DEVELOPMENT OF C3 RECEPTORS ON NORMAL AND MEMORY MARROW CELLS AND CORRELATION OF RECEPTOR APPEARANCE WITH ANTIBODY SYNTHESIS

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ABSTRACT

DEVELOPMENT OF C3 RECEPTORS ON NORMAL AND MEMORY MARROW CELLS AND CORRELATION OF RECEPTOR APPEARANCE WITH ANTIBODY SYNTHESIS

By

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Primed and unprimed bone marrow cells were adoptively transferred into lethally irradiated syngeneic mice. The spleens were tested for the development of complement receptor lymphocytes (CRL) using the erythrocyte-antibody-complement (EAC) rosette assay in the presence and absence of antigen challenge.

When normal bone marrow was transferred into mice in the absence of antigen, there was little regeneration of splenic CRL for the first 20 days, but their number rose to near normal levels between days 20 and 25. This same pattern of CRL generation was observed in mice receiving antigen primed cells, but not exposed to further antigen stimulation. When mice were reconstituted with normal bone marrow and then given antigen 3 days prior to assay, compared with non-antigen primed mice, the number of CRL was significantly higher 20 days after reconstitution. Moreover, when mice were reconstituted with antigen prior to assay, development of receptor-bearing cells was initiated sooner.

Even when mice were given a challenging dose of antigen, the results obtained with 1 month memory cells were similar to those with normal bone marrow cells in the presence and absence of antigen stimulation. Cells from animals primed 2 months before transfer gave rise to a greater number of CRL than did cells primed for only 1 month and fewer CRL than those of 3 or 5 month memory cells.

Development of cells bearing the C3 receptor was not influenced in mice that had been thymectomized before irradiation and reconstitution or given thymocytes in addition to normal bone marrow cells.

Characterization of the function of the C3 receptor has not been conclusive. In these studies, differentiating spleen cells in mice irradiated and reconstituted with normal bone marrow were examined in an attempt to correlate development of complement receptor lymphocytes with generation of antibody forming capabilities. Results from previously reported kinetics studies which showed rapid development of splenic CRL between 20 and 25 days after bone marrow reconstitution were used as the basis of this comparison. In adoptive transfer-limiting dilution studies, an inoculum containing 1000-fold more cells was required to deliver one precursor plaque forming cell in 67% of the murine recipients when spleen cells were collected nine days after reconstitution compared with those collected 30 days after adoptive transfer. However, individual precursor cells were equally immunocompetent (with respect to the number of cells required to produce a positive IgM, IgG or IgA plaque response) 20 and 25 days after bone marrow cells were adoptively transferred, even though the proportion of CRL

in the spleens of irradiated, reconstituted animals was much higher 25 days after reconstitution.

Although other investigators have found that C3 receptors can be removed by trypsin treatment or blocked by anti-C4, these treatments did not alter the *in vitro* plaque forming capabilities of spleen cells.

Either limiting dilutions or set concentrations of normal and long term B memory bone marrow cells were CRL depleted using BSA gradients and were transplanted into irradiated recipient mice in combination with non-limiting numbers of thymocytes (required for antibody production) followed by injection with SRBC. Plaque assays demonstrated that CRL depletion of normal bone marrow cells slightly increased the number of cells required to give at least one precursor B lymphocyte needed to yield a clone of antibody-forming cells. Removal of CRL from memory bone marrow cells lowers the number of cells required for a positive plaque response in addition to reducing increased PFC numbers found during the memory response.

In conclusion, these studies indicate that antigen stimulation and induction of bone marrow memory cells alter CRL formation. The C3 receptor does not appear to be involved in regulation of the quantity or class of antibody production in a normal plaque response. However, it probably is involved in the activities of long term B memory cells of the bone marrow.

DEVELOPMENT OF C3 RECEPTORS ON NORMAL AND MEMORY MARROW CELLS AND CORRELATION OF RECEPTOR APPEARANCE WITH ANTIBODY SYNTHESIS

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LIST OF ABBREVIATIONS

Ab	antibody
Ag	antigen
AgAb	antigen-antibody complex
AgAbC	antigen-antibody-complement complex
BCF	C57BL/10 x C3H/He
C	complement
cer	ceramide
CLL	chronic lymphocytic leukemia
CRL	complement receptor lymphocytes
CTX	monocyte chemotactic factors
CVF	cobra venom factor
DNP_5BSA	$dinitrophenol_5$ bovine serum albumin
EA	erythrocyte-antibody complex
EAC	erythrocyte-antibody-complement complex
Fab anti-mouse Ig	papain fragment of rabbit antibodies to mouse Ig
FITC	fluorescein isothiocyanate
Gal	galactosyl
GalNAc	N-acetylgalactosaminyl
Glc	glucosyl
G_{D1a}	Gal(NANA)-GalNAc-Gal(NaNa)-Glc-cer
G_{D2}	Gal-NAc-Gal(NANA-NANA)-Glc-cer

G_{M1}	Gal-GalNAc-Gal(NANA)-Glc-cer
H-LA	histocompatability complex of man
H-2	histocompatability complex of the mouse
Ig	immunoglobulin
Ig^{+}_{-} Cr^{+}_{-}	cells with or without immunoglobulin and/or C3 receptors
IP	intraperitoneally
IV	intravenously
MEM	minimal essential medium
MF	mitogenic lymphokines
NANA	N-acetylneuraminyl
PBA	polyclonal B cell activators
PBS	phosphate-buffered saline
PFC	plaque forming cells
PMN	polymorphonuclear leukocytes
PPD	purified protein derivative
SRBC	sheep red blood cells
VGB	veronal-buffered saline

INTRODUCTION

Bone marrow derived lymphocytes (B cells) possess receptors for the third component of complement. Since the detection of C3 receptors, investigators have studied them in an attempt to elucidate their probable role in activation of B cells. Most of these studies have been aimed at relating the C3 receptor to humoral immunity, although some authors have considered the possibility that it may function elsewhere.

The literature review has been organized to provide background information on the discovery of the C3 receptor as well as reports related to its function. This was done to facilitate objective consideration of C3 receptor involvement in humoral immunity through evidence reported in the literature and that presented herein.

Studies included in this dissertation were stimulated by initial investigations which demonstrated rapid C3 receptor development following a prolonged incubation period. Initial experiments examined the ability of external factors to alter receptor development. These were followed by studies comparing the plaque forming capabilities of developing cells before and after the appearance of the C3 receptor.

Other brief studies concerning the function of this receptor consider 1) the *in vitro* role of the C3 receptor in plaque formation, 2) the possible interaction of the receptor with θ antigen, and

3) pilot studies using another approach to consider the possibility of the C3 receptor being involved in the long term B memory response.

Research results have been presented in the form of two papers to be submitted for publication (Articles 1 and 2) and three appendices (Appendices A, B and C).

LITERATURE REVIEW

Receptor Discovery

Initial investigations concerning complement receptors were studies of erythrocyte immune adherence receptors (Nishioka et al., 1963). Uhr and Phillips (1967) then presented evidence suggesting that complement receptors were present on lymphocytes. They reported that antigen-antibody-complement complexes (AgAbC) had the ability to interact with certain populations of lymphocytes, and that participation of complement in this reaction was critical (Uhr, 1966; Uhr and Phillips, 1967). Subsequent studies by Lay and Nussenzweig (1968) using C5 deficient complement for rosette formation limited receptor activity to the first four complement components. These authors also described some properties of the erythrocyte-antibody complement (EAC) rosette assay for complement receptors, such as temperature dependence of rosette formation and ability of CRL rosettes to maintain themselves in the absence of Mg^{++} (Lay and Nussenzweig, 1968). EAC complexes, EAC4 and EAC43, prepared with purified human components were used to demonstrate the specificity of this receptor (Bianco et al., 1970). Although rosette formation was minimal with EAC4, when EAC43 were used, normal levels of rosettes were observed. This was interpreted as an indication that receptor specificity was for the third component of complement.

Presence on B Lymphocytes

Arguments in favor of the proposal that CRL and non CRL constitute two distinct cell populations were presented by Bianco et al. (1970). Briefly, the points of his paper were as follows: 1) In the presence of normal mouse serum, CRL bind to nylon wool and lymphocytes from the thymus do not. 2) CRL and non CRL have different densities; CRL are found in the less dense portions of a BSA gradient. 3) CRL and Ig bearing cells are either the same population of cells or the CRL are a subpopulation because: (a) depletion of the CRL population also produces decreased numbers of Ig bearing cells and (b) the two populations occur with the same frequencies in various lymphoid organs. 4) The distribution of CRL in various lymphoid organs and tissues is distinctly different from the distribution of non CRL. 5) Neither Ig receptors nor C3 receptors are present on plasmacytes. 6) The pattern of localization of CRL among the lymphoid organs is not random.

Dukor et al. (1970, 1971) showed that EAC would adhere differentially to frozen sections of mouse lymphoid organs depending upon the number of CRL present. Erythrocytes sensitized with complement failed to bind to "thymus-dependent" areas of peripheral lymphoid organs, or to the thymus, but bound "to the follicular areas and the marginal zone of the spleen and in the true cortex of lymph nodes" (Dukor et al., 1970). In another investigation, these authors demonstrated that thymectomized, irradiated, bone marrow reconstituted mice could regenerate CRL in their lymph nodes (Dukor et al., 1971). Bianco and Nussenzweig used preferential depletion of either theta-bearing cells or CRL and discovered that

remaining numbers of cells corresponded closely with those theoretically expected if all of the cells remaining were of the other cell type (Bianco and Nussenzweig, 1971).

In conclusion, it is generally accepted that CRL are B cells or a subpopulation of B lymphocytes. Although the same population of cells is found to possess Ig, Fc and C3 receptors, these markers are located on separate molecules (Bianco and Nussenzweig, 1971; Abrahamsohn et al., 1974; Möller, 1974; Parish and Hayward, 1974a,b). This was determined by several different methods including double labeling (Möller, 1974) and differential capping (Parish and Hayward, 1974a).

Parish and Hayward (1974b) also reported that a small population of CRL exists which lacks surface Ig and that a substantial population of Ig bearing cells exists that does not have C3 receptors. Since their reports, others have also observed $Ig^{+}Cr^{-}$ and $Ig^{-}Cr^{+}$ cells (Ross and Polley, 1975). Fc and C3 populations may overlap somewhat (Parish and Hayward, 1974b); however, B cell mitogens have been demonstrated to selectively activate lymphocytes which differ with regard to the type of receptor which they carry (Möller, 1974).

Presence on Macrophages

Early on, it was recognized that macrophages, polymorphonuclear leukocytes (PMN) and monocytes also possess receptors for C3 (Nelson, 1965; Huber et al., 1968; Lay and Nussenzweig, 1968), but that these receptors were different from the C3 receptors found on B lymphocytes. Absence of Mg^{++} from the system causes macrophage EAC rosettes to dissociate but does not affect lymphocyte EAC rosettes

(Lay and Nussenzweig, 1968). Also, anti-plasma cell serum does not affect the macrophage, PMN or monocyte C3 receptor, but does affect lymph node cells bearing a C3 receptor (Nariuchi and Matuhasi, 1974).

Assays for the C3 Receptor

The EAC Rosette Assay

The EAC rosette assay was the first method used for C3 receptor detection (Lay and Nussenzweig, 1968; Bianco et al., 1970). In most systems indicator cells were prepared with purified components because incubation of erythrocyte-antibody complexes (EA) with whole complement causes lysis of the red blood cells (Bianco et al., 1970; Eisen, 1973). Mouse complement, however, does not lyse red blood cells and need not be purified before use (Bianco et al., 1970; Eisen, 1973). In the EAC rosette assay, the activated C3 on the surface of erythrocytes sensitized with antibody and complement, interacts with the C3 receptors on the surface of CRL through multiple attachments with long cytoplasmic projections (Chen et al., 1972), resulting in rosette formation. This assay has been modified for use in observation of two receptor types (i.e., Fc and C3) (Möller, 1974). SRBC were conjugated with fluorescein isothiocyanate (FITC) prior to preparation of EAC or EA and cells bearing specific receptors could be distinguished depending upon positive or negative fluorescence of these rosettes (Möller, 1974).

Zymosan-C3 Complexes

Zymosan-C3 complexes have been used in an assay that is very similar to the EAC rosette assay. Here, however, zymosan rather

than antigen-antibody complexes activates complement (Eisen, 1973). Zymosan-C3 rosettes and E rosettes can be counted in the same preparation, making it easier to control for T cell rosettes (Mendes et al., 1974).

Indirect Immunofluorescent Staining

Indirect immunofluorescent staining techniques have been developed to observe C3 receptors on human lymphocytes (Theofilopoulos et al., 1974a,b). In this assay lymphocytes are incubated with normal human serum, washed, and incubated with anti-C3 that had been conjugated with FITC. They used radioiodinated C3 and C3b to directly demonstrate binding to C3 receptors (Theofilopoulos et al., 1974a,b).

Immunoadherence Assay

Immunoadherence assay results have been found to correlate with rosette formation and can be substituted for the rosette assay when only qualitative results are required (Dierich et al., 1974).

Specificity of the C3 Receptor

Complement Specificities

When C3 receptors were demonstrated on B lymphocytes, they were originally thought to be specific for C3b (Bianco et al., 1970). It was soon realized that two receptors were present on most lymphocytes (Ross et al., 1973; Okada and Nishioka, 1973; Dierich et al., 1974), one for C3b and another for C3b inactivator-cleaved C3b (C3d) (Ross et al., 1973; Ross and Polley, 1975). The C3b receptor which is antigenically similar to the immune adherence receptor found on

erythrocytes was found to be totally independent of the C3d receptor (Dierich et al., 1974; Ross and Polley, 1975).

At times it has been suggested that there might be a receptor on lymphocytes specific for C4 (Ross and Polley, 1975; Cooper, 1969; Bokisch and Sobel, 1974). However, it has been shown that this reactivity is due to a cross reaction with that part of the C3b receptor specific for C3c, and that only two kinds of complement receptors are found on B lymphocytes, those for C3b and C3d (Ross and Polley, 1975).

C3b and C3d receptors can be specifically measured through the use of EAC1-3b and EAC1-3d in which the SRBC-antibody preparations were reacted with purified complement components (Ross and Polley, 1975). Although purified mouse complement components are not available, mouse EAC specific for either C3b or C3d can be prepared by varying the length of time that EA are incubated with the normal mouse serum. Ten minute incubations produce primarily EAC1-3b whereas 30 minute incubations, as used in most studies published to date, produce primarily EAC1-3d (Bianco, 1975). This is due to the very potent C3b inactivator present in mouse serum (Dierich et al., 1974). Their work suggests that all studies in the mouse system have probably been primarily investigations of the C3d receptor.

Species Specificity

The presence of two receptors for C3 renders results concerning species specificity more difficult to interpret. The C3d receptor seems to be relatively species independent with respect to all mammals tested (Bianco et al., 1970; Dierich et al., 1974).

However, the interaction between ^{human}EAC1-3d and mouse lymphocytes is rather weak (Dierich et al., 1974), and ^{mouse}EAC (which are actually ^{mouse}EAC1-3d) do not form rosettes with chicken lymphocytes (Bianco et al., 1970). With respect to the C3b receptor, ^{human}EAC43 adhere to rabbit, guinea pig and human lymphocytes, but not to mouse lymphocytes (Bianco et al., 1970; Dierich et al., 1974).

Binding of AgAbC Complexes to Receptors

AgAbC complexes bind to B cells only via the C3 receptors (Theofilopoulos et al., 1974). Adherence of cellular complexes to these receptors is dependent upon their ability to aggregate into small patches (Dierich and Reisfeld, 1975). It has been suggested that microaggregation of these receptor sites may be required for activation of receptors.

Function of the C3 Receptor in Humoral Immunity

Follicular Localization

Bianco and co-workers (1970) found that they could enrich a population of cells for plaque forming cells (PFC) by depleting them of complement receptor lymphocytes, indicating that C3 receptors are probably not present on antibody-producing cells. This information along with the observation that CRL could bind antigen-antibody-complement complexes led to the hypothesis that C3 receptors might be involved in follicular localization of the B lymphocyte that would eventually differentiate into plasma cells (Brown et al., 1970; Dukor et al., 1970; Hanna et al., 1971). Bianco et al. (1971) described follicular localization as "a mechanism designed to

concentrate antigenic material in strategic regions of lymphoid organs, in their pathway for the induction or maintenance of immunity" and postulated that both antigen localization and lymphocyte accumulation in the follicles of lymphoid organs might involve complement and the C3 receptor.

A Non-Clonal Binding, Clonal Selection Hypothesis of Antibody Formation

In 1973, other members of this group (Eden et al., 1973) presented evidence that soluble immune complexes, ^{125}I BSA-anti mouse BSA-mouse complement (AgAbC), could bind to B lymphocytes via a different receptor (presumably the C3 receptor) than that responsible for binding of antigen-antibody (AgAb) complexes. They also pointed out that soluble immune complexes would not only bind at 37°C but that significant binding would also occur at 0°C (Eden et al., 1973) to this same C3 receptor (Nussenzweig et al., 1973). These complexes could be released from the cell by means of a complement-dependent mechanism involving the alternate complement pathway (Miller, G. W., et al., 1973) or by addition of excess antigen or papain fragments of rabbit antibodies to mouse Ig (Fab anti-mouse Ig) (Nussenzweig et al., 1973). Based on this finding, it was believed that complement might serve as a regulator of interactions between immune complexes and cell membranes (Miller and Nussenzweig, 1974). Nussenzweig proposed that this C3 receptor might be more directly involved in initiation of the immune response. This hypothesis, for interaction between lymphocytes and soluble immune complexes, was a "non-clonal binding, clonal selection hypothesis of antibody formation." In it, Nussenzweig et al. (1973)

proposed that AgAbC complexes interacted nonspecifically with C3 receptors of all B cells which would then produce multiple and "nonrelevant" antibodies of specificites predetermined for each cell. Cells could then be induced to divide by a second specific signal provided by interaction with antigen (within the complex) to membrane-bound antibody on the surface of the cell.

The C3 Receptor as the Second Signal for B Cell Activation

The background information provided by Nussenzweig's group and a publication by Pepys (1972) suggesting that the C3 receptor might play a role in antigen presentation prompted Dukor and Hartmann to publish a hypothesis outlining what they thought might be happening with respect to the role of the C3 receptor in humoral immunity (Dukor and Hartmann, 1973). Their model is similar to that proposed by Nussenzweig et al. (1973) in that it also proposes a two signal mechanism for B cell activation in which the C3 receptor is responsible for nonspecific activation of these cells. The primary differences, however, are: 1) Dukor and Hartmann see antigen as the first, "specific" signal and C3 (not necessarily in an immune complex) as the second signal. 2) In Nussenzweig's model, all cells activated via the first signal (interaction with the C3 receptor) produce antibody and undergo blast transformation only when stimulated with Ag, whereas in Dukor and Hartmann's model, cells do not produce antibody unless the second signal has been activated. They extend their hypothesis to include mechanisms of activation of this receptor with both T-dependent and T-independent antigens. They point out that many polyclonal B cell activators (PBA) and T-independent

antigens can cleave C3 and that this might in fact be what makes them T-independent. T-dependent antigens might in some way interact with T cells and cause them to either directly or indirectly cleave C3 and activate the "second signal."

C3 Receptors on Plasma Cells

If C3 receptors are responsible for activation of B lymphocytes, then plaque forming cells might be expected to possess C3 receptors. Both Bianco et al. (1970) and Parish and Hayward (1974b) used CRL depletion to demonstrate the absence of C3 receptors on 19S PFC. Ramasay and Williams (1975), however, developed a sensitive assay using both SRBC and fowl erythrocytes and determined that a small population of direct PFC does possess C3 receptors. This suggests that although most 19S PFC do not have C3 receptors, they may have had them and lost them as they differentiated, since they no longer required interaction with C3. 7S plasma cells do maintain the C3 receptors on their surface, however (Parish and Hayward, 1974b).

Testing the Hypothesis

Pepys and Feldmann (Pepys, 1972; Feldmann and Pepys, 1973; Pepys, 1974) utilized Cobra Venom Factor (CVF) and other C3 reactive agents to suggest the dependence of T-dependent antigens on C3. However, they were unable to demonstrate any dependence of T-independent antigens or B cell mitogens (Janossy et al., 1973) on complement.

Parish and Hayward (1974c) depleted rat thoracic duct lymphocytes of various lymphocyte populations to examine functions displayed by these cells and found that the $Ig^{+}Cr^{+}$ (immunoglobulin receptor^{+} C3 receptor^{+}) cells were required for a 7S plaque response. They could

not make a conclusion about the 19S plaque response because cells from the irradiated hosts produced 19S antibody.

These studies are provided as a basis for the belief that in some instances (for T-dependent or IgM plaque responses), C3 is necessary for activation of an antibody response. Support for evidence which precludes cleavage of C3 as the mechanism by which T-independent antigens activate B cells is provided by several investigators who have demonstrated B cell mitogens and T-independent antigens that do not activate C3. Some of these are hyaluronic acid, polyglutamic acid, DNP_5BSA (Pryjma et al., 1974), LPS from *Salmonella* mR345 (Janossy et al., 1973) and DNP-Ficoll (Moisier et al., 1974). The action of pneumococcal polysaccharide (SIII) is controversial since Pryjma et al. (1974) report its inability to cleave C3 and Dukor et al. (1974) find the opposite. Other investigators also reported that cell mediated or mitogen-triggered plaque responses required the presence of C3 (Bitter-Suermann et al., 1973; Dukor et al., 1974). In addition to this, they proposed that T-independent antigens and mitogens could serve as bypass activators and allow production of antibodies against T-dependent antigens in the absence of T cells. It is interesting to note that these authors not only found this to be the case, but found that CVF would act as a bypass activator of the C3 receptor and was both a B and T cell mitogen. Although both these authors and Pepys and Feldmann use their evidence in partial support of the hypothesis presented by Dukor and Hartmann (1973), it is difficult to reconcile these two sets of results since such different observations were made with

respect to the action of CVF, and since their conclusions depend so heavily on that issue.

Hartmann and Bokish (1975) provided evidence that interaction of lymphocytes with isolated C3b caused stimulation of DNA synthesis and blast transformation and views presented in a review article by Hartmann (1975) indicated that in light of the information that was available at that time he did not feel compelled to change his hypothesis.

Most recently, Waldmann and Lachmann (1975) have reported that CVF has no effect on the response *in vitro* of cells cultured in media that is serum free or contains human or fetal calf serum. When normal mouse serum was treated with CVF, however, results similar to those observed by Pepys and Feldmann (Pepys, 1972; Pepys, 1974; Feldmann and Pepys, 1974) were obtained. Rather convincing experiments were carried out in an attempt to demonstrate that this effect was due to contamination of CVF with phospholipase A and show that purified CVF, although anti-complementary, did not alter the plaque response *in vitro*.

They also performed several other experiments (anti-C3 treatment of cells, addition of C3b-inactivator, etc.), all of which demonstrated a lack of involvement of C3 or the C3 receptor in activation of an immune response.

The "One Non-Specific Signal" Hypothesis of B Cell Activation

Möller and Coutinho disagree with the two signal model of B cell activation (Coutinho and Möller, 1975). They propose a "one non-specific signal" hypothesis and they do not believe that Ig

receptors serve as that specific signal for B cell activation (Möller and Coutinho, 1975; Möller, 1975; Coutinho, 1975). Evidence presented by these investigators indicates that neither Fc nor C3 receptors alone or in combination serve this function, but all three B cell receptors (Ig, Fc and C3) possess passive focusing function (Möller and Coutinho, 1975).

Other Possible Functions of the C3 Receptor

Lymphokine Synthesis by B Cells

The C3 receptor has been linked to the production of monocyte chemotactic (CTX) and mitogenic (MF) lymphokines (Wahl et al., 1974; Mackler et al., 1974). This lymphokine production appears to be induced by cross linking of Fc, C3 and/or Ig receptors (Wahl et al., 1974).

Cytotoxicity

Cells involved in antibody mediated cytotoxicity apparently possess C3 receptors (van Boxel et al., 1972, 1973; Perlmann and Perlmann, 1970; Möller and Suchag, 1972; Formen and Möller, 1973). Recently it has also been reported that these cells may be involved in a non-thymus derived cell cytotoxic effector mechanism which is independent of antibody (O'Neill et al., 1975). This is contradictory to results reported by Perlmann et al. (1975), who find no cytotoxicity with CRL in the absence of antibody.

These results can be explained if C3 is involved in the mechanism responsible for triggering Ab mediated cytotoxicity. If

this is the case, then O'Neill et al. (1975) may have activated this mechanism by substituting zymosan-C complexes for AgAbC complexes.

The C3 Receptor as a Diagnostic Tool

Although the lymphocytes of most patients with chronic lymphocytic leukemia (CLL) do not have Ig receptors on their surfaces, in many cases they have been found to possess C3 receptors (Michlmayr and Huber, 1970; West and Herberman, 1974). The ability of these human hematopoietic cells to form EAC rosettes has provided a useful method for diagnosing some of these leukemias as being of B lymphocyte origin (Papenhausen et al., 1975).

C3 Receptors on Human Lymphoblastoid Cell Lines

Characteristics of the C3 receptor can be easily studied with human lymphoblastoid cell lines. The one that has been most extensively studied is the Raji line derived from a Burkitt lymphoma (Bokisch and Theofilopoulos, 1973; Theofilopoulos et al., 1974a). Various cell lines seem to differ in the types of receptors that they have. Several combinations were observed and results varied depending upon whether they were obtained with EAC or soluble immune complexes (Theofilopoulos et al., 1974b).

Genetic Control of C3 Receptor Expression

Although Dierich et al. (1974) reported that expression of the C3 receptor does not appear to be related to H-2 or HL-A phenotypes or antigenic determinants of β2-microglobulin, Gelfand and collaborators provide compelling evidence to the contrary (1974a,b). They found that development of CRL varies between mouse strains. Depending

upon the relative rate of this development, they labeled strains as either "low CRL" or "high CRL" strains. They also reported that the relative rate of appearance during normal development is similar to that in irradiated mice which have been reconstituted with syngeneic bone marrow (1974a). This same group then continued these studies to determine that the rate of appearance of CRL seems to be under the control of two independent genes, one of which is linked to the mouse H-2 complex (Gelfand et al., 1974b).

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DEVELOPMENT OF C3 RECEPTORS ON B LYMPHOCYTES DERIVED FROM NORMAL AND MEMORY MARROW CELLS

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Abbreviations used in this paper: EA, erythrocyte-antibody complex; EAC, erythrocyte-antibody-complement complex; C, complement; CRL, complement receptor lymphocyte; SRBC, sheep red blood cells; BCF_1 mice, C57BL/10 x C3H/He mice; MEM, minimal essential medium; PBS, phosphate-buffered saline; VGB, veronal-buffered saline; IV, intravenously; IP, intraperitoneally; PFC, plaque forming cells.

ABSTRACT

Primed and unprimed bone marrow cells were adoptively transferred into lethally irradiated syngeneic mice. The spleens were tested for the development of complement receptor lymphocytes (CRL) using the erythrocyte-antibody-complement (EAC) rosette assay in the presence and absence of antigen challenge.

When normal bone marrow was transferred into mice in the absence of antigen, there was little regeneration of splenic CRL for the first 20 days, but their number rose to near normal levels between days 20 and 25. This same pattern of CRL generation was observed in mice receiving antigen primed cells, but not exposed to further antigen stimulation. When mice were reconstituted with normal bone marrow and then given antigen 3 days prior to assay, compared with non-Ag primed mice, the number of CRL was significantly higher 20 days after reconstitution. Moreover, when mice were reconstituted with antigen prior to assay, development of receptor-bearing cells was initiated sooner.

Even when mice were given a challenging dose of antigen, the results obtained with 1 month memory cells were similar to those with normal bone marrow cells in the presence and absence of antigen

stimulation. Cells from animals primed 2 months before transfer gave rise to a greater number of CRL than did cells primed for only 1 month and fewer CRL than those of 3 or 5 month memory cells.

Development of cells bearing the C3 receptor was not influenced in mice that had been thymectomized before irradiation and reconstitution or given thymocytes in addition to normal bone marrow cells.

INTRODUCTION

Many B lymphocytes bear a membrane receptor for the third component of complement (1). Mechanisms of B cell activation have been proposed which involve either passive or active participation of the C3 receptor. Bianco et al. (2) suggested that the receptor could passively act as an antigen concentrating mechanism. Dukor and Hartmann (3) hypothesized that activation of the C3 receptor could be a "second signal" required to turn on a humoral immune response. More recent reports (4,5) suggest that this might be the case for T-dependent antigens, but may not hold for T-independent antigens. This potential role of the C3 receptor in B lymphocyte activation has recently been used as justification of studies related to receptor development (6). If binding of cleaved C3 to the receptor is involved in B cell memory, C3 receptors would probably be generated more rapidly on cells that have been antigen primed.

In the present study, bone marrow cells were used in adoptive transfer studies to examine the production of complement receptor lymphocytes on differentiating B cells. Extremely long-lived memory cells are produced when mice are antigen-primed, as described by

Miller and Cudkowicz (7). Long term B memory cells of the marrow not only produced higher numbers of antigen specific B cells, but also generated clones of plasma cell progeny that had as great as three times the normal burst size (7,8,9). This memory differs from shorter term B lymphocyte memory found in other organs. We now extend these observations by studying C3 receptor development on precursor B lymphocytes from antigen-primed donor mice. Complement receptor lymphocytes arose earlier when using antigen-primed bone marrow cells as the source of long term B memory cells than when using normal bone marrow cells. Thymectomy or T cell reconstitution of recipients did not alter the development of the C3 receptor.

MATERIALS AND METHODS

Animals. C57BL/10 x C3H/He (BCF_1) female mice were from Cumberland View Farms, Clinton, TN, or from Health Research Labs, West Seneca, NY.

Irradiation. Ten- to twelve-week-old mice received 900 rads of whole body irradiation from the ^{60}Co γ-irradiation source in the Department of Food Science at Michigan State University. The animals were rested for at least four hours before reconstitution.

Thymectomy. Four-week-old mice were thymectomized according to the methods of Miller (10). They were allowed to rest for at least one month prior to irradiation. When thymectomized mice were killed, the mediastinum was examined macroscopically for the presence of thymus remnants. No such remnants were found.

Antigen priming and challenge. Long term memory bone marrow cells were collected from mice primed intravenously with sheep erythrocytes prior to use (7). Mice were primed for 1 to 5 months depending upon the stage of memory differentiation to be tested in the various experiments. In addition, certain groups of experimental mice were antigen stimulated with sheep erythrocytes administered intravenously three days before their spleen cells were assayed.

Cell suspensions. Bone marrow cells from tibias and femurs of 10- to 12-week-old normal or antigen primed mice were gently aspirated in Eagle's minimum essential medium (MEM) in Hanks' salts with a syringe and a 25 ga needle. Cells were then passed through a 27 ga needle to produce a dispersed cell suspension.

Thymuses from 6- to 8-week-old mice were teased with wide-tipped forceps. Thymocytes were then dispersed using needles of progressively finer (21 to 27) gauge. For assay, recipient spleens were suspended in a similar fashion.

All cell suspensions were washed once by centrifugation (170 x g) in MEM and were then diluted to the appropriate concentrations to be injected. Mice received various combinations of 1×10^6 bone marrow cells/ml, 5×10^7 thymocytes/ml and 5×10^8 SRBC/ml.

Injections. One milliliter volumes of all cell suspensions were injected intravenously (IV) into lateral tail veins, using 27 ga needles. Fifty units of heparin were given intraperitoneally (IP) from 5 to 20 min prior to IV injection of thymocytes.

Preparation of EA and EAC. Indicator cells were prepared according to the methods of Bianco et al. (1). Sheep red blood cells (SRBC) washed three times in phosphate buffered saline (PBS) were incubated for 30 min at 37°C with 19S goat antibody to the Forsmann antigen. These cells were then washed three more times in PBS and half of the erythrocyte-antibody (EA) preparation was diluted to 1×10^8 cells/ml in MEM. The remaining cells were made up to a 5% suspension in veronal buffered saline (VGB) according to the methods of Rapp and Borsos (11). This EA suspension was then diluted in equal volumes of 10% serum from normal mice in VGB, and incubated for 30 min at 37°C. Mouse serum was collected and maintained on ice and either used within the hour or frozen at -20°C and used within the week. These EAC were then washed three times in VGB and resuspended to 1×10^8 cells/ml in MEM.

Rosette formation. Equal volumes of EA and EAC (1×10^8) and spleen cell suspension (2×10^6) were mixed and rotated on a Bellco Glass rocker platform at approximately 30 rpm for 30 min at 37°C. After incubation, tubes containing rosette suspensions were placed on ice and all were counted within four hours.

Counting rosettes. Lymphocytes surrounded by two or more erythrocytes were counted as rosettes. Cells from each spleen were incubated separately with EA and EAC. The difference between the percent of EA and EAC rosettes per spleen was obtained in order to account for rosettes due to possible cellular interactions with erythrocytes or antibody. This control was required because rosette formation varied slightly between individual animals. Indicator preparations were

also examined prior to use in order to be certain that EA or EAC clumps were not recorded as false rosettes. Approximately 600 lymphocytes were counted for each spleen assayed and the number of rosettes was recorded.

Calculations and statistics. The mean percentage of CRL of at least four normal spleens was determined on each assay day. CRL levels ranged between 20 and 30% for normal animals as influenced by variations in individual EA and EAC preparations. The proportion of the normal CRL level in the spleens of experimental mice were standardized daily with the mean normal value.

The Student's t test was used, with t values of $p<0.05$ being considered significant.

RESULTS

Irradiated recipient mice were reconstituted with precursor complement receptor lymphocytes from the bone marrow (12). Since normal levels of bone marrow CRL in the mouse reportedly range from 5 to 8% (12,13). Initial experiments included the use of CRL depleted (1) bone marrow cells. Although CRL levels in bone marrow were observed at expected levels, splenic CRL development from depleted bone marrow was not noticeably different from that with complete bone marrow (unpublished results) and this technique was not continued.

BCF_1 mice lethally irradiated and reconstituted with normal bone marrow cells but not challenged with antigen developed complement receptor-bearing cells between days 10 and 20 (Figure 1). A marked increase from 22 to 90% CRL occurred between days 19 and 25.

Figure 1. Percent of the normal CRL level in spleens of mice that (a) have been irradiated and reconstituted with 1×10^6 normal bone marrow cells or (b) have been irradiated and not reconstituted. Brackets indicate standard errors. In (a), an average of 13 mice are represented for each point of the graph. In (b), an average of 5 mice are represented for each point. Fewer mice per group resulted because 50% of those mice irradiated and not reconstituted died before they could be assayed.

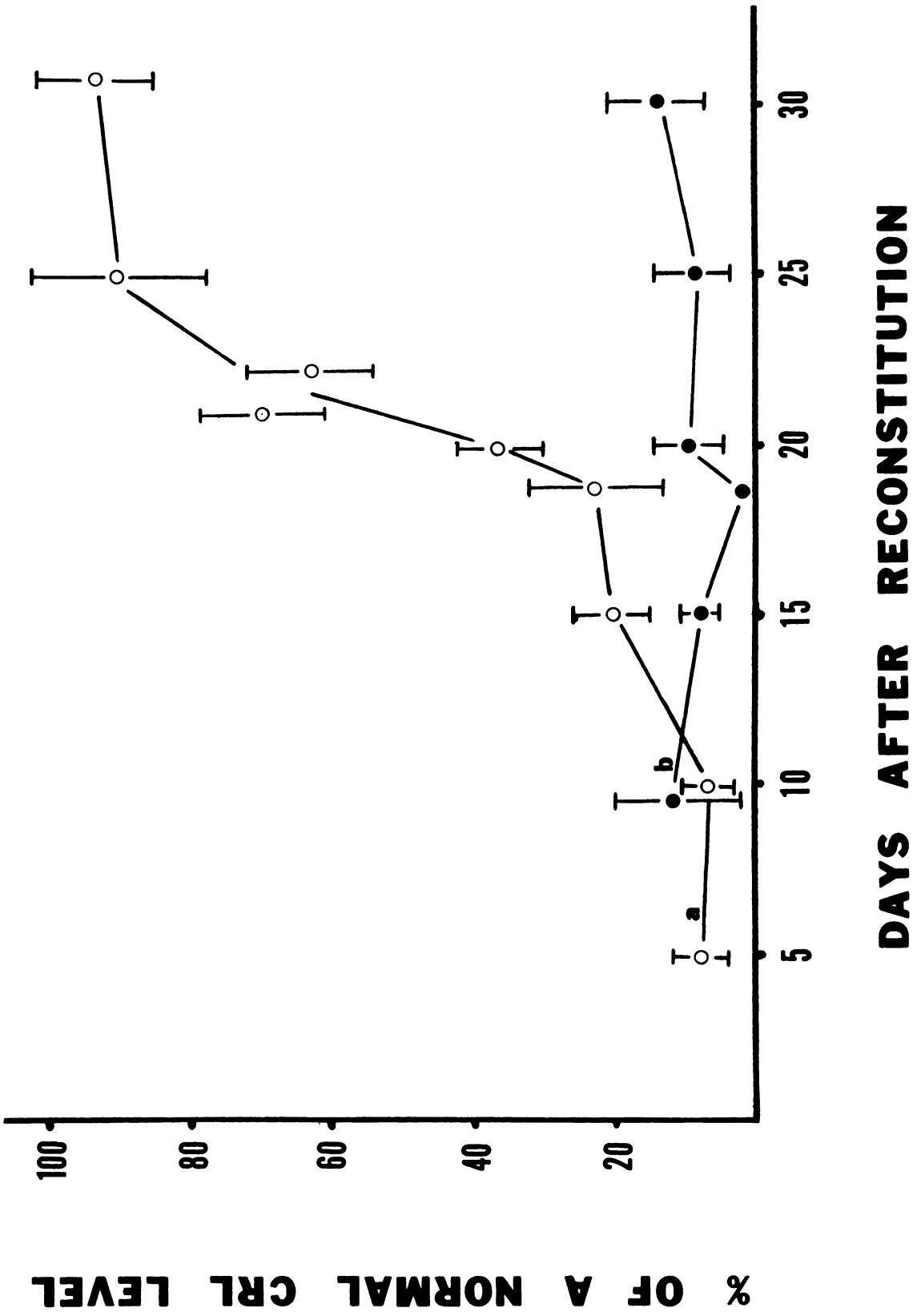
% OF A NORMAL CRL LEVEL
100
80
60
40
20
DAYS AFTER RECONSTITUTION
5
10
15
20
25
30
a
b

Figure 1

As a control, mice were irradiated and not reconstituted (Figure 1). Fifty percent of unreconstituted mice died before they could be assayed; most of these died prior to day 15. Assay of the CRL levels in the spleens of surviving mice indicated that cells bearing complement receptors did not develop in the absence of bone marrow cells in the time interval tested. Animals given memory bone marrow from mice primed with SRBC, but not stimulated with antigen before assay, showed a similar pattern of CRL development (Table 1). In both cases increases significant to the 99.5% confidence level occurred between days 10 and 15, 15 and 20, and 20 and 25, with the increases bewteen days 20 and 25 being more than twice as great as those at earlier times.

When irradiated, reconstituted mice were given antigen prior to assay, increases of complement receptor-bearing cells in their spleens proceeded as before for 15 days (Figure 2). Large increases in the number of CRL occurred between 15 and 20 days, 5 days earlier than before. Differences between these results and those obtained without antigen stimulation were significantly different at the 99.5% confidence level on day 20.

If mice were given bone marrow cells from donors primed once with SRBC, three or five months prior to use, more CRL developed 15 days after reconstitution than if normal bone marrow cells had been given. However, CRL levels did not exceed those for normal mice on day 20 (Figure 2). CRL development of receptor-bearing cells from memory precursors was more linear and a significantly greater number of CRL were present on day 15. Nearly normal levels of CRL were reached by day 20 when cells were given a challenging antigen dose

Table 1. Proportion of the normal CRL level in spleens of mice that were irradiated and reconstituted with normal or memory bone marrow cells and not challenged with antigen before assay

Days after reconstitution	Normal bone marrow	5 month memory bone marrow
5	0.07 (5)* ± 0.04@	0.03 (6) ± 0.01
10	0.03 (5) ± 0.03	0.02 (4) ± 0.02
15	0.21 (20) ± 0.05	0.23 (5) ± 0.07
20	0.37 (22) ± 0.07	0.28 (12) ± 0.10
25	0.90 (18) ± 0.12	0.83 (12) ± 0.12
30	0.92 (10) ± 0.09	0.88 (5) ± 0.06

None of the differences between experimental and control groups were significant when tested with a Student's t test.

*Number of mice per group.

@Standard error.

Figure 2. Percent of the normal CRL level in spleens of mice that had been irradiated and reconstituted with (a) normal bone marrow, (b) normal bone marrow and challenged with 5 x 10^8 SRBC 3 days prior to assay, (c) 5 month memory cells and challenged with antigen 3 days prior to assay, or (d) 3 month memory cells and challenged with antigen prior to assay. Brackets indicate standard error. Except for those points with an asterisk (*), which represent 8 and 5 mice, respectively, each point represents from 15 to 25 mice. Differences between (a or b) and (c or d) on day 15 as well as differences between (a) and (b, c or d) on day 20 were significant to a $p<0.005$ when tested with a Student's t test. None of the differences between (c) and (d) on any given day were significant.

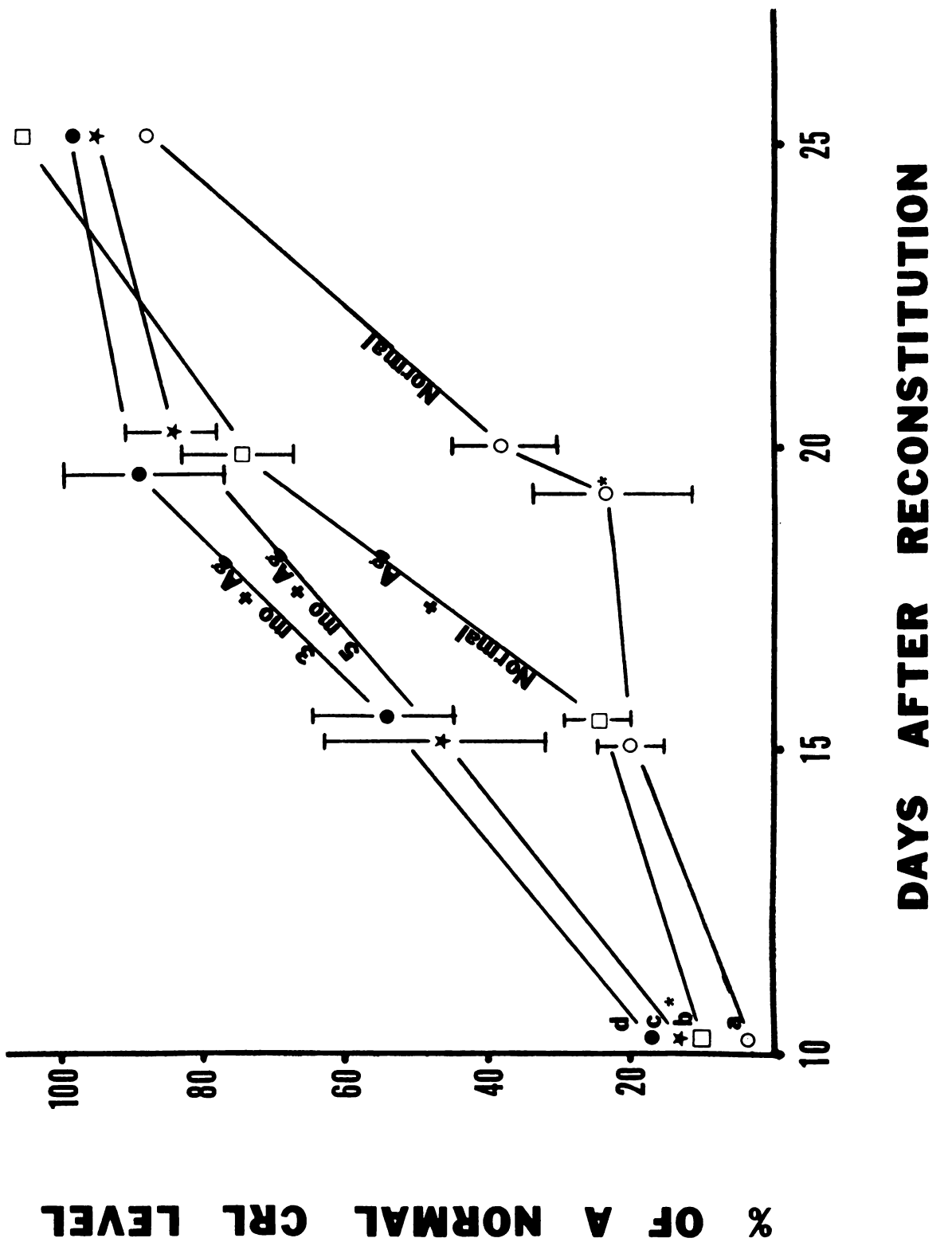
% OF A NORMAL CRL LEVEL
100
80
60
40
20
10
15
20
25
DAYS AFTER RECONSTITUTION
Normal
Normal + Δε
5 mo + Δε
3 mo + Δε
a
b
c
d

Figure 2

prior to assay, regardless of whether normal or memory cells were examined. Values for 3 and 5 month memory, plus antigen stimulation, were not significantly different.

When antigen was administered prior to assay, CRL development varied depending upon the stage of memory bone marrow cells used for adoptive transfer (Figure 3, A). An examination of day 15 results indicated no differences between CRL levels generated from 1 month memory cells than from normal bone marrow cells. Two month memory CRL levels were, however, almost as high as three month levels. Differences between numbers of receptor-bearing lymphocytes generated from 1 and 3 month memory bone marrow cells were significant when tested with a Student's t test.

When day 20 results were compared for various groups (Figure 3, B), mice given 1 month memory cells and stimulated with antigen 3 days prior to assay were found not to produce the rapid increase of receptor-bearing cells that had been observed with normal bone marrow cells. Although 2 month memory cells generated more CRL than 1 month memory cells on day 15, the number of CRL in the spleens of mice given 2 month memory did not change between days 15 and 20. Three and five month memory cells, however, did give rise to a higher number of splenic CRL. Differences between the percent of a normal CRL response for 1 and 2 month memory were not significant, although differences between 1 or 2 month memory and 3 or 5 month memory were significant at the 95% confidence level.

Two approaches were used in examination of the possible role of the thymocyte in CRL development. In the first set of experiments animals were thymectomized and allowed to rest for one month before

Figure 3. Percent of a normal CRL response when mice were irradiated and reconstituted either with normal bone marrow or various stages of memory bone marrow. In every case, 5×10^8 SRBC were given as antigen 3 days prior to assay. (A) Day 15 after reconstitution. (B) Day 20 after reconstitution. Brackets indicate standard error. In (A), values for normal bone marrow and 1 month bone marrow were significantly different than values for 3 and 5 month bone marrow. In (B), values for 1 and 2 month memory were significantly different when tested with a Student's t test from values for 3 and 5 month memory bone marrow cells.

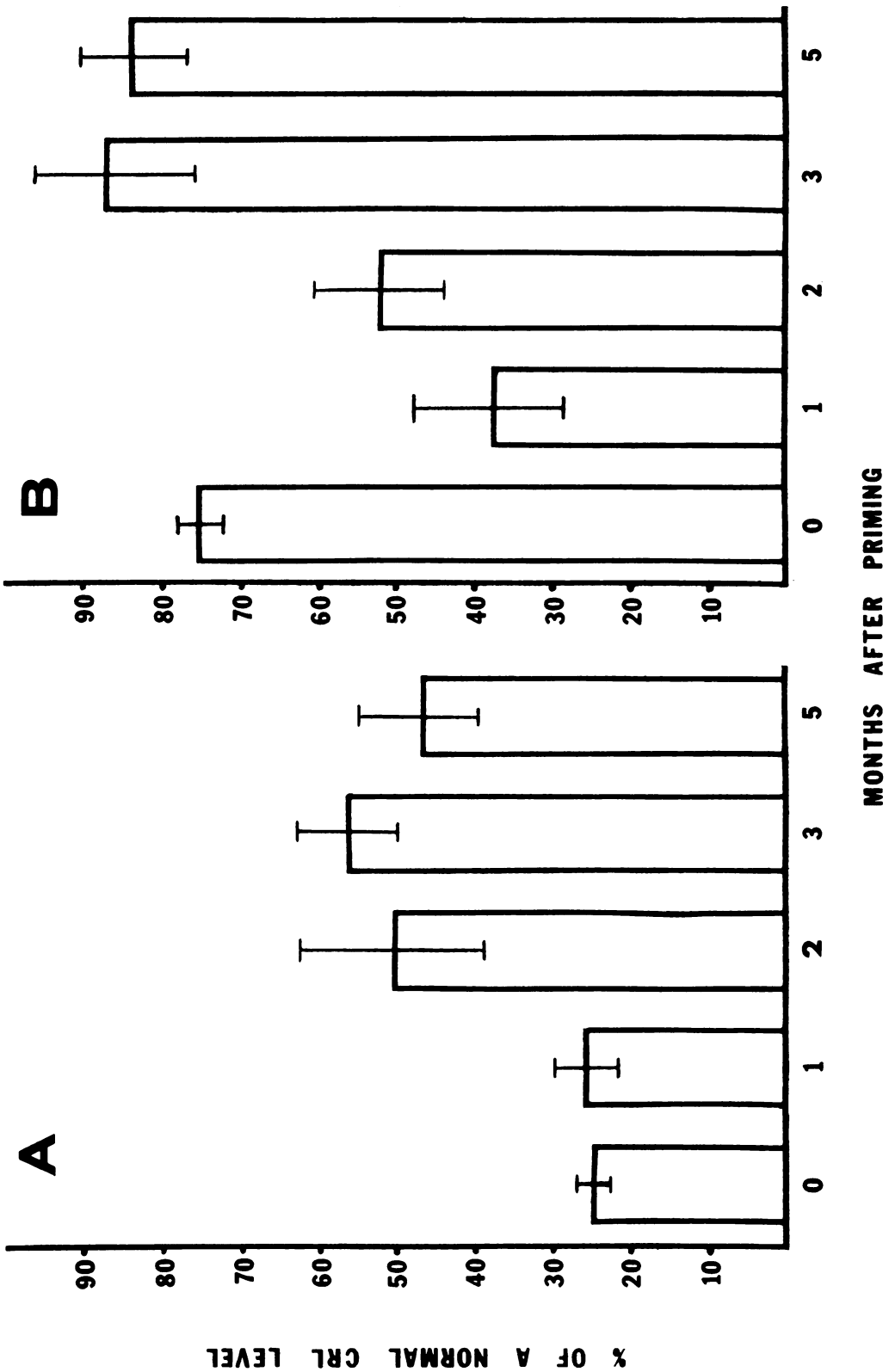
A
B
% OF A NORMAL CRL LEVEL
MONTHS AFTER PRIMING
0
1
2
3
5
10
20
30
40
50
60
70
80
90

Figure 3

they were irradiated and reconstituted with bone marrow cells. This protocol did not delay development of receptor-bearing cells (Table 2). CRL levels increased abruptly between days 20 and 25 as they had when normal mice had been used for transfer. Upon assay, thymectomized mice were autopsied for evidence of thymic regeneration; none was ever found. In the second set of experiments, groups of mice were then irradiated and given 5×10^7 thymocytes in addition to 1×10^6 bone marrow cells. Complement receptor lymphocytes did not appear earlier in the presence of thymocytes (Table 2) since the increase in CRL again occurred between days 20 and 25.

DISCUSSION

Delay of CRL development in irradiated (C57BL x C3H)F_1 mice given bone marrow and not stimulated with antigen would qualify these mice as a "low CRL" strain as defined by Gelfand et al. (6). Since the ability of various strains of mice to generate splenic CRL appears to be under genetic control, and C57BL/6 is defined as a "low CRL" strain (6), our results appear consistent with published observations, and indicate that C3H might also be a "low CRL" strain since a C57BL x C3H cross produces a "low CRL" strain.

It is interesting to compare CRL development from normal bone marrow with that of IgM, IgG, and IgA production capabilities published by van Muiswinket et al. (14). Although (C57BL x C3H)F_1 mice are not used in their studies, (DBA/2 x C57BL/Rij)F_1 female mice would be expected to produce similar CRL regeneration profiles since this mouse is also an F_1 progeny of two "low CRL" parents (6). They found that IgM-PFC are present immediately after transplantation.

Table 2. The role of the thymocyte in development of CRL from bone marrow cells injected into irradiated mice

Days after reconstitution	Proportion of the normal CRL level in spleens of mice	
	Thymectomized, irradiated and bone marrow reconstituted	Irradiated and reconstituted with bone marrow and thymocytes
10	---	0.08 ± .01* (8)@
15	---	0.22 ± .04 (18)
20	0.33 ± .05 (5)	0.28 ± .08 (10)
25	0.79 ± .17 (5)	0.74 ± .03 (12)
30	1.22 ± .17 (5)	---
35	1.16 ± .16 (5)	---

Both groups of mice were given 900 Rads of ^{60}C γ-irradiation and transplanted with 1 x 10^6 normal bone marrow cells. Mice in the left column were thymectomized prior to irradiation. Those in the right column received 5 x 10^7 thymocytes IV. Rosette assays were done as in other experiments. Thymectomized mice were autopsied for evidence of thymic regeneration and none was ever found.

*Standard error.

@Number of mice per group.

Our results indicate that CRL are absent or, at most, present in low levels at this time, indicating that the C3 receptor is not involved in IgM plaque formation. van Muiswinket et al. (14) report detection of IgG-B cells between 13 and 16 days after transplantation. This corresponds to the time when the number of splenic CRL of transplanted animals increases from less than 10% to slightly more than 20% of a normal CRL level. IgA-B cells are reported to arise 22 days after adoptive transfer. This corresponds to a time when nearly 70% of the normal number of CRL are present. Both the number of B cells as described by van Muiswinkel et al. (14) and the number of CRL have reached normal levels 30 days after bone marrow transfer.

The similarity between the time of appearance of IgG and IgA-B cells and generation of graded numbers of CRL suggests that as IgG or IgA specific B cells acquire C3 receptors, they obtain the ability to produce and secrete IgG or IgA antibody, respectively. It is also possible that complement receptors arise passively at these times due to generation of IgG and IgA PFC. CRL development in this case might not reflect an alteration in B cell function. In order to directly determine the role of C3 receptors in humoral immunity, experiments either removing C3 from the system (4,5) or blocking the receptor (15,16) are required. Experiments are under way to examine the ability of differentiating cells to produce an antibody response before and after generation of the C3 receptor.

Although CRL levels appear to be under genetic control, immunological factors such as antigen stimulation or adoptive transfer with memory cells are capable of influencing this development. When reconstituted animals are given antigen prior to splenic assay, CRL

in the spleen appear sooner, indicating that antigen challenge facilitates generation of CRL in the spleen. If these animals are reconstituted with long term B memory cells (8) and stimulated with antigen 3 days prior to assay, regeneration of CRL occurs more rapidly than when normal bone marrow is used for adoptive transfer. This indicates that the presence of C3 receptor-bearing cells at sites responsible for induction of antibody and generation of memory cells (17) may not be coincidental.

Experiments to determine which stages of long term B memory cells (9) induce earliest regeneration of CRL levels in the spleen suggest that 3 month memory cells had the greatest CRL generation capacity. Five month memory levels were not significantly different from 3 month levels, but were consistently lower, indicating a slight decline in memory capabilities. One month memory cells are not different from normal bone marrow when considered up to 15 days after reconstitution, and by 20 days they were less capable of CRL production when both groups of mice were given antigen prior to assay. One possible interpretation would be that one month after injection of mice with SRBC, those cells that respond to antigen have begun to differentiate into long term B memory cells. These cells had been committed and could no longer respond to antigen as normal bone marrow cells would have, but had not completed differentiation into long term B memory cells. Two months after antigen challenge, memory bone marrow cells had gained the ability to produce CRL earlier than normal bone marrow. However, the population of "normal" bone marrow had not yet been replenished and CRL levels did not continue to increase on day 20. This interpretation implies

that two populations of lymphocytes are involved in generating CRL. One population, found in normal bone marrow cells, produces an increasing CRL response 20 days after antigen priming. Another population, found in long term memory cells, is responsible for earlier generation of CRL. This early generation of CRL by memory cells seems to occur only upon antigen stimulation. A comparison of 5 month memory and normal bone marrow without antigen fails to demonstrate a difference in CRL development.

It is interesting to compare cells capable of early CRL generation to the sensitized marrow cells reported by Miller and Cudkowicz (9). This work suggests that if bone marrow cells are primed with antigen 2 months earlier, more cells are required to produce a positive response in 63% of the animals tested (according to Poisson statistics, the spleens at this point were seeded by one precursor unit) than when normal bone marrow cells were tested. Three month memory cells required only a fraction of the number of cells needed for a positive plaque response from normal marrow. By 3.5 months more cells were required than with marrow that had been primed 2 months earlier. Since we found that memory properties of CRL precursors (in the same strain of mice, as observed on day 15) of 2 and 5 month memory cells were not significantly different from 3 month memory cells, it would seem reasonable to propose that increased CRL producing capabilities occur before PFC memory. PFC memory appears to be shorter lived and declines more rapidly than memory properties of CRL precursors. Affinity studies are now in order in which we could examine the effect of removal of CRL from 5 month

memory cells on the affinity of antibody produced by the progeny plasma cells of these long term B memory lymphocytes.

For some antigens, T lymphocytes (18) or specific (19,20) or nonspecific (21) T cell products are needed for immunological triggering of B lymphocytes into metabolically active immunoglobulin secreting plasma cells. Knowledge of this requirement for T cell-B cell interaction coupled with cognizance of the ability of adoptively transferred bone marrow cells to replenish the thymus of irradiated mice when the thymus is left intact (22) led to the supposition that T cell interaction might also be required for development of CRL. To determine whether this might indeed be the case, mice were thymectomized before irradiation and reconstitution. This presumes that the environment required to cause stem cells to differentiate into thymocytes would be removed, and if a T cell-B cell interaction were required for CRL development, then receptor-bearing cells would arise more slowly in thymectomized, irradiated, and reconstituted mice. Our experimental results indicate that this is not the case, as CRL were detected at the same time in thymectomized mice as in normal mice which had undergone the same treatment. Although no evidence of thymic regeneration could be found when these mice were autopsied, as an additional assurance that T cell interaction was not required, other experiments were performed where thymocytes were added to bone marrow cells before adoptive transfer. This was done with the assumption that if T cells did affect the development of bone marrow cells into CRL that CRL would now arise more rapidly. CRL development, however, was not significantly different from that in thymectomized or normal mice reconstituted with bone marrow alone. These results

indicate a lack of involvement of the thymocyte in complement receptor lymphocyte development.

In conclusion, we have shown that various stages of long term B memory lymphocytes give rise to CRL more rapidly than unprimed bone marrow cells. This is only true when animals are challenged with antigen prior to assay and occurs regardless of the presence or absence of T lymphocytes. An understanding of the role of memory cells in CRL development may eventually lead to a mechanism for utilizing long term B memory. Delineation of C3 receptor development from precursor CRL of the bone marrow may also help to determine the role of the C3 receptor in immunity.

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COMPARATIVE ABILITY OF DIFFERENTIATING CELLS TO PRODUCE AN ANTIBODY RESPONSE BEFORE AND AFTER C3 RECEPTOR DEVELOPMENT

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Abbreviations used in this paper: CRL, complement receptor lymphocytes; C, complement; SRBC, sheep red blood cells; PBS, phosphate-buffered saline; MEM, Eagle's minimal essential medium; IV, intravenously; IP, intraperitoneally; PFC, plaque forming cells; EAC, erythrocyte-antibody-complement complex; EA, erythrocyte-antibody complex.

ABSTRACT

Characterization of the function of the C3 receptor has not been conclusive. In these studies, differentiating spleen cells in mice irradiated and reconstituted with normal bone marrow were examined in an attempt to correlate development of complement receptor lymphocytes (CRL) with generation of antibody forming capabilities. Results from previously reported kinetics studies which showed rapid development of splenic CRL between 20 and 25 days after bone marrow reconstitution were used as the basis of this comparison. In adoptive transfer-limiting dilution studies, an inoculum containing 1000-fold more cells was required to deliver one precursor plaque forming cell in 67% of the murine recipients when spleen cells were collected nine days after reconstitution compared with those collected 30 days after adoptive transfer. Individual precursor cells were equally immunocompetent (with respect to the number of cells required to produce a positive IgM, IgG or IgA plaque response) 20 and 25 days after bone marrow cells were adoptively transferred, even though the proportion of CRL in the spleens of irradiated, reconstituted animals was much higher 25 days after reconstitution.

INTRODUCTION

Controversy continues over the possible role of the C3 receptor as a second nonspecific signal required to activate antibody production by B lymphocytes as proposed by Dukor and Hartmann (1). Pepys and Feldmann (2,3,4) supported this hypothesis indicating that agents which destroy C3 do reduce T-dependent antibody production but do not alter T-independent responses. Although depletion of CRL from splenic lymphocytes did not alter the number of direct PFC (5,6,7), Parish and Hayword (5) reported that this lowers the indirect plaque response. Recent work suggested that a small population of those cells responsible for direct plaques does possess C3 receptors (8). Waldmann and Lachmann (9), however, have been unable to demonstrate a requirement for C3 in the response, and they dispute previously reported results indicating a role for the C3 receptor in humoral immunity. Our previous results also did not support any decrease in plaque responses observed when C3 receptors were removed from cells with trypsin (10) or blocked with anti-C4 (11) *in vitro* (12).

By examining the plaque forming capabilities of differentiating cells before and after development of complement receptors, the involvement of this receptor in humoral immunity can be assessed, minimizing the possibility that results might be altered by external factors. Our studies indicated that lymphocytes which possess only a small portion of the CRL normally present were fully immunocompetent with respect to IgM, IgG, and IgA plaque forming capabilities.

MATERIALS AND METHODS

Animals. C57BL/10 x C3H/He (BCF_1) female mice were from Cumberland View Farms, Clinton, TN, or from Health Research Labs, West Seneca, NY.

Irradiation. Ten- to 12-week-old mice received 900 Rads of whole body irradiation from the ^{60}Co γ-irradiation source in the Department of Food Science at Michigan State University. These animals were rested for at least four hours before reconstitution.

Antigen. Sheep red blood cells (SRBC) were obtained as a sterile suspension in Alsever's solution from the Williams Sheep Farm, Mason, MI. Cells were washed three times in phosphate-buffered saline (PBS) before use.

Cell suspensions. Bone marrow cells from tibias and femurs of 10- to 12-week-old mice were gently aspirated in Eagle's minimum essential medium (MEM) in Hanks' salts with a syringe and a 25 ga needle. Cells were then passed through a 27 ga needle to produce a single cell suspension.

Thymocytes were from 6- to 8-week-old mice. Spleens excised immediately prior to injection or assay were dissected free of fascia, teased with wide forceps, and cells were dispersed using needles of progressively finer (21 to 27) gauge.

All cell suspensions were washed once (170 X g) in MEM and then diluted to the appropriate concentrations for transplantation.

Injections. All cells were suspended in 1 ml for intravenous (IV) injection into lateral tail veins, using 27 ga needles. Fifty units

of heparin were given intraperitoneally (IP) from 5 to 20 min prior to IV injection of thymocytes.

In the first adoptive transfer, 10^6 cells/mouse were injected into lethally irradiated mice. No thymocytes or antigen were transplanted at this time. In the second adoptive transfer, varying numbers of spleen cells from these bone marrow reconstituted mice were administered along with 5×10^7 normal thymocytes to each mouse. SRBC (5×10^8) were injected on the following day.

EAC rosette assay. This assay was used to evaluate the percent of CRL in pooled spleens of bone marrow reconstituted animals used in the second adoptive transfer. Methods are essentially those of Bianco et al. (6) as modified by Baum and Miller (14).

Spleen cells from at least four mice were isolated in suspension for the second adoptive transfer. Cells were assayed before reconstitution to determine the proportion of a normal CRL level present. In every case CRL levels were similar to those reported previously (14), constituting approximately 30% of a normal population of receptor-bearing cells on day 20 and 90% of a normal level on day 25.

Assays for plaque-forming cells. The number of plaque-forming cells (PFC) in spleens of irradiated, reconstituted mice was determined by the Jerne hemolytic plaque assay, as modified for use with glass slides (15,16,17,18). Agarose (L. Industrie Biologique Francaise S. A., Gennevilliers, France, lot number 2321) was boiled for 30 min in glass distilled water, diluted to 0.5% in MEM with Hanks' salts, and maintained at 63°C in 0.4 ml aliquots. Erythrocytes, 30%, 0.05 ml, and spleen cell suspensions, 0.1 ml, were added to the agarose, mixed,

and poured onto microscope slides that had previously been dipped into 0.1% agarose and dried. After solidification, the slides were inverted and placed on special trays (16) for plaque development. Slides assaying for direct PFC (presumably releasing IgM antibody) were incubated for 1 hr at 37°C in a humid atmosphere containing 8% CO_2. Fresh frozen guinea pig complement (Grand Island Biological Co., Grand Island, NY) diluted 1:10 in MEM with glutamine (2 mM) and penicillin streptomycin (10^4 units) was added and incubation was continued for 2 to 3 hr. For facilitation of indirect PFC (presumably releasing either IgG or IgA antibody), the slides were incubated for 1 hr and goat anti-mouse IgG (7S) globulins (Meloy Labs, Springfield, VA, lot number 41353) or goat anti-mouse IgA (Meloy, as above, lot number 51714) antisera were added. The anti-IgG was used at dilutions that inhibited approximately 90% of the direct plaques and provided maximum development of indirect plaques. Anti-IgA did not inhibit direct plaques and was used at a dilution that provided development of maximum numbers of plaques. After another hour of incubation, the slides were transferred to a clean tray, diluted guinea pig complement was added, and incubation was continued for another 3 hr. Plaques were counted at 7X magnification with indirect fluorescent illumination.

Since the anti-IgG inhibited development of most but not all direct PFC, corrections were necessary to calculate the number of indirect PFC in each slide (total number of PFC minus noninhibited direct PFC). Correction factors were applied according to Wortis et al. (19). The number of anti-IgA plaques was calculated by subtracting the total number of direct plaques from indirect IgA

plaques as described by Walters and Jackson (20). PFC counts were obtained from duplicate slides.

RESULTS

The role of the C3 receptor in the plaque response of differentiating cells. CRL development in mice irradiated and given 10^6 normal bone marrow cells (previously presented [21] and Table 1) was used in order to evaluate plaque forming capabilities of these developing spleen cells. This was accomplished by transplanting limiting dilutions of differentiating spleen cells (from mice reconstituted with bone marrow cells 9, 20, 25, or 30 days prior to cell collection, in combination with 5×10^7 thymocytes, into irradiated recipients, followed in one day by inoculation of 5×10^8 SRBC (Figure 1).

Comparison of spleen cells from mice reconstituted 9 and 30 days after the initial transplantation displayed large differences in the number of cells required for a positive plaque response for both direct and indirect assays (Table 2). However, since many other differentiation events could have occurred in this interval, we could not be certain that this difference in plaque forming capabilities was due to the presence of complement receptors on approximately 86% more lymphocytes. In order to establish the role of the C3 receptor with respect to humoral immunity, the time interval between assays was shortened, localizing plaque formation to a period when CRL developed most rapidly (14).

Cells were collected from mice 20 and 25 days after they were transplanted with marrow cells. These times correspond with those when approximately 37 and 90% of normal complement receptor

Table 1. Percent of a normal CRL level present in spleens of mice after adoptive transfer of 10^6 bone marrow cells*

Days after reconstitution	Percent of normal CRL level
9	6.2 ± 1.3 (8)†
20	37.0 ± 6.5 (22)
25	90.0 ± 12.1 (18)
30	92.0 ± 9.1 (14)

*These results were previously reported (20).

†Number of mice per group.

@Standard error.

Figure 1. The experimental protocol used for double reconstitution experiments used to examine plaque forming capabilities of differentiating spleen cells.

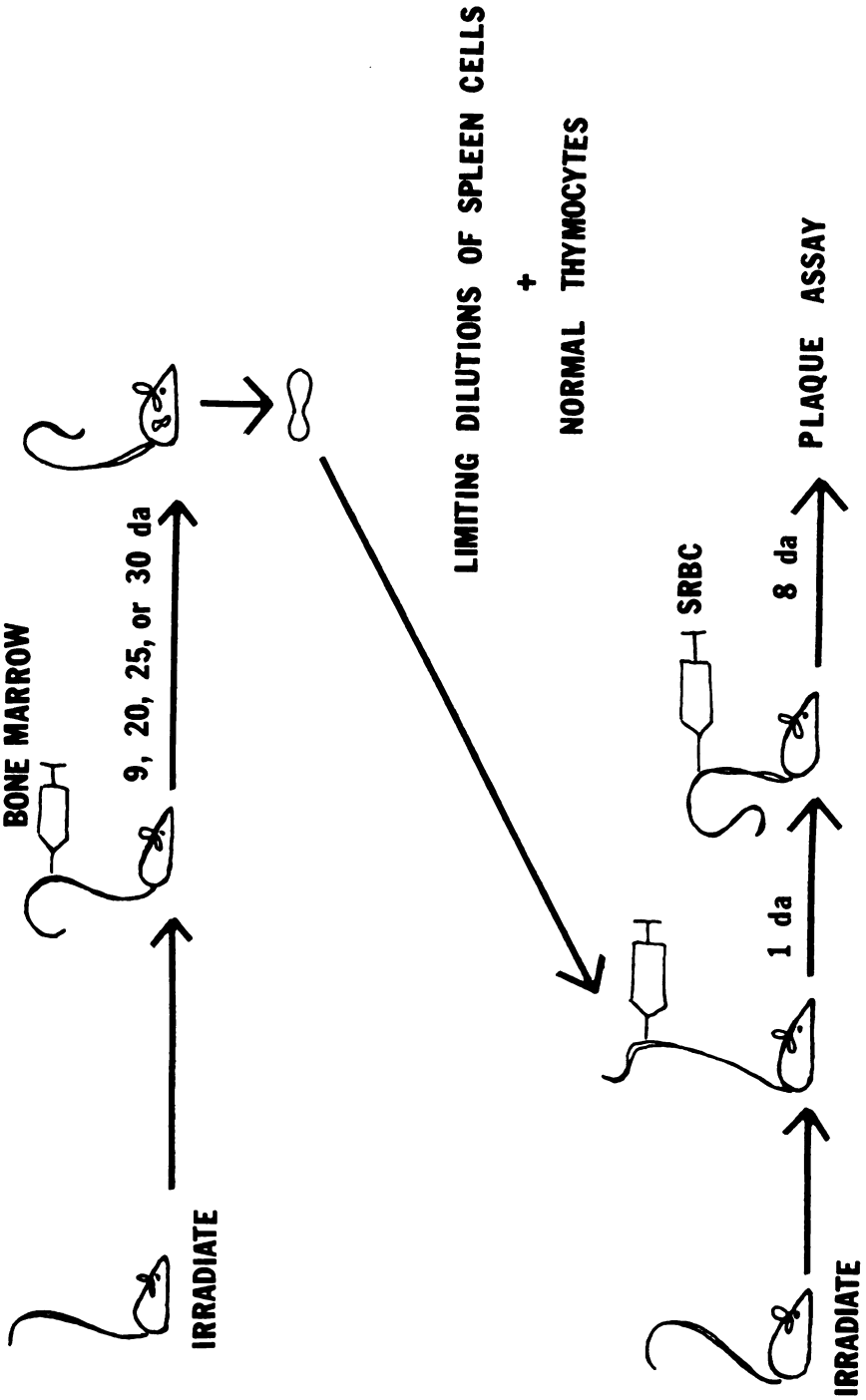
IRRADIATE
BONE MARROW
9, 20, 25, or 30 da
LIMITING DILUTIONS OF SPLEEN CELLS
+
NORMAL THYMOCYTES
IRRADIATE
1 da
SRBC
8 da
PLAQUE ASSAY

Figure 1

Table 2. The percent of recipient spleen cells displaying a positive PFC response to SRBC after reconstitution with 5 x 10^7 thymocytes, 5 x 10^8 SRBC, and graded numbers of spleen cells from bone marrow reconstituted mice. A comparison of day 9 to day 30

	Day 9		Day 30	
	Direct	Indirect	Direct	Indirect
$\geq 2.5 \times 10^6$	100(24)†	50*(24)	---	---
1.5×10^6	67(6)	0 (6)	---	---
1.0×10^6	43(7)	0 (7)	100(6)	17(6)
1.0×10^5	17(6)	0 (6)	95(20)	25(20)
$1.0\text{-}2.5 \times 10^4$	---	---	88(33)	27(26)
$5.0\text{-}6.3 \times 10^3$	---	---	80(20)	25(20)
1.0×10^3	---	---	29(7)	14(7)

*Not an average of all above; every dilution tested gave 50% positive spleens.

†Number of mice per group.

lymphocyte levels were present (Table 1). A sample of pooled spleen cells was assayed with the EAC rosette assay to be certain that they contained the expected proportion of C3 receptor-bearing cells prior to their use in limiting dilution assays. These double reconstitution experiments clearly demonstrate that the number of cells required to produce both direct and indirect (both IgG and IgA) plaques is not altered by the absence of more than half of the CRL normally present (Table 3).

DISCUSSION

The difference in the plaque forming capabilities of cells 9 and 30 days after bone marrow reconstitution can be explained in several ways. Unknown differentiation events occurring during this time period could be responsible for development of antibody forming capabilities; CRL development may not be complete enough and may be the required differentiation event. If CRL development is responsible for this difference, then total plaque forming capabilities must require more than 6% of a normal CRL level and less than the 37% present on day 20, since by day 20, although a normal number of CRL are expressed, sufficient numbers for total function are present.

In a previous study (12), attempts were made to compare the development of C3 receptors with IgM, IgG and IgA plaque forming capacities published by van Muiswinkel et al. (21). Since strains of mice used in both cases appeared to be "low CRL" strains (22), correlation of these results was feasible. In the strain of mice studied by van Muiswinkel et al., development of IgA antibodies occurs only after 22 days of differentiation (20). IgA plaque forming

Table 3. The percent of recipient spleen cells displaying a positive PFC response to SRBC after reconstitution with 5 x 10^7 thymocytes, 5 x 10^8 SRBC, and graded numbers of spleen cells from bone marrow reconstituted mice. A comparison of day 20 to day 25

Number of spleen cells	Percent of positive* spleens when 2nd reconstitution was 20 days after 1st			Percent of positive spleens when 2nd reconstitution was 25 days after 1st		
		Indirect			Indirect	
	Direct	IgG	IgA	Direct	IgG	IgA
$\geq 10^6$	100(12)@	100(12)	---	---	---	---
2.5 x $10^{5\dagger}$	100(22)	77(22)	---	---	---	---
2.5 x 10^4	100(21)	67(21)	88(9)	100(12)	50(12)	88(8)$^{\Delta}$
2.5 x 10^3	83(12)	33(12)	---	73(22)	55(22)	---
2.5 x 10^2	71(35)	40(35)	19(26)	53(34)	21(28)	18(28)
2.5 x 10^1	43(7)	29(7)	---	17(12)	15(13)	---

†These actually represent dilutions from 1-5 x 10^x except for

$^{\Delta}$which represents from 1 x 10^4 to 5 x 10^3.

@Numbers in parentheses indicate the number of mice per group.

*Recipient spleens with >200 direct and >100 indirect PFC were scored as positive.

These results consist of a summary of 4 separate experiments.

capabilities in BCF_1 mice, on the other hand, arise earlier, being as good 20 days after bone marrow reconstitution as 25 days after transplantation. Even though both strains of mice in question may well be "low CRL" strains, there is most probably some differentiation event other than C3 receptor development responsible for differences in temporal development of plaque forming capabilities.

In most attempts to evaluate the role of the C3 receptor in humoral immunity, C3 is either removed from the system (2,3,4) or cells bearing the receptor are depleted from the population to be tested (5,6,7). Neither of these methods provides a direct evaluation of the ability of the C3 receptor to function in humoral immunity. Although C3 may be capable of interacting with the receptor, this does not preclude the possibility of interaction with other molecules. It has also been suggested that blockage of C3 receptor function by some reagents used to remove C3 may be artifactual and not due to removal of C3 (9). Depletion of cells bearing C3 receptors could produce misleading results if antibody forming capabilities are a function of those cells removed that is unrelated to the presence of the receptor. Thus, the presence of C3 receptor on cells responsible for IgG production does not necessitate involvement of the C3 receptor in that function.

The method used for evaluation of C3 receptor function in this publication eliminates questions of inappropriate interference in the mechanism of antibody production, since cells are simply evaluated before and after receptor development. This method does not illustrate a difference in plaque forming capabilities of spleen cells when examined at intervals prior to and subsequent to the time of most

rapid CRL development. This suggests that either 1) C3 development is not required for humoral immunity or 2) the number of CRL present after 20 days of differentiation is sufficient.

Although quantities of antibody produced at these stages of CRL development are not different, it would be interesting to investigate the affinity properties of antibody arising from precursor cells during various stages of CRL differentiation.

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APPENDICES

APPENDIX A

INABILITY TO DEMONSTRATE A ROLE FOR THE C3 RECEPTOR IN THE ANTIBODY RESPONSE *IN VITRO*

ABSTRACT

Although other investigators have found that C3 receptors can be removed by trypsin treatment or blocked by anti-C4, these treatments did not alter the *in vitro* plaque forming capabilities of spleen cells.

INTRODUCTION

Incubation of spleen cells with appropriate concentrations of trypsin removes the C3 receptors and leaves the antibody receptors intact (Theofilopoulos et al., 1974; Lay and Nussenzweig, 1968; Parish and Hayward, 1974b). Anti-C4 also blocks EAC rosette formation (David, 1975). Even though other methods are used to investigate the function of the C3 receptor, neither of these reagents has been used in an attempt to examine the effects of blockage or removal of the receptor on its proposed function in the humoral response.

Contradictory findings have been reported regarding the role of the C3 receptor in immunity (Pepys, 1972; Pepys, 1974; Pepys and Feldmann, 1974; Möller and Coutinho, 1975; Waldmann and Lachmann, 1975; Dukor et al., 1974; Bitter-Suermann et al., 1973). Previous studies *in vitro* suggest that this receptor was probably not required for normal antibody production (Baum and Miller, 1976b). This

report substantiates that finding by demonstrating that substances which reportedly interfere with the C3 receptor do not alter the plaque forming capabilities of B lymphocytes.

MATERIALS AND METHODS

Spleen cell suspensions. Spleens were excised aseptically from 9- to 15-week-old (C57BL/10 x C3H/He)F_1 female mice. They were teased with wide forceps and cells were dispersed in Eagle's minimum essential medium (MEM) in Hanks' salts using needles of progressively finer (21 to 27) gauge.

Trypsin treatment. Spleen cell suspensions were incubated in a 0.01% trypsin solution in MEM for 15 min at 37°C, as described by Theofilopoulos et al. (1974). Cells were washed three times before culturing.

Anti-C4 treatment. Goat anti-human C4 (Meloy Labs, Springfield, VA) was added to spleen cell cultures in tenfold dilutions. Goat anti-rabbit IgG was used as a control.

Culture techniques. Spleen cells were cultured in Marbrook chambers as described by Marbrook (1967) and modified by Miller and Esselman (1975).

Plaque asssay. The plaque assay of spleen cells maintained in Marbrook chambers was performed as described by Jerne (1963) and modified by Miller and Cudkowicz (1970).

Antigen. Sheep red blood cells (SRBC) were obtained as a sterile suspension in Alsever's solution from the Williams Sheep Farm, Mason, MI. Cells were washed aseptically three times in sterile phosphate-buffered saline (PBS) before use.

EAC rosette assay. The EAC rosette assay for detection of CRL was performed according to the methods of Bianco et al. (1970) as modified by Baum and Miller (1976a).

RESULTS

Treatment of spleen cells from BCF_1 mice with 0.01% trypsin for 30 min at 37°C removed a sufficient number of C3 receptors from the surface of B lymphocytes to block CRL rosette formation with EAC 75% of the time. This was tested on three occasions. Although anti-C4 reportedly blocks C3 receptors, we could not substantiate this effect by interference with the EAC rosette assay. Regardless of this, the effects of anti-C4 on the plaque response *in vitro* were examined in order to determine whether this reagent might yet interact with the receptor in a fashion that would preclude plaque formation.

In separate experiments where spleen cells were removed by trypsin treatment, the *in vitro* plaque response was slightly though not significantly higher than that of untreated cultures. Unpublished *in vivo* experiments where irradiated mice were reconstituted with trypsin treated or normal spleen cells and given thymocytes and antigen also failed to demonstrate a reduced plaque response.

Cultures incubated with anti-C4 or a control antiserum at either 1/10 or 1/100 dilutions produced similar numbers of plaques.

Those incubated with anti-C4 again were slightly but not significantly higher than responses obtained from normal cultures or cultures incubated with a control antiserum.

DISCUSSION

Trypsin treatment did not totally eliminate EAC rosette forming cells; therefore, the small number of cells which maintained C3 receptors on their membranes could have been responsible for maintenance of function. It seems likely that if C3 receptors are required to turn on antibody forming cells, removal of enough receptors to prevent rosette formation in over 75% of the cells that normally bear them would also alter the direct plaque response. These results can only be discussed with respect to direct PFC since IgG PFC could not be detected in spleen cell cultures and may have been dependent upon CRL.

Inability to block the plaque response with anti-C4 may have resulted from incomplete blockage of the C3 receptor as evidenced by inability to block the EAC rosette assay. This could reflect the species specificity of the receptor, as goat anti-human C4 and mouse lymphocytes were used.

The antiserum control in these experiments was included due to the observation by Waldmann and Lachmann (1975) that anti-C3 blocked plaque formation only due to a nonspecific interaction of the Fc portion with the Fc receptors on B cells. Since anti-C4 did not block plaque formation, this was not a problem in our system.

In most attempts to evaluate the function of the C3 receptor, C3 is either removed from the system (Pepys, 1972, 1974; Feldmann

and Pepys, 1974) or cells bearing the C3 receptor are depleted from the population to be tested (Bianco et al., 1970; Parish and Hayward, 1974b). Neither of these methods provides a direct evaluation of the ability of the C3 receptor to function in humoral immunity. Use of stearoyl dextran to block C3 receptors (Möller and Coutinho, 1975) did not alter the plaque response *in vitro* of polyclonal B-cell activators (PBA) to lipopolysaccharide (LPS) or to purified protein derivative (PPD). Our attempts to remove the C3 receptor with trypsin and block its function with anti-C4 substantiate the findings of those who tried to illustrate the involvement or lack of involvement in the most direct fashion and are contradictory to those who believe that the C3 receptor is the "second signal" required for B cell activation.

Table A-1. The number of plaques in a direct Jerne plaque assay when spleen cells are treated with trypsin or incubated with anti-C4

	Number of plaques in direct Jerne plaque assay	
	Experiment I	Experiment II
Untreated	277 ± 39* (10)†	448 ± 36 (9)
Trypsin treated	365 ± 36 (10)	476 ± 39 (8)
1/10 anti-C4	---	478 ± 38 (4)
1/100 anti-C4	---	460 ± 47 (4)
1/10 control serum@	---	434 ± 18 (4)
1/100 control serum	---	438 ± 15 (4)

*Standard error.

†Number of cultures.

@Goat anti-rabbit IgG absorbed with normal spleen cells from BCF_1 mice.

APPENDIX B

INABILITY OF THY-1 (G_{M1} GANGLIOSIDE) TO BLOCK C3 RECEPTOR *IN VITRO*

ABSTRACT

Thy-1 (G_{M1} ganglioside), in the form of cholesterol-lecithin liposomes, when cultured with B lymphocytes, modulate their terminal differentiation. The addition of this ganglioside to spleen cell cultures did not inhibit the ability of these cells to form EAC rosettes.

INTRODUCTION

Miller and Esselman (1975) suggest that Θ antigen present on T lymphocytes is G_{M1} ganglioside, and that this glycolipid may modulate B cell function. In the presence of Thy-1 antigen, B lymphocytes become susceptible to lysis by anti-Thy 1.2 in the presence of complement (Miller and Esselman, unpublished results). Since the C3 receptor may also be involved in regulation of B cell function via one of the possible mechanisms (Dukor and Hartmann, 1973; Nussenzweig et al., 1973) previously suggested, it was proposed that Thy-1 might bind to B lymphocytes by interacting with the C3 receptor.

In order to test this, G_{M1} ganglioside and a control ganglioside found inactive with respect to B cell modulation (Miller and Esselman, 1975) were incubated with spleen cells. These gangliosides were incapable of blocking EAC rosette formation.

MATERIALS AND METHODS

Spleen cell suspensions. Spleen cells were collected as described in Appendix A.

Culture techniques. Spleen cells were cultured in Marbrook chambers as described by Marbrook (1967) and modified by Miller and Esselman (1975).

Ganglioside preparation. G_{M1}, G_{D2}, and G_{D1} ganglioside-cholesterol-lecithin mixtures were obtained from Dr. Walter Esselman, Michigan State University (Miller and Esselman, 1975). G_{D2} and G_{D1a} were used as controls. Prior to use, ganglioside mixtures were reconstituted with 1 ml of sterile 0.85% saline and were warmed and sonicated for 5 min in an ultrasonic cleaner (Mettler Electronics Corp., Anaheim, CA) to allow formation of liposomes. Each culture received 1 µg of ganglioside.

EAC rosette assay. The EAC rosette assay for detection of CRL was performed according to the methods of Bianco et al. (1970) as modified by Baum and Miller (1976a).

Antisera and cytotoxicity tests. Anti-Thy-1.2 (0.05 ml) (Bionetics, Kensington, MD) at a titer capable of killing 25% of the spleen cells was added to 0.1 ml of spleen cells at a density of 2×10^7 cells/ml. Cells were incubated at 37°C for 30 min; 0.05 ml of absorbed guinea pig complement was added one-half hour later and incubation was continued for one hour. Viability was determined by trypan blue exclusion. Cytotoxicity index was calculated using the formula $\frac{100\ (a - b)}{a}$

where (a) is the cytotoxicity of normal control cells cultured similarly to experimental cells and (b) is the cytotoxicity of cells cultured with ganglioside.

RESULTS

CRL levels in spleen cells cultured with ganglioside were not consistent, but CRL values for control gangliosides varied as much as values for G_{M1} (Table B-1). The only consistent change observed was a slight drop in the number of rosette forming cells 4 hr after cells were cultured. This drop was not reflected by a concurrent drop in the cytotoxicity index but was accompanied by a drop in the viability of cells incubated with G_{M1} (i.e., at 4 hr, normal cells were 75% viable, cells incubated with G_{D2} were 78% viable, but those incubated with G_{M1} were only 65% viable). These values were not reflected in cytotoxicity indices because addition of antisera and complement caused complete lysis of dead cells. This was demonstrated in antibody and complement controls, which showed an increase in viability when C or antibody alone were added to cells prior to viability counts.

No consistent changes in cytotoxicity with anti-Thy 1.2 were observed when cells were incubated with G_{M1} or control gangliosides.

DISCUSSION

Quantities of G_{M1} capable of modulating the plaque forming capabilities of spleen cells do not alter the binding of EAC to B lymphocytes. This indicates that G_{M1} probably does not bind via the C3 receptor. It is possible, however, that G_{M1} binds to the C3 receptor in quantities capable of modulating plaque formation but

Table B-1. The percent of a normal CRL level in spleen cells cultured with ganglioside

Time (hr)	Experiment I	Experiment II		Experiment III		Average	
	G_{M1}	G_{M1}	G_{D2}	G_{M1}	G_{D1a}	G_{M1}	G_{D2} or G_{D1a}
0	--	118	124	--	--	118	124
3	87	80	125	110	90	92±13	108±18
4	52	71	96	88	104	70±16	100± 4
6	--	59	102	126	151	93±33	127±25
7	--	64	106	105	87	85±21	97±10
9	--	94	99	79	90	87± 8	95± 5
24	90	--	--	--	--	90	--
96	77	--	--	--	--	77	--

not great enough to block EAC rosette formation. This could be tested by the addition of greater quantities of ganglioside to purified B lymphocytes.

APPENDIX C

EFFECTS OF CRL DEPLETION ON THE PLAQUE RESPONSE OF NORMAL AND MEMORY BONE MARROW CELLS

ABSTRACT

Either limiting dilutions or set concentrations of normal and long term B memory bone marrow cells were CRL depleted using BSA gradients and were transplanted into irradiated recipient mice in combination with non-limiting numbers of thymocytes (required for antibody production) followed by injection with SRBC. PFC assays demonstrated that CRL depletion of normal bone marrow cells slightly increased the number of cells required to give at least one precursor B lymphocyte needed to yield a clone of antibody-forming cells. Removal of CRL from memory bone marrow cells was found to lower the number of cells required for a positive plaque response in addition to reducing increased PFC numbers found during the memory response.

INTRODUCTION

Investigations concerning the role of the C3 receptor to date examine only those cells of splenic origin (Pepys, 1972, 1974; Feldmann and Pepys, 1974; etc.). Although the bone marrow only possesses a small proportion of C3 receptor-bearing cells (Dukor et al., 1970, 1971), it is the source of antibody precursors (Mitchell and J. F. A. P. Miller, 1968; Nossal et al., 1968; Davies et al.,

1968; H. C. Miller and Cudkowicz, 1970) and may play an important part in synthesis of immunoglobulins (Hijmans, 1975).

Whereas others have observed that removal of splenic CRL lowered the number of indirect plaques and did not alter the number of direct plaques (Bianco et al., 1970a; Möller, 1974; Parish and Hayward, 1974b), we found that CRL depletion of normal or long term memory bone marrow cells lowered both the direct and indirect plaque response of these cells.

MATERIALS AND METHODS

Animals. (C57BL/10 x C3H/He)F_1, (BCF_1) female mice were from Cumberland View Farms, Clinton, TN, or from Health Research Labs, West Seneca, NY.

Irradiation. Ten- to 12-week-old mice received 900 Rads of whole body irradiation from the ^{60}Co γ-irradiation source in the Department of Food Science at Michigan State University. These animals were rested for at least four hours before reconstitution.

Cell suspensions. Bone marrow cells from tibias and femurs of 10- to 12-week-old mice were gently aspirated in Eagle's minimum essential medium (MEM) in Hanks' salts with a syringe and a 25 ga needle. Cells were then passed through a 27 ga needle to produce a single cell suspension.

Thymocytes from 6- to 8-week-old mice were teased with wide forceps. Thymocytes were then dispersed using needles of progressively finer (21 to 27) gauge. For assay, recipient spleens were suspended in a similar fashion.

All cell suspensions were washed once by centrifugation (170 X g) in MEM and were then diluted to the appropriate numbers to be injected in a 1 ml volume per mouse. Varying numbers of bone marrow cells were injected in combination with 5×10^7 thymocytes; 5×10^8 SRBC were injected the following day.

CRL depletion. Bone marrow cells were incubated with EAC in order to allow rosette formation. Cells were incubated with EA as a control. Rosetted cells were then removed on a BSA gradient as described by Bianco et al. (1971).

Antigen. Sheep red blood cells (SRBC) were obtained as a sterile suspension in Alsever's solution from the Williams Sheep Farm, Mason, MI. Cells were washed three times in phosphate-buffered saline (PBS) before use; 5×10^8 cells/ml were given to each mouse as antigen.

Injections. All cells were injected in 1 ml quantities intravenously (IV) into lateral tail veins, using 27 ga needles. Fifty units of heparin were given intraperitoneally (IP) from 5 to 20 min prior to IV injection of thymocytes.

Plaque assay. The number of PFC in spleens of irradiated, reconstituted mice was determined by the use of the Jerne hemolytic plaque assay as modified by others and described by Baum and Miller (1976b). Recipient spleens with greater than 200 direct and 100 indirect PFC were scored as positive when limiting dilution assays were performed.

RESULTS

Restricted numbers of normal bone marrow cells or normal or memory bone marrow cells depleted of CRL were injected into irradiated recipient mice in combination with 5×10^7 normal thymocytes, followed by 5×10^8 SRBC. Greater numbers of cells were required to produce both a direct and an indirect plaque response when bone marrow cells were CRL depleted (Table C-1). The plaque response which results from adoptive transfer of bone marrow cells that were incubated with EA rather than EAC and then run on a BSA gradient was always very similar (i.e., averages were always within a few plaques of each other; percent-positive spleens were always within 10% if the same number of cells were used for reconstitution) to that obtained with normal bone marrow. Preliminary results also indicate that the difference in the number of cells required for a positive plaque response (>200 PFC/spleen) may be even more pronounced when long term memory bone marrow cells are CRL depleted (Table C-1).

Assays were also performed to compare the plaque response obtained with a given number of normal or memory spleen cells to that of memory cells which have been depleted of CRL. These results are only preliminary, but indicate that CRL depletion lowers the plaque response of memory marrow cells by approximately 2500 plaques to at least the level of plaques observed with normal bone marrow cells.

DISCUSSION

Results of this investigation indicate that although the C3 receptor does not seem to be required in the plaque response of the spleen (Waldmann and Lachmann, 1975; Baum and Miller, 1976b), cells

Table C-1. Percent of positive spleens in mice irradiated and reconstituted with normal or CRL depleted bone marrow cells

Number of cells	Normal bone marrow*		Normal marrow CRL depleted		Memory bone marrow CRL depleted
	Direct	Indirect	Direct	Indirect	Direct
4×10^6	---	---	80(5)	80(5)	---
2×10^6	---	---	80(5)	60(5)	---
1×10^6	94(18)@	67(18)	72(25)	39(25)	17(6)
5×10^5	100(22)	50(22)	75(12)	6(17)	0(6)
2.5×10^5	85(13)	38(13)	36(11)	36(11)	0(4)
1.25×10^5	82(17)	29(17)	50(18)	33(12)	0(5)
6.25×10^4	70(10)	20(10)	0(6)	---	0(6)
3.13×10^4	50(18)	28(18)	29(7)	---	---

@Number of mice per group.

*Normal data are from Miller and Cudkowicz (1970); normal bone marrow cells were also tested at 1×10^6 cells/mouse each time an assay was performed.

Table C-2. Plaque response of 10^6 bone marrow cells compared to that of 10^6 memory and CRL depleted memory bone marrow cells

	Number of PFC per spleen
Normal bone marrow	980 ± 260* (6)@
Memory bone marrow	3,486 ± 1,075 (7)
CRL depleted memory bone marrow	873 ± 397 (6)

*Standard error.

@Number of mice per group.

bearing the receptor seem to be involved in the plaque response which results from precursor B lymphocytes of the bone marrow, especially that response caused by "memory" bone marrow cells.

It is not surprising that those who remove splenic CRL find a difference in the plaque response, since they have removed from 20 to 40% of the splenic lymphocytes, and all but approximately 12% of the Ig bearing cells (Parish and Hayward, 1975b). However, it is surprising that removal of from 5 to 8% of the cell population (that CRL population of the bone marrow) of an organ could alter its plaque response. This is especially true in the case of long term memory bone marrow cells, since the induction of memory bone marrow does not increase the number of CRL in the bone marrow (unpublished results); yet removal of these CRL seems to deplete the memory response.

Results concerning the CRL depletion of memory bone marrow cells are not conclusive, but rather indicate that preliminary results with CRL depleted memory cells in conjunction with experiments showing more rapid development of CRL from memory cells demonstrated that the C3 receptor is in some way linked to the long term B memory response of the bone marrow.

These studies provide an indication of the direction that future studies might take, examining the role of the C3 receptor in the long term B memory response of the bone marrow.

SUMMARY

The development of the C3 receptor on bone marrow stem cells of irradiated, reconstituted mice was investigated in order 1) to determine what factors might alter this development and 2) to correlate that development with the generation of antibody forming capabilities. These studies indicated that antigen stimulation and induction of bone marrow memory cells altered CRL formation. The generation of CRL, however, could not be correlated with the development of plaque forming capabilities. Spleen cells appear to be equally immunocompetent in terms of class and quantity of antibody produced immediately prior to development of C3 receptors on differentiating cells as they are after complete receptor development. *In vitro* studies were also performed which support these findings by demonstrating that removal of the C3 receptors from cultured spleen cells did not enhance their plaque response. Nor did quantities of G_{M1} ganglioside capable of modulating the plaque forming capabilities of spleen cells alter the binding of EAC to B lymphocytes.

In conclusion, the C3 receptor does not appear to be involved in regulation of the quantity or class of antibody produced in a normal plaque response. However, it probably is involved in the activities of long term B memory cells of the bone marrow

(described by Miller and Cudkowicz, 1970, 1971, 1972) as demonstrated by the ability of memory cells to alter the development of C3 receptors and by the depletion of the memory response by removal of a very small proportion of the bone marrow cells which bear the C3 receptor.