-down of IgM secretion. This might even be an important mechanism of immune regulation, triggered by excess antigen or antigen-antibody complexes via complement activation. However, the physical form of the antigen may be critical, because we also found that intact SRBC bearing C3b, C3d or both, induced a stronger primary IgM response than SRBC bearing C4 but no C3 (Tables 23 and 24). The differences were moderate but consistent and were seen with both $C3bR^+$ and $C3dR^+$ cells. Thus it may be that circumstances which promote patching and/or capping of C3 receptors shut down IgM secretion for a prolonged period, while those which only allow binding to individual receptors stimulate IgM secretion. Since exogenous C3 added to cultures 24 but not 12 hr before harvest stimulated IgM secretion, an interval of about 15-20 hr must elapse between the binding of C3 fragments to receptors, and enhanced IgM secretion. A much shorter interval seems sufficient to turn off IgM secretion after C3 receptor cross-linking. These results might be considered to differ from those of Horn et al. (1981), who found that the presence of C5b-9 on lysed stroma strongly suppressed the primary but not the secondary IgM response to SRBC. The presence of C5b-9 also suppressed the IgG response, and C3 on the cells was unimportant. However their experiments were all performed in vivo, so it is hard to relate their results to our own. In addition, all the cells which suppressed in their work were injected in the form of

lysed stroma, whereas all the cells which did not suppress seem to have been injected as intact SRBC. It seems possible that this physical difference alone, which was not controlled for, might have influenced the results.

Normal spleen cell cultures seem somewhat deficient in C3, but not in the means necessary to fragment C3. Addition of exogenous murine C3, or bovine C3 in the form of semi-purified C3 or unadsorbed FCS, leads to a 2-3 fold boost in the number of IgM plaque forming cells. One can only speculate as to the magnitude of this boost were it possible to prevent murine C3 secretion in vitro and thus to lower the "background."

Recently much attention has been focused on the stimulatory and/or inhibitory effects of C3 and C5 fragments on antibody production in vitro. Human C5a and C5a-des-arg stimulated the primary response of mouse spleen cells to SRBC in vitro, but had to be added by day 2 of culture for any effect (Goodman et al., 1982). Fleisher and Berger (1983) found that C3a stimulated secretion of polyclonal Ig by human leukocytes in response to pokeweed mitogen, whereas C3b depressed this response. Both effects required the C3 fragments to be present at initiation of culture. On the other hand, Morgan et al. (1982) reported that C3a strongly suppressed human and murine B cell resposes when present from day 0 to day 1. C3a-des-arg was inactive, and no effect was seen if T cells were replaced by TRF.

The above observations and circumstances clearly differ from ours, in that the C3 and C5 fragments must be present from the first day or two of culture, whereas we found exogenous C3 to be stimulatory when added as late as day 4 in a 5 day culture. The suppressive

effect of C3a required in addition that active T cells be present and that serum peptidases be blocked by an inhibitor, whereas our C3 effects are seen without an inhibitor and in the presence of TRF instead of T cells. Our mouse C3 does not contain any C5 fragments because it was prepared from C5-deficient serum.

To summarize the results of this investigation, we have:

1. Developed a sensitive, semi-quantitative functional assay for mouse C3.
2. Demonstrated the accumulation in mouse spleen cell cultures of macrophage-secreted C3, and of a factor (not C3b-INA) which destroys this C3 activity.
3. Devised specific indicator particles which react only with C3b or C3d receptors on mouse spleen cells.
4. Used these indicator particles to fractionate mouse B cells into several sub-populations with very different C3 receptor and functional properties.
5. Shown that B cell C3 receptor expression progresses <u>in vitro</u> and presumably <u>in vivo</u> from C3b receptors on immature cells, capable only of a limited IgM response (to T-dependent antigens), through a relatively short period when both C3b and C3d receptors are found, to a C3d receptor stage which makes strong IgM and IgG responses, and finally to a CR^- stage which makes IgG and very little IgM.

   Thus primary IgM secretion is associated primarily with cells bearing C3d receptors; secondary IgM secretion is associated with $C3dR^+$ and $C3bR^+$ cells; and IgG secretion is associated only with $C3dR^+$ and CR^- cells.

6. Provided evidence that this maturational sequence is moderately accelerated by stimulation with horse RBCs, and more strongly accelerated by lipopolysaccharide (LPS).
7. Shown that the strongest mitogenic and polyclonal antibody responses to LPS are made by CR^+ and $C3dR^+$ cell fractions, with a moderate response by $C3bR^+$ cells and a weaker response by the CR^- fraction.
8. Found that the strongest response to the T-independent antigen DNP-Ficoll is produced by $C3bR^+$ cells, with a moderate response by CR^+, a weak response by CR^-, and no response by $C3dR^+$ cells.
9. Shown that exogenous semi-purified mouse C3 induces a 2 to 3 fold increase in the number of detectable IgM secreting CR^+ cells, while having no effect at all on CR^- cells or on IgG secretion by CR^+ cells. C3 stimulates IgM secretion by both $C3bR^+$ and $C3dR^+$ cells.
10. Demonstrated that this C3 effect can be seen when C3 is introduced as late as day 4 in a 5 day culture; and that it is not seen in the responses to LPS and DNP-Ficoll.
11. Found that antibody against mouse C3 reduces the IgM response of CR^+ fractions to horse RBCs by about 80%, while having no effect on CR^- cells, the IgG response of CR^+ cells, or the response to LPS.
12. Observed that this anti-C3 effect on IgM secretion occurs when the antibody is added as late as 1 hr before harvesting the culture, or as early as zero time followed by removal 12-24 hr later.

This work by itself does not tell us whether stimulation of C3

receptors drives the differentiation of B cells, or whether differentiation leads to a change in C3 receptor expression. However, logic and the *in vivo* experiments of others with complement-deficient individuals and with cobra venom factor suggest that C3 receptor stimulation probably promotes the generation of memory B cells and the switch from production of IgM to IgG and other isotypes. Certainly it is clear that T-dependent IgM secretion is minimal with CR^- cells, and that IgG secretion is not associated with C3b receptors. It seems equally clear that C3 (fragments?) stimulate(s) IgM secretion by CR^+ cells of all types, while having no effect on IgG secretion, IgM secretion by CR^- cells, or the response to T-independent antigens. Cross-linking of C3 receptors may provide a strong negative signal that again affects only IgM secretion by CR^+ cells, and seems to do so by a pathway triggered within a few hours, early in immune induction as well as by a pathway effective only an hour or so before cell harvest and plating. The T-independent antigen DNP-Ficoll stimulated primarily $C3bR^+$ cells, and the resulting antibody response was unaffected by exogenous C3 and anti-C3.

Our work has been independent of possible T cell effects, since T cells were depleted from all fractions, and substituted for with TRF. However, recent work showing that T cells have moderate numbers of C3b and probably C3d receptors (Wahlin *et al.*, 1983; Wilson *et al.*, 1983), points out the strong possibility that C3 may be involved in additional pathways affecting immune regulation. If T helpers were to carry only C3b receptors and T suppressors only C3d receptors, or vice versa, numerous possibilities for control are evident which would be antigen-independent. Certainly the existence on T and B cells of

these different and highly specific receptors for C3 fragments cannot be fortuitous!

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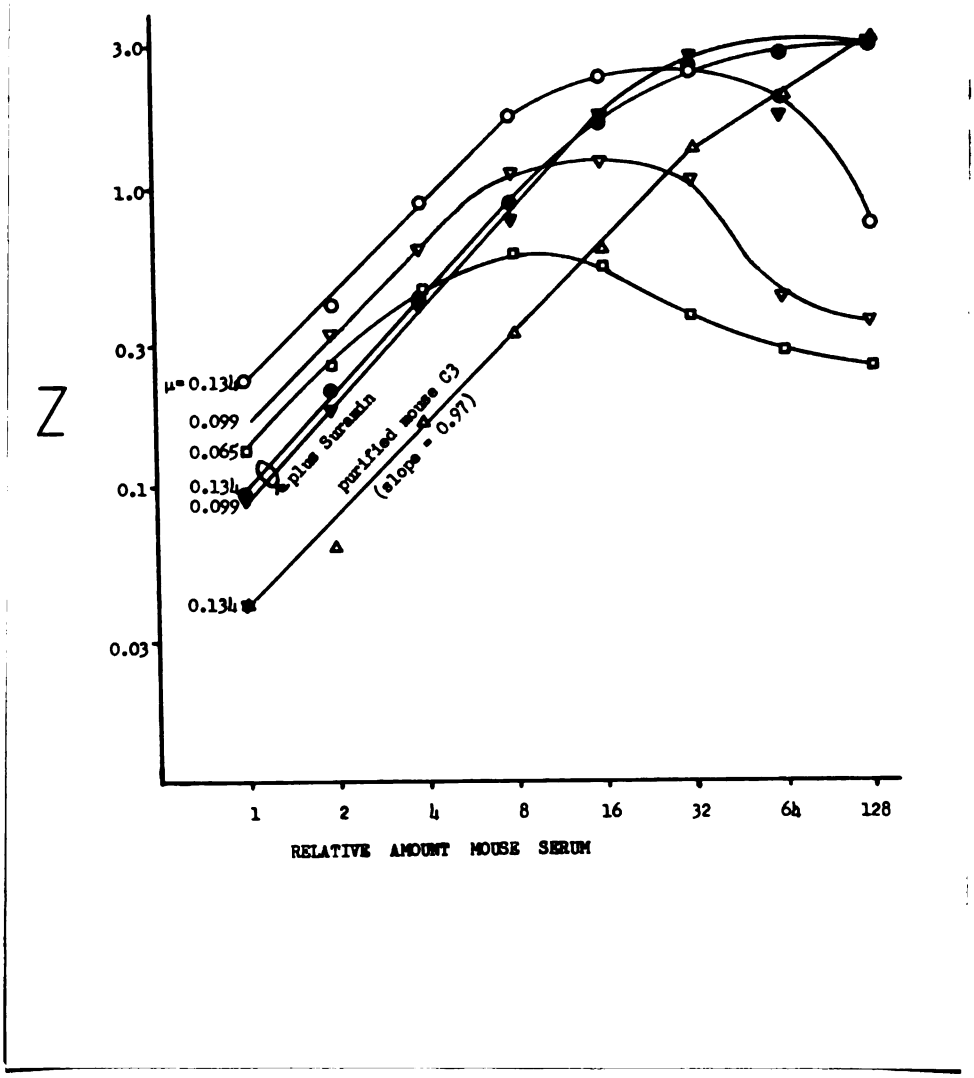

Z
3.0
1.0
0.3
0.1
0.03
μ = 0.134
0.099
0.065
0.134
0.099
0.134
plus Suramin
purified mouse C3
(slope = 0.97)
1
2
4
8
16
32
64
128
RELATIVE AMOUNT MOUSE SERUM

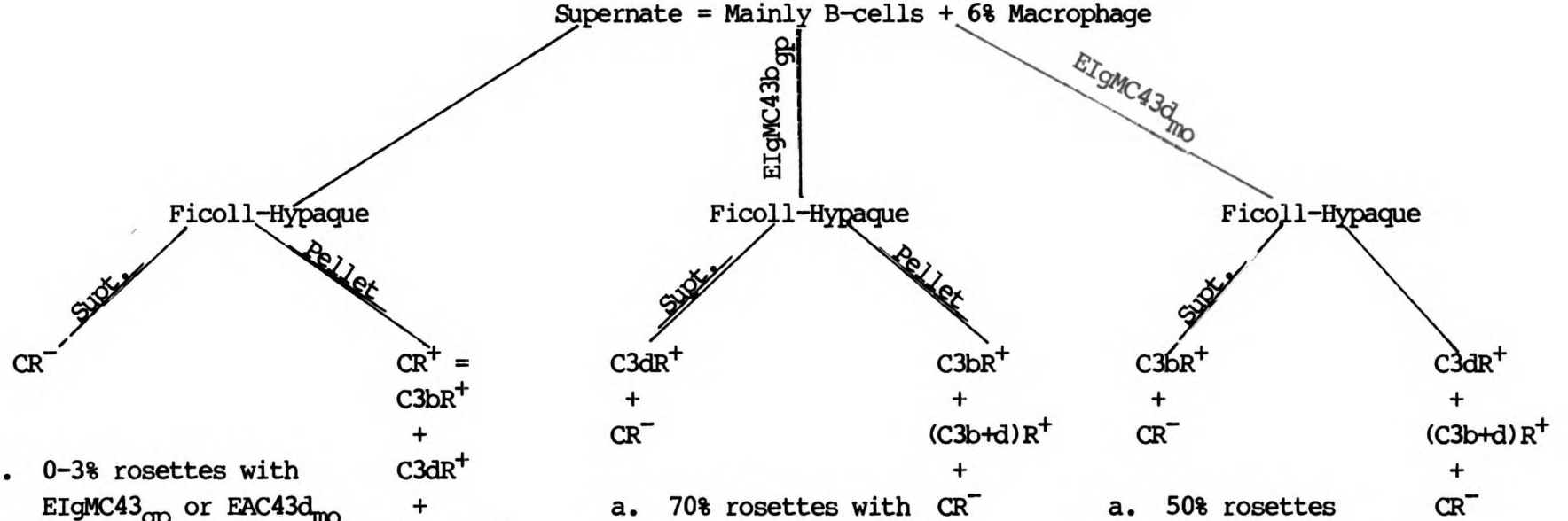
FIGURE II

SEPARATION OF COMPLEMENT RECEPTOR LYMPHOCYTES FROM WHOLE SPLEEN CELL SUSPENSION

BDF1 Spleen Cells + Anti-Thy 1.1 + Rabbit Complement

Ficoll-Hypaque Separation

Supernate = Mainly B-cells + 6% Macrophage



a. 0-3% rosettes with EIgMC43$_{gp}$ or EAC43d$_{mo}$
b. 80% are sIg$^+$
c. 7% are Thy 1.1$^+$
d. 3% are macrophages
e. No response to con A
f. No suppression of CR$^+$ response
g. 2-mercaptoethanol does not induce C3 receptors
h. C3 receptors do not appear during several days of normal culture

a. 70% rosettes with EIgMC43d$_{mo}$
b. 0-2% rosette with EIgMC43b$_{gp}$
c. C' receptors unaffected by 5 x 10^{-5}M 2-mercapto-ethanol

a. 50% rosettes with EIgMC43b$_{gp}$
b. 0-2% rosette with EIgMC43d$_{mo}$
c. 2-mercaptoethanol rapidly reduces C3d receptors, with no loss of C3b receptors

TABLE 1

C3 Titration and the
Effect of Heating on C3 Activity
in Pooled A/J Mouse Serum

Serum Treatment	Serum of C3 dilutions: 1/30	1/100	1/300	1/1000	1/3000	1/10,000	No Serum
unheated	ND[a]	17[b]	45	63	51	23	0
15 min 56°C	1	20	52	60	40	ND	
30 min 56°C	1	19	47	51	33	ND	
semi-purified mouse C3	ND	100	96	88	51	21	

a = not done

b = percent lysis

TABLE 2

Effects of Cobra Venom Factor and Suramin
on C3 Activity in Pooled A/J Mouse Serum

	Serum dilution:						
Serum Treatment	1/5	1/10	1/20	1/200	1/400	1/800	No Serum
Normal Serum	43[a]	41	43	55	61	55	0
CVF-Treated Serum	10	7	7	0	0	0	
Normal Serum & Suramin	91	92	86	ND[b]	ND	ND	
CVF Serum & Suramin	8	6	7	ND	ND	ND	

a = percent lysis

b = not done

TABLE 3

C3 Levels in Sera of Several Mouse Strains

Mouse Strain and Sex		$C3H_{63}$/ml
C3D2F1/J	♀	13,000
A/J (pooled)	♀	14,000
CBA/J	♀	20,000
BDF/1	♀	20,000
BALB/c	♀	21,000
CBA/N	♀	22,000
C57Bl/6	♂	22,000
CBA/N	♂	27,000
C57Bl/6	♀	34,000
C3HeB/FeJ	♀	55,000

TABLE 4

Conglutination, Immune-Adherence, and Spleen Cell Rosetting with Mouse Complement-Treated Red Cells ($EIgMC_{mo}$)

I-A Hemagglutination

EIgM Treatment:	$1x10^8$[a]	$5x10^7$	$2.5x10^7$	$1.25x10^7$	$6.25x10^6$	$3.12x10^6$	$1.5x10^6$	$7.8x10^5$	Percent Conglutination	Rosettes
None	0[b]	0	0	0	0	0	0	0	control	2[c]
Mo Serum 10sec 37°C	0	0	0	0	0	0	0	0	4	2
Mo Serum 30sec 37°C	2	2	2	2	0	0	0	0	0	23
Mo Serum 1min 37°C	4	4	4	4	4	4	2	2	75	23
Mo Serum 2min 37°C	4	4	4	3	2	0	0	0	86	29
Mo Serum 5min 37°C	4	4	4	3	3	0	0	0	98	30
Mo Serum 10min 37°C	4	4	4	3	3	0	0	0	98	31
Mo Serum 15min 37°C	4	4	4	3	3	0	0	0	92	35
Mo Serum 17min 37°C	4	2	2	2	2	0	0	0	ND[d]	32
Mo Serum 30min 37°C	4	2	2	2	0	0	0	0	81	21
Mo Serum 60min 37°C	0	0	0	0	0	0	0	0	68	16
Mo Serum 120min 37°C	0	0	0	0	0	0	0	0	0	16

[a] Number of $EIgMC_{mo}$ per tube. The fewer the cells required for hemagglutination, the more C3b there is per cell.

[b] Hemaglutination Patterns (0 to 4+) with Human RBCs.

[c] Percent of Spleen Cells Rosetting with $EIgMC_{mo}$

[d] Not done

TABLE 5

Immune-Adherence and Spleen Cell Rosette Formation with $EIgMC43b_{gp}$ or $EIgMC43d_{gp}$

Indicator Cells	Decayed 90 min at 37°C in EDTA-GVB	Treatment $\bar{c}$ Heated serum[b]	Immune-Adherence						Percent Rosettes	
			25[a]	12.5	6.2	3.1	1.6	0.8	Mouse Spleen	G.P. Spleen
EIgMC4	-	untreated	0	0	0	0	0	0	1	1
$EIgMC43b_{gp}$	-	untreated	4	4	4	4	4	0	32	27
$EIgMC43b_{gp}$	+	untreated	4	4	4	4	0	0	28	25
$EIgMC43b_{gp}$	+	5min 0°C	4	4	4	Lost	Lost	0	6	28
$EIgMC43b_{gp}$	+	120min 37°C	0	0	0	0	0	0	1	21

[a] Number of EIgMC43b per tube x 10^6

[b] 1/2 ml $_{gp}$ serum 1/30 (HI, 56°C 1hr) per $2x10^8$ cells

[c] Converts $EIgMC43b_{gp}$ to $EIgMC43d_{gp}$

TABLE 6

Kinetic Analysis of EIgMC43b$_{gp}$ Degradation by Heated Serum

Heated Serum[a]	Conditions		I-A Hemagglutination					Percent Lysis by C1+C2+ C5-C9	Percent Rosettes with	
	Min	°C	25[b]	12.5	6.2	3.1	1.6		mouse spleen cells	guinea pig spleen cells
None	10	37°	4	4	4	3	1	100	32	26
None	60	37°	4	4	4	3	0	100	26	24
None	180	37°	4	4	4	2	0	100	14	24
GPS 1:30	1	0°	4	4	4	3	0	84	0	27
GPS 1:30	10	0°	4	4	4	3	1	85	0	29
GPS 1:30	60	0°	4	4	4	2	0	32	0	30
GPS 1:30	180	0°	4	4	3	0	0	27	0	28
GPS 1:30	1	37°	4	4	4	3	0	38	7	20
GPS 1:30	10	37°	4	4	4	3	0	22	3	24
GPS 1:30	60	37°	3	0	0	0	0	7	1	22
GPS 1:30	180	37°	0	0	0	0	0	3	2	20
MoS 1:8	1	37°	4	4	4	3	0	100	14	27
MoS 1:8	10	37°	4	4	4	3	0	63	1	28
MoS 1:8	60	37°	4	4	4	2	0	44	0	28
MoS 1:8	180	37°	1	0	0	0	0	7	0	19

[a] Serum diluted in EDTA-.037 buffer

[b] Number of EIgMC43b per tube x 10^6

TABLE 7

C3 Receptor Properties of Mouse Spleen Cell Fractions
Separated By Different Positive and Negative Rosette Selection Procedures

Cell Fraction Tested	Percent Lymphocytes Rosetting with: EIgMC43b$_{gp}$	EIgMC43d$_{mo}$	EIgMC$_{mo}$	Percent Lymphocytes not Rosetting with: EIgMC$_{mo}$	percent Macrophages
T Cell-Depleted Spleen Cells	18	36	54	46	7
EIgMC$_{mo}$ Rosetting Supernate (= negatively selected CR$^-$ cells)	0	0	ND	100	3
EIgMC$_{mo}$ Rosetting Pellet (= positively selected C3bR$^+$ + C3dR$^+$ + C3b+dR$^+$ + CR$^-$ cells)	ND[a]	ND	70	30	6
EIgMC43b$_{gp}$ Rosetting Supernate (= negatively selected C3dR$^+$ + CR$^-$ cells)	0	71	ND	28	3
EIgMC43b$_{gp}$ Rosetting Pellet (= positively selected C3bR$^+$ + C3b+dR$^+$ + CR$^-$ cells)	71	23	ND	48	2
EIgMC43d$_{mo}$ Rosetting Supernate (= negatively selected C3bR$^+$ + CR$^-$ cells)	51	2	ND	48	2
EIgMC43d$_{mo}$ Rosetting Pellet (= positively selected C3dR$^+$ + C3b+dR$^+$ + CR$^-$ cells)	8	82	ND	15	5

[a] Not done

TABLE 8

Changes in Cell Surface Complement Receptors During Culture Without Antigen

Days in Culture	Cell Fraction	% Rosettes EIgMC43b$_{gp}$	% Rosettes EIgMC43d$_{mo}$
1	Whole Spleen Cells	12	23
2	Whole Spleen Cells	25	23
3	Whole Spleen Cells	23	15
4	Whole Spleen Cells	19	8
1	T Cell-Depleted Spleen Cells	32	35
2	T Cell-Depleted Spleen Cells	41	28
3	T Cell-Depleted Spleen Cells	38	28
4	T Cell-Depleted Spleen Cells	31	16
1	CR^- Cells	2	0
2	CR^- Cells	2	5
3	CR^- Cells	1	6
4	CR^- Cells	1	2
1	CR^+ Cells	6	4
2	CR^+ Cells	45	5
3	CR^+ Cells	43	2
4	CR^+ Cells	45	5

TABLE 9

Changes in Cell Surface Complement Receptors
During Culture of Whole Mouse Spleen Cells
with Horse Red Blood Cells or Lipopolysaccharide

Days in Culture	Stimulator	% Rosettes EIgMC43b$_{gp}$	% Rosettes EIgMC43d$_{mo}$
0	Horse RBCs	16	22
1	Horse RBCs	11	28
2	Horse RBCs	15	18
3	Horse RBCs	11	5
4	Horse RBCs	11	4
0	Lipopolysaccharide	16	22
1	Lipopolysaccharide	3	27
2	Lipopolysaccharide	2	28
3	Lipopolysaccharide	1	31
4	Lipopolysaccharide	0	24

TABLE 10

Changes in the Cell Surface Complement Receptors of $C3bR^+$, $C3dR^+$ and CR^- cells during culture with and without HRBC or LPS.

Days of Culture	Conditions	$C3bR^+$ Cells[a]		$C3dR^+$ Cells[b]		CR^- Cells[c]	
		C3bR[d]	C3dR[e]	C3bR	C3dR	C3bR	C3dR
0	Start of Culture	71	23	8	82	0	2
1	No Antigen	4	8	0	5	1	1
2	No Antigen	5	39	1	8	ND[f]	ND
3	No Antigen	6	8	1	12	0	2
1	HRBC	0	42	2	6	1	0
2	HRBC	3	15	2	15	ND	ND
3	HRBC	2	5	0	6	1	8
1	LPS	4	40	1	30	1	0
2	LPS	3	45	5	39	ND	ND
3	LPS	10	12	0	37	0	4

[a] positively selected (pellet) (also contains cells with both kinds of C3 receptors)

[b] positively selected (pellet) (also contains cells with both kinds of C3 receptors)

[c] negatively selected (supernatant)

[d] percent rosetting with $EIgMC43b_{gp}$

[e] percent rosetting with $EIgMC43d_{mo}$

[f] not done

TABLE 11

Changes in the Cell Surface Complement Receptors of Both Positively and Negatively selected Cells Cultured with HRBC

Days of Culture	Positively Selected $C3bR^+$ Cells		Negatively Selected $C3bR^+ + CR^-$		Positively Selected $C3dR^+$ Cells		Negatively Selected $C3bR^+ + CR^-$	
	C3bR[a]	C3dR[b]	C3bR	C3dR	C3bR	C3dR	C3bR	C3dR
1	11	20	25	24	2	13	2	28
2	3	10	40	36	1	1	2	53

[a] Percent rosettes with $EIgMC43b_{gp}$

[b] Percent rosettes with $EIgMC43d_{mo}$

TABLE 12

Effect of 5×10^{-5} M 2-mercaptoethanol in the culture medium on the expression of lymphocyte complement receptors

	Cell Fraction			
	$(C3bR^+ + CR^-)$		$(C3dR^+ + CR^-)$	
Culture Conditions (1hr 37°C)	C3bR[a]	C3dR[b]	C3bR	C3dR
without 2-mercaptoethanol	47	2	0	79
with 2-mercaptoethanol	48	39	0	81

[a] Percent rosettes with $EIgMC43b_{gp}$

[b] Percent rosettes with $EIgMC43d_{mo}$

TABLE 13

C3 Production and Destruction by Mouse Spleen Cell Cultures

CULTURE COMPOSITION[a]	HOURS IN CULTURE							
	24		48		72		120	
	C3 activity	C3-destroying activity	C3 activity	C3-destroying activity	C3 activity	C3-destroying activity	C3 activity	C3-destroying activity
Semi-purified Mouse C3 1/500	77[b]	ND[c]	59	ND	51	ND	31	ND
Mouse C3 1/500 + 10% FCS	79	ND	25	ND	12	ND	1	ND
Spleen Cells	74	+[d]	43	++[e]	41	+	47	+
Spleen Cells + 10% FCS	63[f]	-	0	+	6	+	45	+
Spleen Cells + 10% FCS + HRBC	69	-	0	+	17	+	33	-
Spleen Cells + LPS	78	-	46	++	82	++	ND	ND
Spleen Cells + 5% FCS	71	-	15	+	8	++	ND	ND
Spleen Cells + 5% FCS + LPS	60	-	2	+	18	+	ND	ND

[a] all cultures contained RPMI-1640

[b] percent lysis by 0.2 ml of undiluted culture supernate

[c] Not Done

[d] Supernate destroyed 15-40% of mouse C3 offered

[e] Supernate destroyed > 40% of mouse C3 offered

[f] Supernate destroyed < 15% of mouse C3 offered

TABLE 14

C3 Content and C3 Destroying Activity of 48 Hour Supernates from Con A Stimulated Mouse Cell Preparations

Cell Type	C3 Content	C3 Destruction
Macrophages	60[a]	[b]51 vs 24
T Cells	1	51 vs 35
B Cells	1	51 vs 48

[a] Percent lysis

[b] Percent lysis with mouse C3 dilution before and after contact with culture supernate for 3 hr at 37°C

TABLE 15

Test of Culture Supernates for C3b-inactivator activity

Substrate		Supernate	percent lysis by C1 + C2 + C5 - C9
$EIgMC43b_{gp}$	+	GVB^{++}	29
$EIgMC43b_{gp}$	+	RPMI-1640	31
$EIgMC43b_{gp}$	+	Heat-inactivated mouse serum 1/10	5
$EIgMC43b_{gp}$	+	Whole spleen culture supernate	29
$EIgMC43b_{gp}$	+	T Cell enriched culture supernate	30
$EIgMC43b_{gp}$	+	T Cell enriched culture supernate	29
$EIgMC43b_{gp}$	+	T Cell enriched culture supernate	28

TABLE 16

Specificity of Mouse C3 Inactivating Substance
Produced by Cultured Mouse Spleen Cells

C3 Source	Treatment	Residual C3 Activity (% Lysis)
Mouse C3	RPMI-1640	75
Mouse C3	BDFI Supernate	51
Guinea pig C3	RPMI-1640	80
Guinea pig C3	BDFI Supernate	82
Bovine C3	RPMI-1640	61
Bovine C3	BDFI Supernate	59

TABLE 17

Production of C3 and C3 Inactivating Substance by CBA/N Mouse Spleen Cells in Culture

Mouse Strain and Sex	Antigen	C3 Activity	C3 Inactivation[a]
CBA/N ♂	None	68[b]	75 vs 72
CBA/N ♂	LPS	66	75 vs 69
CBA/N ♀	None	32	75 vs 70
CBA/N ♀	LPS	34	75 vs 58
BDFI[c] ♀	LPS	28	75 vs 51

[a] Change in C3 activity (percent lysis) after incubating semipurified mouse C3 with culture supernate for 3 hr at 37°C

[b] % Lysis with undiluted culture supernate

[c] Mouse strain with no B cell defect

TABLE 18

Reconstitution of the Primary Immune Response to Horse RBCs by Addition of TRF to Anti-Thy1.1 - Treated Spleen Cells

Cell Population	Conditions	Plaques Per Culture
Whole spleen	No Ag	6[a]
Whole spleen	HRBC	840
T cell-depleted spleen	No Ag	4
T cell-depleted spleen	HRBC	17
T cell-depleted spleen	HRBC + TRF 0.1 ml	2441
T cell-depleted spleen	HRBC + TRF 0.15 ml	3540

[a] Average of duplicate plaque assays on triplicate cultures

TABLE 19

Requirement for both TRF and macrophages
to reconstitute a primary sheep RBC response by various cell fractions

Culture Contents	Plaques per culture
MØ alone	3
MØ + TRF[a]	0
MØ + TRF + SRBC	3
T Cell-Depleted Spleen alone	0
T Cell-Depleted Spleen + SRBC	6
T Cell-Depleted Spleen + MØ[b] + SRBC	0
T Cell-Depleted Spleen + TRF + SRBC	94
T Cell-Depleted Spleen + TRF + MØ + SRBC	297
CR^+ Cells alone	7
CR^+ Cells + MØ + TRF	6
CR^+ Cells + MØ + SRBC	1
CR^+ Cells + TRF + SRBC	8
CR^+ Cells + TRF + MØ + SRBC	229
CR^- Cells alone	3
CR^- Cells + MØ + TRF	3
CR^- Cells + MØ + SRBC	3
CR^- Cells + TRF + SRBC	7
CR^- Cells + TRF + MØ + SRBC	126

[a] 0.15 ml TRF added per culture

[b] 8×10^4 MØ added per culture

TABLE 20

Kinetics of the primary response of mouse spleen cell fractions to horse RBCs

Cell Fraction	Ab Response	Plaques per Culture on Day: 4	5	6	7
T Cell-Depleted Spleen	IgM	346	920	548	324
	IgG	ND	0	24	14
CR^+ Cells	IgM	465	1488	524	438
	IgG	ND	0	32	38
CR^- Cells	IgM	12	57	79	64
	IgG	ND	15	10	20
$C3bR^+$ Cells	IgM	447	1744	553	410
(pos. selected)	IgG	ND	0	0	36
$C3bR^+$ + CR- Cells	IgM	149	80	237	45
(neg. selected)	IgG	ND	4	0	35
$C3dR^+$ + CR- Cells	IgM	484	2085	580	461
(pos. selected)	IgG	ND	0	0	11
$C3dR^+$ + CR- Cells	IgM	319	939	502	389
(neg. selected)	IgG	ND	4	0	66

* All cultures supplemented with macrophages and TRF.

TABLE 21

The Secondary antibody response of mouse spleen cell fractions to horse RBCs

Cell Fraction*	Ab Response	Plaques per Culture on Day: 2	3	4	5	6	7
EXPERIMENT I							
Whole Spleen	IgM	5	56	559	244	54	232
	IgG	51	168	418	382	281	389
T Cell-Depleted Spleen	IgM	14	116	486	383	91	36
	IgG	84	135	279	289	299	86
CR^+ Cells	IgM	13	21	734	523	31	659
	IgG	12	29	18	1679	69	112
CR^- Cells	IgM	8	0	58	5	29	17
	IgG	164	47	332	709	70	53
$C3bR^+$ Cells	IgM	9	116	387	143	309	61
(pos. selected)	IgG	10	20	21	7	18	9
$C3dR^+$ Cells	IgM	18	46	1733	1029	765	315
(pos. selected)	IgG	41	86	188	1300	637	474
EXPERIMENT II							
$C3bR^+ + CR^-$ Cells	IgM			420			
(neg. selected)	IgG				160		
$C3dR^+ + CR^-$ Cells	IgM			628			
(neg. selected)	IgG				1240		

* All cultures supplemented with macrophages and TRF.

TABLE 22

Primary IgM response of sham-rosetted T cell-depleted spleen cells to horse or sheep RBCs (rosetting stroma removed or not removed)

RED CELL ROSETTING PARTICLES:		Antigen	Plaques per Culture on Day 5* EXP I	EXP II	
EIgMC4	(Stroma removed)	None	10	6	(SRBC plate)
"	"	None	14	2	(HRBC plate)
"	"	SRBC	544	677	
"	"	HRBC	510	598	
$EIgMC43b_{gp}$	"	None	9	8	(SRBC plate)
"	"	None	6	15	(HRBC plate)
"	"	SRBC	228	324	
"	"	HRBC	740	883	
$EIgMC43d_{mo}$	"	None	7	7	(SRBC plate)
"	"	None	6	4	(HRBC plate)
"	"	SRBC	510	443	
"	"	HRBC	960	1240	
$EIgMC_{mo}$	"	None	3	4	(SRBC plate)
"	"	None	8	2	(HRBC plate)
"	"	SRBC	620	1266	
"	"	HRBC	740	898	
EIgMC4	(Stroma not removed)	None	5	10	(HRBC plate)
"	"	HRBC	345	490	
$EIgMC43b_{gp}$	"	HRBC	76	170	
$EIgMC43d_{mo}$	"	HRBC	9	84	
$EIgMC_{mo}$	"	HRBC	70	110	

* All cultures contained T cell-depleted mouse spleen cells + macrophages + TRF

TABLE 23

Primary IgM response of T cell-depleted spleen cells to sheep RBCs bearing complement components

Antigen	Plaques per culture on Day 5*	
	EXP I	EXP II
-	12	-
E (SRBC)	836	940
EIgMC4	745	822
$EIgMC43b_{gp}$	1589	1260
$EIgMC43d_{mo}$	1541	1480
$EIgMC_{mo}$	1635	1290

* All cultures supplemented with macrophages + TRF.

TABLE 24

Primary IgM response of positively and negatively selected cells challenged with sensitized sheep RBCs coated with either C3b or C3d

Cell Fraction	Antigen	Plaques per culture on Day 5* EXP I	EXP II
$C3bR^+$ Cells (positively selected)	None	210	14
" "	E(SRBC)	1090	940
" "	EIgMC4	1109	990
" "	$EIgMC43b_{gp}$	1675	1240
" "	$EIgMC43d_{mo}$	1368	1190
$C3dR^+$ Cells (positively selected)	None	197	26
" "	E(SRBC)	808	1020
" "	EIgMC4	889	960
" "	$EIgMC43b_{gp}$	1309	1640
" "	$EIgMC43d_{mo}$	1423	1850
$C3bR^+ + CR^-$ Cells (negatively selected)	None	22	4
" "	E(SRBC)	368	290
" "	EIgMC4	556	320
" "	$EIgMC43b_{gp}$	1051	960
" "	$EIgMC43d_{mo}$	1103	1080
$C3dR^+ + CR^-$ Cells (negatively selected)	None	12	3
" "	E(SRBC)	412	580
" "	EIgMC4	524	672
" "	$EIgMC43b_{gp}$	553	610
" "	$EIgMC43d_{mo}$	1049	720

* All cultures supplemented with macrophages and TRF.

TABLE 25

Test for suppressor cells in the CR^- cell population

Cell Fraction	Antigen	IgM plaques per culture on Day 5*
CR^+ (1×10^6)	-	22
" (2×10^6)	-	3
" (1×10^6)	+	917
" (2×10^6)	+	152
CR^- (1×10^6)	-	5
" (1×10^6)	+	178
CR^+ (1×10^6) + CR^- (1×10^6)	-	3
CR^+ (1×10^6) + CR^- (1×10^6)	+	751
CR^+ (1×10^6) + CR^- (5×10^5)	+	543

* All cultures supplemented with macrophages + TRF.

TABLE 26

Effect of mouse or bovine C3 on the primary IgM Response to horse RBCs of B cell subpopulations[a]

Cell Fraction	medium only	25 ul Mo C3	25 ul Bov C3	HRBC	25 µl GVB[b]+ HRBC	50ul GVB + HRBC	25 ul Mo C3+ HRBC	50 ul Mo C3+ HRBC	25 ul Bov C3+ HRBC	50 ul Bov C3+ HRBC
CR^-	8	9	7	178	166	182	ND	180	ND	151
CR^+	13	10	9	917	849	ND[c]	2380	2488	2030	2064
pos. selected $C3bR^+$	14	7	5	884	488	20?	1084	1280	674	556
pos. selected $C3dR^+$	7	20	24	1084	1112	988	2292	2376	2262	2456

[a] Numbers are IgM plaques per culture. All cultures supplemented with macrophages + TRF.

[b] control for the gelatin veronal buffer in which C3 was stored

[c] Not Done

TABLE 27

The effect of mouse C3 and zymosan adsorption of FCS on the primary IgM response to horse RBCs

Cell Fraction*	HRBC	FCS	FCS-Z[a]	Mo C3	IgM plaques per culture
CR^+	-	+	-	Buffer only[b]	24
CR^+	-	+	-	+	20
CR^+	-	-	+	Buffer only	16
CR^+	-	-	+	+	34
CR^+	+	+	-	-	1132
CR^+	+	+	-	Buffer only	1624
CR^+	+	+	-	+	3046
CR^+	+	-	+	-	508
CR^+	+	-	+	Buffer only	816
CR^+	+	-	+	+	1208
CR^-	-	+	-	Buffer only	10
CR^-	-	+	-	+	10
CR^-	+	+	-	-	314
CR^-	+	+	-	Buffer only	262
CR^-	+	+	-	+	238

[a] FCS adsorbed with zymosan (see Methods & Materials)

[b] control for the gelatin-veronal buffer in which C3 was stored

* All cultures supplemented with macrophages and TRF.

TABLE 28

Effect of Mouse C3 on the primary IgM response of negatively selected cells to horse RBCs

Cell Fraction*	HRBC	Mo C3	IgM Plaques per culture
$C3bR^+ + CR^-$	-	-	3
$C3bR^+ + CR^-$	+	-	72
$C3bR^+ + CR^-$	+	Buffer only**	35
$C3bR^+ + CR^-$	+	+	299
$C3dR^+ + CR^-$	-	-	10
$C3dR^+ + CR^-$	+	-	222
$C3dR^+ + CR^-$	+	Buffer only	236
$C3dR^+ + CR^-$	+	+	687

* All cultures supplemented with macrophages + TRF.

** Control for the gelatin-veronal buffer in which C3 was stored.

TABLE 29

Primary IgM response to horse RBCs when mouse C3 and antigen are added at different times after initiation of the culture

Cell Fraction	Time HRBCs added	Time C3 added	Macrophage added	TRF added	Plaques per Culture on Day 5
T cell-depleted spleen	None	None	+	+	4
"	0 time	None	+	-	9
"	0 "	0 time	+	-	12
"	0 "	None	+	+	826
"	0 "	Buffer control	+	+	903
"	0 "	0 time	+	+	1647
"	24 hr	None	+	+	900
"	24 hr	24 hr	+	+	1730
"	48 hr	None	+	+	302
"	48 hr	48 hr	+	+	359

TABLE 30

Effect of mouse C3 added at varying times on the primary IgM response to horse RBCs

Cell Fraction*	Antigen	time C3 added	Plaques per culture on Day 5
EXPERIMENT I			
T cell-depleted spleen	-	None	5
"	+	None	820
"	+	0 time	1760
"	+	24 hours	1705
"	+	48 hours	1703
"	+	72 hours	1635
"	+	96 hours	1692
EXPERIMENT II			
T cell-depleted spleen	-	None	16
"	+	Buffer control	1146
"	+	0 time	3344
"	+	96 hours	2982
"	+	12 hr before harvest	863
"	+	5 min before harvest	1125

TABLE 31

Effect of mouse C3 on the secondary immune response to horse RBCs

Cell Fraction	Antigen	Mo C3	IgM Plaques* Per culture	IgG Plaques* per culture
$C3bR^+$ (pos. selected)	-	-	4	51
" "	+	-	288	181
" "	+	Buffer control	56	129
" "	+	+	968	161
$C3bR^+ + CR^-$ (neg. selected)	-	-	19	3
" "	+	-	341	105
" "	+	Buffer control	468	56
" "	+	+	964	66
$C3dR^+$ (pos. selected)	-	-	7	36
" "	+	-	238	1459
" "	+	Buffer control	49	1544
" "	+	+	102	1484
$C3dR^+ + CR^-$ (neg. selected)	-	-	6	58
" "	+	-	150	1862
" "	+	Buffer control	81	2478
" "	+	+	42	2534
CR^- (neg. selected)	-	-	3	64
" "	+	-	53	662
" "	+	Buffer control	62	692
" "	+	+	29	720

* Cells harvested on day 5. All cultures supplemented with macrophages + TRF.

TABLE 32

Effect of anti-mouse C3 antibody on the primary IgM response of T cell-depleted spleen cells to horse RBCs

Antigen	Anti-C3 from:	Time anti-C3 added	IgM plaques per culture on Day 5*
-	-	-	9
+	-	-	1327
+	Rabbit[a]	24 hr	248
+	Rabbit	48 hr	270
+	Rabbit	72 hr	281
+	Rabbit	96 hr	306
+	Rabbit	12 hr before harvest	255
+	Rabbit	1 hr before harvest	279
+	Goat[b]	24 hr	312
+	Goat	48 hr	327
+	Goat	72 hr	320
+	Goat	96 hr	377
+	Goat	12 hr before harvest	315
+	Goat	1 hr before harvest	408

* Cell cultures supplemented with macrophages + TRF.

[a] Rabbit anti-mouse C3, 25 ul

[b] Goat anti-mouse C3 F(ab')2, 25 ul

TABLE 33

Effect of different anti-C3 preparations on the primary IgM response of CR^+ and CR^- cells to horse RBCs

Cell Fraction[1]	Antigen	Anti-C3 or Control	IgM Plaques per culture on Day 5
CR^+	-	-	11
"	+	+	1338
"	+	normal rabbit serum	1325
"	+	rabbit anti-Bov C3[2]	249
"	+	rabbit anti-Mo C3	287
"	+	normal goat serum	1398
"	+	goat anti-MoC3F(ab')$_2$ 1/5	204
"	+	goat anti-MoC3F(ab')$_2$ 1/10	312
CR^-	-	-	9
"	+	-	201
"	+	normal rabbit serum	197
"	+	rabbit anti-Bov C3	171
"	+	rabbit anti-Mo C3	180
"	+	normal goat serum	191
"	+	goat anti-Mo C3F(ab')$_2$ 1/5	198
"	+	goat anti-Mo C3F(ab')$_2$ 1/10	183

[1] All cultures supplemented with macrophages + TRF.

[2] All antibody fractions containing preservatives were dialyzed against RPMI-1640 prior to use.

TABLE 34

Effect of adsorbing the rabbit anti-mouse C3 antiserum with mouse spleen cells. Primary response to horse RBCs

Cell Fraction[1]	Antigen	Anti-C3 or Control	IgM Plaques per Culture on Day 5
CR^+	-	-	48
"	+	-	1582
"	+	adsorbed normal rabbit serum	1584
"	+	adsorbed rabbit anti-Mo C3	174
CR^-	-	-	17
"	+	-	290
"	+	adsorbed normal rabbit serum	253
"	+	adsorbed rabbit anti-Mo C3	263

[1] All cultures supplemented with macrophages + TRF.

TABLE 35

Lack of cytotoxicity of anti-C3 antisera for mouse spleen cell fractions

Cell Fraction	Reaction Mixture[a]	Cell Viability
CR^+	Medium alone	86%
CR^+	Medium + Complement[b]	85%
CR^+	Medium + Rabbit anti-MoC3[c]	86%
CR^+	Medium + Goat anti-MoC3F(ab')$_2$[d]	87%
CR^+	Medium + Rabbit anti-MoC3 + C'	84%
CR^+	Medium + Goat anti-MoC3 + C'	86%
CR^-	Medium alone	84%
CR^-	Medium + Complement	85%
CR^-	Medium + Rabbit anti-MoC3	85%
CR^-	Medium + Goat anti-MoC3	89%
CR^-	Medium + Rabbit anti-MoC3 + C'	84%
CR^-	Medium + Goat anti-MoC3 + C'	87%

[a] Culture medium + antiserum incubated 90 min 37°C

[b] Lowtox rabbit complement 1:5

[c] 25 ul of undiluted rabbit anti-MoC3

[d] 25 ul of goat anti-MoC3F(ab')$_2$ 1:5

TABLE 36

The effect of goat anti-mouse C3 $F(ab')_2$ on the secondary response of CR^+ and CR^- cell fractions to horse RBCs

Cell Fraction*	Ag	Serum Added	Ab Class	PPC on Day 5
CR^+	-	-	IgM	30
CR^+	-	-	"	580
CR^+	+	normal goat[a]	"	535
CR^+	+	goat anti-C3[b]	"	65
CR^+	-	-	IgG	56
CR^+	+	-	"	1690
CR^+	+	normal goat	"	1720
CR^+	+	goat anti-C3	"	1650
CR^-	-	-	IgM	4
CR^-	+	-	"	31
CR^-	+	normal goat	"	22
CR^-	+	goat anti-C3	"	26
CR^-	-	-	IgG	19
CR^-	+	-	"	960
CR^-	+	normal goat	"	920
CR^-	+	goat anti-C3	"	942

* All cultures supplemented with macrophages + TRF.

[a] 25 ul normal goat serum

[b] 25 ul anti-mouse C3 $F(ab')_2$

TABLE 37

Effect of removing anti-mouse C3 by washing the cultures at varying times.

Culture Contents at 0 time[a]	5-day IgM plaques of cultures washed at: 24hr	48hr	72hr	96hr	"Rescue" after[b] 96 hr wash: Buffer	MoC3[c]
Medium only	N.D.	14	10	15		
HRBC	1232	1180	1268	1166	1104	2301
HRBC + Normal Rabbit Serum	1200	1126	1229	1191		
HRBC + Normal Goat Serum	1199	1147	1094	1155		
HRBC + Rabbit anti-MoC3	255	270	247	309		374
HRBC + Goat anti-MoC3 1:5	321	312	313	296		344
HRBC + Goat anti-MoC3 1:10	338	347	335	329		365

[a] All cultures contained T cell-depleted spleen cells plus macrophages + TRF.

[b] Cultures were washed 2 times with culture medium and then resuspended in fresh medium plus HRBC.

[c] Mouse C3 was added to some cultures after 96 hr wash.

TABLE 38

The effect of removing anti-mouse C3 by washing at short intervals after culture initiation, and adding mouse C3

Culture Contents at Time 0[a]	Culture treatment	IgM PPC on Day 5
Medium only	-	91
HRBC	-	1550
HRBC + Buffer[b]	-	1551
HRBC + MoC3	-	3078
HRBC + Buffer (added at 24 hr)	-	1541
HRBC + MoC3 (added at 24 hr)	-	3177
HRBC + normal goat serum	-	1609
HRBC + " " " + buffer	-	1577
" + " " " + MoC3	-	3281
" + goat anti-mouse C3F(ab')$_2$	-	366
HRBC + normal goat serum	wash after 30 min	1383
" " " "	" " " " + MoC3	2613
" " " "	wash after 3 hr	1565
" " " "	" " " " + MoC3	2645
" " " "	wash after 12 hr	1560
" " " "	" " " " + MoC3	3169
" " " "	wash after 24 hr	1497
" " " "	" " " " + MoC3	2453
HRBC + goat anti-mouse C3F(ab')$_2$	wash after 30 min	1397
" " " "	" " " " + MoC3	3309
" " " "	wash after 3 hr	1397
" " " "	" " " " + MoC3	3149
" " " "	wash after 12 hr	673
" " " "	" " " " + MoC3	1505
" " " "	wash after 24 hr	298
" " " "	" " " " + MoC3	313

[a] All cultures contained T cell-depleted spleen cells + macrophages + TRF.

[b] gelatin-veronal buffer (control for C3)

TABLE 39

Correlation of C3 secretion by cultured cells with the negative effect of anti-mouse C3 on the primary IgM response to horse RBCs

EXPERIMENT A:

Titration of mouse C3 in macro culture supernates

Time after culture initiation	% Lysis
1 hr	21%
3 hr	24%
6 hr	27%
12 hr	30%
18 hr	39%
24 hr	48%

EXPERIMENT B:

Removal of anti-C3[a] by washing after varying intervals of culture.

Contents at 0 time[b]	Culture Treatment	PFC Day 5
Medium only	-	6
HRBC	-	950
HRBC + anti-C3[a]	wash after 1 hr	970
HRBC + anti-C3	wash after 3 hr	1020
HRBC + anti-C3	wash after 6 hr	945
HRBC + anti-C3	wash after 12 hr	345
HRBC + anti-C3	wash after 24 hr	120
HRBC + MoC3-> 1 hr + anti-C3 -> 2 hr	wash after 3 hr	920
HRBC + 24 hr supernate -> 1 hr + anti-C3 -> 2 hr	wash after 3 hr	145

[a] Goat anti-mouse $C3F(ab')_2$

[b] All cultures contain T cell-depleted mouse spleen cells + macrophages + TRF.

TABLE 40

The effect of antigen concentration on the primary IgM response to HRBCs, with or without added mouse C3.

Cell Fraction	Amount of Antigen	Mouse C3 Added	PPC Day 5[a]
T cell-depleted spleen	none	-	10
" " " "	normal (=5 x 10^4 cells)	-	956
" " " "	10X normal	-	1026
" " " "	50X normal	-	486
CR^+	none	-	10
"	normal	-	2078
"	10X normal	-	2094
"	50X normal	-	1988
CR^-	none	-	8
"	normal	-	308
"	10X normal	-	32
"	50X normal	-	36
$C3bR^+$ + $C3bC3dR^+$	none	-	33
" "	normal	-	355
" "	1/10 normal	-	284
$C3dR^+$ + $C3bC3dR^+$	none	-	8
" "	normal	-	1026
" "	1/10 normal	-	886
CR^+	none	-	4
"	1/10 normal	-	840
"	1/10 normal	+	1590
"	normal	-	960
"	normal	+	1840
"	10X normal	-	1050
"	10X normal	+	1980

[a] All cultures supplemented with macrophages + TRF.

TABLE 41

Mouse C3 can not replace TRF in the primary IgM response of T cell-depleted spleen cells to horse RBCs

TRF	Antigen	Mouse C3	Plaques per culture on Day 5*
-	+	-	9
+	+	-	822
+	+	+	1643
-	+	+	12

* All cultures supplemented with macrophages.

TABLE 42

Kinetics of the polyclonal mitogen response to lipopolysaccharide

	stimulation index on day:			
Cell Fraction*	1	2	3	4
Whole spleen	9	41	81	10
T cell-depleted spleen	3	38	220	24
CR^+	36	70	186	20
CR^-	4	29	8	2
$C3bR^+$ (pos. selected)	14	84	30	17
$C3bR^+ + CR^-$ (neg. selected)	3	39	14	8
$C3dR^+$ (pos. selected)	17	132	311	62
$C3dR^+ + CR^-$ (neg. selected)	12	79	37	29

* All cultures supplemented with macrophages and 10 ug LPS/culture.

TABLE 43

Polyclonal antibody response of different cell fractions to LPS

Cell Fraction	Mitogen*	**IgM plaques per culture on day:	
		3	4
Whole spleen	LPS	60	28
T cell-depleted spleen	LPS	62	280
CR^+	LPS	63	308
CR^-	LPS	22	14
$C3bR^+$ (pos. selected)	LPS	62	172
$C3bR^+ + CR^-$ (neg. selected)	LPS	40	84
$C3dR^+$ (pos. selected)	LPS	36	394
$C3dR^+ + CR^-$ (neg. selected)	LPS	23	229

* Macrophages and 10 ug of mitogen added per culture.

** IgM plaques on fluorscein-SRBC plates.

TABLE 44

Effect of mouse C3 on the polyclonal mitogen response of various cell fractions to LPS

Cell Fraction	LPS	Mouse C3	Corrected CPM
T cell-depleted spleen	-	-	702
" " " "	+	-	50,398
" " " "	+	C3 buffer control	46,398
" " " "	+	+	19,955
CR^+	-	-	2,200
"	-	C3 control buffer	6,398
"	-	+	3,384
"	+	-	51,350
"	+	C3 buffer control	54,490
"	+	+	48,635
CR^-	-	-	784
"	+	-	13,881
"	+	C3 buffer control	10,527
"	+	+	11,330

TABLE 45

Effect of mouse C3 on the polyclonal antibody response of various cell fractions to LPS

Cell Fraction	LPS	Mouse C3	IgM PPC on Day 5
T cell-depleted spleen	-	-	6
" " " "	+	-	616
" " " "	+	C3 buffer control	577
" " " "	+	+	595
CR^+	-	-	105
"	+	-	1992
"	+	C3 buffer control	1996
"	+	+	1004
CR^-	-	-	59
"	+	-	649
"	+	C3 buffer control	829
"	+	+	711

TABLE 46

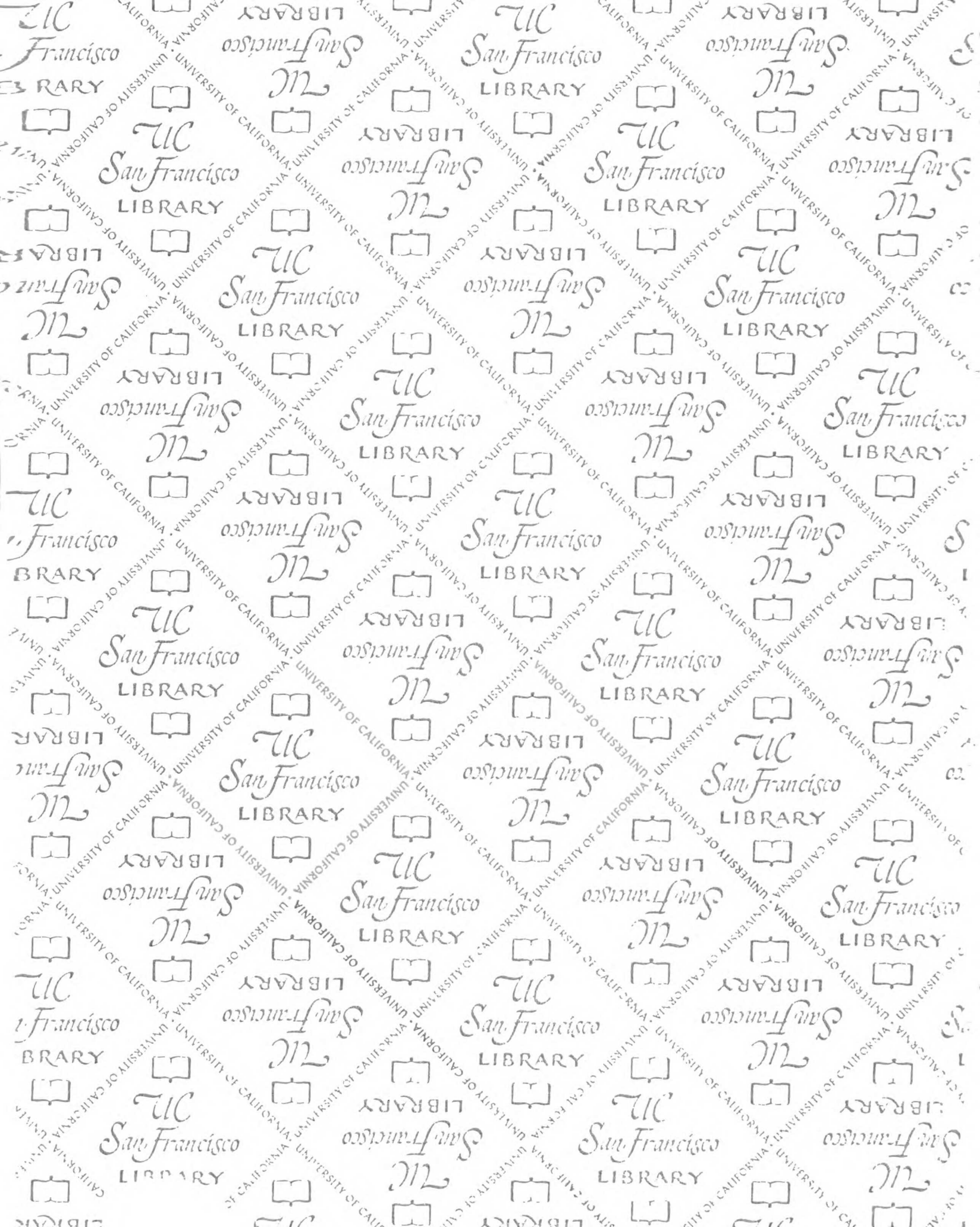
Effect of goat anti-mouse C3 on the polyclonal mitogen response of various cell fractions

Cell Fraction	LPS	Culture Addition	Stimulation Index
CR^+	+	-	138
"	+	normal goat serum	76
"	+	goat anti-MoC3F(ab')$_2$	90
CR^-	+	-	7.9
"	+	normal goat serum	5.4
"	+	goat anti-MoC3F(ab')$_2$	5.3

TABLE 47

T-independent response of various cell fractions to DNP-FICOLL

Cell Fraction	Mouse C3	IgM anti-DNP plaques per culture on Day 4
T cell-depleted spleen	-	57
CR^+	-	37
CR^-	-	12
$C3bR^+$ (pos. selected)	-	79
$C3bR^+$ (pos. selected)	+	70
$C3bR^+ + CR^-$ (neg. selected)	-	33
$C3dR^+$ (pos. selected)	-	1
$C3dR^+$ (pos. selected)	+	5
$C3dR^+ + CR^-$ (neg. selected)	-	4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