Anti-immunoglobulin Induced Proliferation and Differentiation of Murine Lymphocytes

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Abbreviations

AECM-Ficoll	N-(2-aminoethyl) Carbamylmethylated-AECM-Ficoll
ATXBM	Adult Thymectomized Irrodiated Bone Marrow Reconstituted
AFC	antibody forming cell
Anti-Ig	anti-immunoglobulin
Con A	conconavalin A
DNP-AECM-Ficoll	2,4-dinitrophenylated-AECM-Ficoll
DXS	dextran sulfate
FcR	receptor for the F_c portions of immunoglobulin
FGG	fowl gamma globulin
2-Me	2-mercaptoethanol
LPS	lipopolysaccharide
NNP	4-hydroxy-3,5-dinitrophenylacetyl
NIP	4-hydroxy-3-iodo-5-nitrophenylacetyl
NIP-AECM-Ficoll	4-hydroxy-3-iodo-5-nitrophenylacetylated-AECM-Ficoll
NCPS	Normal Guinea Pig Serum
NRG	normal rabbit IgG
NWSM	norcordia water-soluble mitogen
PBA	polyclonal B-cell activator
PFC	plaque forming cell
PHA	phytohemmagglutinin
PPD	purified protein derivative of tubercule bacteria
PTA	polyclonal T-cell activator
RMG	rabbit anti-mouse globulins
RMF	rabbit anti-mouse IgG $F(ab)_2$
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TDA	thymic-dependent antigen
TIA	thymic-independent antigen
TNP	2,4,6-trinitrophenylated
TNP-BA	TNP-Brucella abortus
TRF	T cell Replacing Factor

ABSTRACT

The feasibility of utilizing anti-immunoglobulin/surface immunoglobulin interactions as the basis for a model of B-lymphocyte activation was examined. When normal mouse spleen cells were treated with the protein A purified IgG fractions of a) rabbit anti-normal mouse immunoglobulin (RMG) or b) rabbit anti-normal mouse IgG $F(ab')_2$ (RMF) incorporation of ^3H-thymidine was inhibited relative to untreated control cultures containing 5% fetal calf serum (FCS) and increased relative to cultures receiving normal rabbit IgG (NRG). In contrast, cultures treated with the $F(ab')_2$ of RMF or RMG showed a marked increase in thymidine incorporation (15-30x) relative to control cultures receiving either FCS or the $F(ab')_2$ of NRG. The mitogenic response was attributed to anti-Ig specificity since the stimulatory activity of RMG $F(ab')_2$ was removed by passage through normal mouse IgG-Sepharose but not by passage through ovalbumin coupled Sepharose. These experiments also showed that the mitogenesis induced was not due to contamination of the anti-Ig preparations with endotoxin. The possibility of protein A contamination of the rabbit $F(ab')_2$ being responsible for the activation was also excluded since 1) $F(ab')_2$ of RMG purified using Bio-Gel P-150 showed similar levels of stimulation to those observed with protein A purified $F(ab')_2$, 2) NR $F(ab')_2$ processed with protein A failed to induce proliferation and 3) NR IgG coupled to Sepharose 4B which binds protein A failed to remove the activity any more effectively than OA-Sepharose 4B, which does not bind protein A.

The anti-Ig $F(ab')_2$ induced mitogenesis showed a minimal requirement for T cells as (a) cultures of spleen cells treated with rabbit anti-mouse brain theta or AKR anti C3H thymocytes and complement and (b) spleen cells

from ATXBM mice had levels of incorporation comparable to those observed in whole spleen cultures. FCS was not an essential component of the medium although higher levels of ^{3}H-thymidine incorporation were observed when FCS was present.

The concentrations of $F(ab')_2$ which were mitogenic resulted in the total removal of sIg from the B lymphocytes, and the sIg were not subsequently re-expressed. A 48 hour exposure to $F(ab')_2$ of anti-Ig was required for a near optimal mitogenic response at 48-72 hours after the initiation of the cultures.

Efforts to demonstrate polyclonal antibody induction by immunofluorescence or by monitoring for anti-TNP antibody forming cells showed the $F(ab')_2$ mitogenesis to be inhibitory to the background levels of antibody formation which were observed in cultures receiving only FCS. The addition of a Con A induced T cell replacing factor $TRF_{Con\ A}$ in the presence of anti-Ig $F(ab')_2$ also failed to cause an increase in antibody formation.

An examination of the immune reactivity of cells treated with the $F(ab')_2$ of anti-Ig showed that such cells had a reduced capacity to respond to subsequent challenge with either LPS or DNP-AECM-Ficoll. The generation of LPS induced PFC was decreased by 50-80% relative to control cultures and the LPS mitogenesis was reduced by 50% relative to cultures treated with LPS alone. The data collectively suggested that treatment of cells with mitogenic doses of $F(ab')_2$ resulted in a suppressive effect on the B cells.

When cells were cultured with the $F(ab')_2$ of anti-Ig at concentrations 50-100 x lower than those required for the induction of mitogenesis, a small but significant increase in the number of anti-TNP or anti-SRBC PFC

recovered per culture was observed. Efforts to increase this response with $TRF_{Con\ A}$ have met with limited success.

The possibility of enhancing the response with a view to facilitating biochemical studies of lymphocyte activation are discussed.

Introduction

1. GENERAL INTRODUCTION

The cells of the immune system play a key role in the discrimination between self and non-self at the cellular and molecular levels. That this capacity to identify and react with foreign cells and substances is essential for the well-being of the host is clearly demonstrated in cases where the host is immunologically compromised such as in naturally occurring immunodeficiency states or during induced immunosuppression. Also, there exist states of enhanced or auto-reactivity which represent a threat to the well-being of the host, i.e. allergy, contact hypersensitivity and autoimmunity. Clearly the ability to manipulate the immune response to selected antigens would be of great medical benefit and this is one of the key reasons for the current interest in the subject of immunology. However, there are several important properties of the immune system which make it potentially one of the most useful systems for the examination of various aspects of eukaryotic cell biology. (1) The cells of the lymphoid system exist largely as free entities suspended in blood or lymph, this allows for their isolation en masse with minimal trauma to the cells. Furthermore, the development of the conditions for their maintenance in vitro and the induction of various types of immune reactions has facilitated a detailed examination of the cellular events involved in these phenomena in a relatively controlled environment. (2) Lymphocytes are divisible into two lines of differentiation (T and B cells) each with their distinctly different surface markers and functional capabilities. Each of these two lines can in turn be subdivided on the basis of differentiation antigens (Ly,Lyb,Ia) and effector functions (memory, helper, suppressor, cytotoxic). (3) Numerous

methods exist for the isolation of the various cell pools, e.g. the selective enrichment or depletion of specified populations. This allows for recombination experiments to be done on various populations and the identification of phenomena involving cellular collaboration. (4) Study of the immune system of mice has allowed a considerable amount of information to be amassed on the genetic control of immune reactions. With the ever increasing numbers of syngeneic and congeneic strains of mice it has been possible to identify the region of the genome known as the major histocompatibility complex as being responsible for the expression of many different immune phenomena (Ir genes, CML and MLR genes, cellular collaboration). An offshoot of the development of inbred strains has been the discovery of several mutant lines (CBA/N, C3H/HeJ, Nu/Nu, NZW) which are operationally defective in certain immune reactions. Also, allotypy has been demonstrated in several surface markers (Ig, Thy1) thus permitting for the preparation of antisera against specific cell surface components. Some of these antisera have proved useful in identifying structures which may be defective or absent in the mutant strains. All of the above points illustrate the unique advantages offered by using lymphoid cells in the development of experimental systems involving several different aspects of cell biology.

The majority of the recirculating lymphocytes are small cells in G_0 phase of the cell cycle. However, upon contact with the appropriate stimuli these cells rapidly proliferate and differentiate along various predetermined developmental pathways to generate effector cells and under appropriate conditions memory cells. There are very few mammalian cell systems in which it is feasible to study the events involved in the transmission of membrane/ligand generated signals to the nucleus of the

cell. The manifestations of such events would involve the repression/expression of the genes responsible for the phenotypic changes which are subsequently expressed. Thus lympocyte populations provide a model system for an examination of the modes of plasma membrane/nuclear information transfer.

An immune response requires several levels of cellular interaction in its generation and regulation. Many of the events have been shown to require identity of histocompatability antigens between the collaborating cell types. Although it is currently unclear as to whether this requirement relates solely to the form in which antigen is initially presented and subsequently recognized, it is known that cellular collaboration is essential. It seems quite probable that the modes of recognition involved in an immune response will be similar to those involved in fertilization, organogenesis, and embryogenesis. An understanding of the way in which lymphocytes communicate by direct interaction or via soluble mediators may be of critical importance in the elucidation of the principles of cellular recognition and interaction.

An examination of immune phenomena has already contributed significantly to cell and molecular biology. However one very severe restriction is evident in the study of lymphocyte molecular biology, namely the low numbers of cells reactive to any given antigenic determinant. The outstanding feature of the immune system is its ability to respond rapidly and in an exponential fashion to antigenic challenge. Following withdrawal of the stimulus the response decays but increased numbers of primed cells remain which are capable of responding rapidly to a secondary challenge. In this way a large repertoire of cells of different specificities can be maintained by the host with minimal metabolic expenditure.

A selected population of lymphoid cells will respond only when the appropriate stimulus is applied to it. This means that although a lymphocyte population can be isolated in a homogeneous state with regard to the state of differentiation or potential effector function only a small fraction will respond on challenge with a given antigen. Thus the system may be unsuitable for biochemical analysis since background levels may mask intracellular changes during activation. Several approaches have been employed in an effort to circumvent this problem of the low frequency of antigen specific cells.

(a) Considerable insight into the mechanism of immunoglobulin synthesis, transport and secretion was achieved through the study of myeloma cell lines. These cells are derived from tumours and represent the products of the expansion of a single clone. Since the binding specificity of some myeloma proteins were known it was possible to examine the relationship between the specificities of the secreted products and the surface receptors. However, since the myeloma cells are transformed, the growth characteristics and properties of these cells need not be representative of their normal analogue. Myeloma lines have been shown to spontaneously produce mediators which are secreted by normal cells following activation; it therefore is highly unlikely that these transformed cells will be useful in the study of early activation events as they appear to be in a continuous state of activation.

(b) Polyclonal activators are compounds which stimulate various populations of lymphocytes to divide and in some cases differentiate without the involvement of the antigen receptor. These activators have been used to induce blast transformation in a large fraction of normal lymphocytes. This approach has contributed significantly to the current

knowledge of the early metabolic requirements and biochemical events of activation as well as to the later transcriptional and translational events. Critics of this approach have argued that mitogens appear to be capable of replacing the requirements for one or more cell types in the generation of certain immunological responses. It therefore seems that the polyclonal activator supplies signal(s) which are equivalent to the multiple distinct signals supplied by several different cell types during an antigen specific response. Thus the net result of exposure to a polyclonal activator is similar to that observed in an antigen specific case, however the underlying inductive events may be markedly different. Indeed mitogen receptors are functionally defined on the basis of ligands which inhibit the activation of lymphocytes by the mitogen. This method does not define a unique molecular species but rather a range of molecules which share the characteristic that they bind the mitogen. It is therefore quite feasible that the activation by mitogens requires interaction of mitogen with several distinct molecular species all supplying different signals which contribute to the activation of the lymphocyte. It should be stressed that the above interpretation of the action of mitogens is not accepted unanimously and will be discussed later.

(c) The most ambitious approaches to the study of activation have involved the isolation of cells on the basis of the specificities of their antigen receptors. This has proved to be practical only for the isolation of B lymphocytes as the T cell receptor appears to be less fully displayed, more labile or more easily lost under the conditions successfully employed for B cell isolation. Alloreactive T cells however have been isolated on monolayers of allogeneic target cells. The difficulty with this type of scheme is that the frequencies of antigen reactive cells are so low

and that the procedures for their isolations do not give quantitative yields making it currently unfeasible to obtain sufficient numbers of cells to facilitate the biochemical characterization of antigen dependent activation. However, methods of cloning lymphocytes have improved radically over the last five years and it may be possible to select for specific clones by expanding them in the presence of antigen to supply large quantities of antigen reactive cells with characteristics more closely resembling those of secondary lymphocytes than "virgin" cells.

The previous discussion was intended to demonstrate some of the areas in which the study of lymphocytes may be able to make significant contribution to the understanding of mammalian cell and molecular biology in general. Some of the limitations of the systems in current use were pointed out in an effort to identify the need for polyclonal models of antigen specific stimulation.

At this point it is appropriate to diverge and to examine some of the characteristics of the various cell populations involved in the generation of an antibody response.

T Lymphocytes

T lymphocytes in the mouse are operationally defined by the presence of the Thy 1 differentiation antigen and the absence of sIg (157). There are numerous other surface markers for example GIX, Tla, Ly-1, Ly-2,3, Ia, FcR (206). Most of the markers have not proven to be useful in the description of functional groups of T cells as they are not sufficiently restricted in their distribution among the T cells. However, the Ly antigens (24) have been used extensively to associate the Ly phenotypes of T cell subsets with functional specificities (33). More recently, Ia

antigens on T cells (177) have been used to further subdivide T cell groups and an even greater potential is offered by the observation that FcR's expressed on a fraction of T cells are blocked by anti Ia sera of different specificities suggesting that several distinct populations of FcR^+ T cells bearing different Ia antigens exist (207).

T cells are responsible for a diverse series of activities ranging from the initiation of immune reactions to the suppression of these responses. It has been tentatively proposed that T cells can be divided into four categories: the initiator T cell (T_i), the helper T cell (T_H), the cytotoxic/effector T cell (T_C) and the suppressor T cell (T_S) (205). Each of these groups may be further subdivided on the basis of which type of reaction is being regulated, i.e. humoral or cellular responses. T_H cells play an obligatory role for most, though not all, immune reactions. Thus there is a T_H for B cell antibody formation T_{HB} (63) and one for the generation of killer/effector T_{HC} cells (150). These two helper populations appear to be distinct groups as indicated by their surface phenotypes, T_{HB} expressing the antigens Ly-1, Ia and T_{HC} having Ly-1, Ia, FcR surface markers. T_i cells appear to be involved in the early inductive events of immune reactions, they apparently do not directly give rise to the effector cells and they are different from T_H cells (36) in tissue distribution and kinetics of appearance. These cells are $Ly\text{-}1^+$, $Ly\text{-}2,3^+$, Ia^+ and possibly FcR^+. T_C cells are characterized by their capacity to cause <u>in vitro</u> cell mediated lympholysis (CML) and may be the same cells responsible for DTH <u>in vivo</u>, however, the phenotype of cytotoxic cells is given as $Ly\text{-}1^{+}$, $Ly\text{-}2,3^{+}$ for cells reactive with syngeneic targets, and $Ly\text{-}1^{\mp}$, $Ly\text{-}2,3^{\pm}$ for cells reacting with allogeneic target cells. It is apparently difficult to assign exact

phenotypes to T_c cells as it is possible that the Ly-1^+, Ly-$2,3^+$ cells may give rise to Ly-1^-, Ly-$2,3^+$ cytotoxic cells, thus the Ly phenotype of T_c will depend upon which stage of activation the T_c cells are phenotyped. T_s are capable of preventing the expression of an immune response, possibly by interacting with the T_H or the effector cell (70). Both non-specific and antigen specific suppression have been observed and it appears that the two types of cells involved can be identified on the basis of their surface markers. The cells suppressing non-specifically for both antibody formation (128) and MLR (162) are Ly-1^+ Ly-$2^+,3^+$ whereas the antigen specific suppressors are Ly-1^-, Ly-$2^+,3^+$, I-J^+ (137). There is evidence for considerable heterogeneity in all four categories of T cells and although the above scheme accommodates most of the data to date, it is not unequivocally established that the Ly phenotypes correlate exactly with a particular type of T cell function. It would seem highly improbable that two cell types which perform similar functions in humoral and cellular responses will always express similar surface structures. The receptors involved in the two phenomena must perform different functions. Moreover, if it is assumed that some of the antisera used to characterize these different populations are reacting with functional markers, then it follows that the markers should be restricted in their distribution.

Several of the antigen specific activities of T cells (T_H or T_S) have been shown to be replaced by the addition of soluble products derived from antigen treated cells (54). Although not all of these factors have been formally shown to derive from T cells and others have been assigned a macrophage origin (173), it is generally assumed that in most cases the factors are of T cell origin. In several instances

it has been demonstrated that these T cell factors bear an antigen combining site (210) and I region gene products (208), the latter often being of the same specificities as those present in the cell populations which are responsible for the production of the factor. Study of these factors will likely lead to the eventual isolation and elucidation of the events involved in lymphocyte activation and regulation.

Recently it has become apparent that the T cell recognizes antigen in association with MHC coded gene products (H-2K/H-2D) for viral and hapten modified self (229) and Ir-gene products especially on the macrophage surface for the generation and recall of T_H or T_C cells (194). These observations have led to the proposal of numerous models of the T cell recognition unit(s)(49). 1) The altered self hypothesis predicts that the T cells recognize antigens in association with determinants coded for in the MHC region, i.e., the single complex receptor combines with the altered MHC product and results in the activation of the T lymphocyte.

2) The dual recognition model postulates that there are two distinct receptor units on a reactive T cell, one which recognizes self MHC coded products and a second with specificity for antigens not coded for by self MHC genes. Thus it is apparently necessary to recognize self for the generation of a response against a foreign antigen. It has been suggested that the T cell receptors which recognize self may be different for T_H and T_C. T_H cells would recognize I-region products whereas T_C cells require K/D region products (218) if this proves to be correct it would account for the differences in histocompatibility requirements for T_H/MØ, T_H/B and T_C/target cell interactions.

The nature of the T cell antigen receptor has proven to be an area

of considerable controversy. That antigen specific T cells exist is not disputed, as adoptively transferred primed T cell populations readily provide a demonstration of antigen specific T cell function. However, the actual visualization of antigen binding T cells has met with varied success (83,16). The possible reasons for this variability have been presented by several groups as it has been found that T cells show much more restricted conditions for antigen binding than B cells do. Hence, T cells bind antigen much more efficiently at 37°C than at 4°C. Furthermore, their binding capacity is markedly affected by the presence of metabolic inhibitors such as sodium azide. The binding of antigen was also inhibited by the appropriate anti-H2 sera but not by anti-Ia sera, suggesting a close association between the K/D coded antigens and the T cell receptor. The presence of 7s IgM on the surface of T cells has been reported (114), however the general consensus would now appear to be that conventional immunoglobulin of any class is not on the surface of T cells.

Considerable potential for insight into the molecular nature of T cell receptors derives from the demonstration of similar idiotypes on the surface of both B and T cells (107). More recently, the observations that the T cell receptor may share allotypic determinants with the V_H region offers even greater feasibility as to the possibility of bulk isolation of T cell receptors, thus permitting for their biochemical analysis (108).

B Lymphocytes

Cells of this class are responsible for the production of antibodies and are characterized by the presence of endogenously synthesized surface immunoglobulin (sIg). The majority of mature B cells have demonstrable

7s IgM and sIgD which are of identical idiotype to the immunoglobulin which the progeny of these cells will produce following stimulation. Numerous surface markers have been identified on murine B lymphocytes (FcR, C3R, Ia, Lyb antigens) but the majority of these markers are not sufficiently restricted in their distribution on these cells to be of use in identifying functional B cell subpopulations (18,80). However, these markers have been used in conjunction with sIg isotypes to follow B cell ontogeny where the sequential appearance of IgM, C3R, IgD and Ia antigens occurs (141).

Indications of B cell heterogeneity were first obtained when the characteristics of B cells responding to TIA's were compared with those activated by TDA's (75). T independent antigen responsive cells have been shown to be larger than TDA reactive cells in primed animals when sedimentation profiles were compared for IgM responses (75). In contrast, IgG producing cells gave nearly identical patterns for TDA and TIA. TIA activatable cells produce smaller clone sizes than TDA reactive cells while both cells secrete antibody of identical idiotype and affinity when tested against TD and TI forms of the phosphorylcholine determinants (154). Some data also suggest that TDA^+ cells bear a receptor for the third component of complement while those responding to DNP-Ficoll and a small proportion of cells responding to TDA's lack the receptor for C3 (112). Both populations responded to LPS by proliferating but their capacity to generate AFC was not reported. The data collectively demonstrate that there are separate populations which respond to TDA's and TIA's and that these populations are mutually exclusive (94). However, these observations do not allow for an unequivocal answer as to whether or not

these responses represent the activation of distinct lineages of B cells or of B cells which are at different stages of development along the same pathway.

Another approach for demonstrating B cell heterogeneity has been to examine the mitogen responsiveness of these cells. Gronowicz and Coutinho (81) found that the B cell mitogens LPS, DXS, and PPD produced markedly different patterns of development. DXS exposure resulted in high levels of proliferation with little differentiation, LPS caused high levels of both proliferation and differentiation, while PPD induced mainly differentiation to AFC. The differential effects of the various mitogens appeared to be a function of the responding cell populations rather than the mitogens delivering qualitatively different signals to the same cells. Further evidence for different subsets of B cells being involved came from the observations that the mitogens had additive effects when both were present in the cultures. The possibility that these populations were at different stages of development along the same differentiation pathway was supported by the observation that sequential sensitivity to the mitogens were demonstrable. Using the 5-bromo-deoxyuridine technique for the selective killing of dividing cells, Bona et al (20) were able to show that there were discrete populations of murine B cells responsive to $NWSM^+$ and to LPS^+, dextran sulfate reactive cells (DxS^+) were present in both of these populations. There did not appear to be a DxS^+ population which did not react to LPS or NWSM. The specificities of the I region coded surface antigens were also examined, LPS responding cells were found to be $I\text{-}A^+$, $I\text{-}C^+$, $NSWM^+$ cells were mostly $I\text{-}A^+$, $I\text{-}C^+$ but a significant proportion (25%) were $I\text{-}C^-$ as determined by cytotoxicity methods.

The organ distribution of lymphocyte responsiveness also indicates that B cells are heterogeneous in their requirements for activation (93). Cells isolated from lymph nodes or Peyer's patches showed predominantly a proliferative response to LPS and PWM, blood lymphocytes on the other hand appeared to respond poorly to these mitogens although this may be dependent upon the levels of challenge by environmental antigens. A large proportion of splenic B cells were induced to proliferate and differentiate to AFC. These results suggest that B cells are a heterogeneous population containing subsets of cells responsive to different mitogens, and that the types of responses induced by a mitogen can vary within a given population.

Subdivisions within the B cell pool have also been demonstrated using surface immunoglobulin isotypes as markers. Vitetta et al (216) were able to determine the responses of two sets of cells, those bearing $\mu^+/\mu^+\delta^+$ and $\mu^-\delta^+$ surface isotypes were separated by selectively depleting splenic populations of μ^+ cells by treatment with anti-IgM and complement. They observed the μ^+ cells ($\mu^+\delta^-/\mu^+\delta^+$) contributed all of the immunoglobulin synthesis which was observed in stimulated cultures containing the unfractionated splenic B cell population. The $\mu^+/\mu^+\delta^+$ cells and the $\mu^-\delta^+$ cells contributed only to the proliferative response. Using an affinity column, Scott isolated splenic δ^+ cells from 3 week old mice in which the proportions of $\mu^+\delta^-$ and $\mu^+\delta^+$ cells are approximately equal (182). Limiting dilution analysis of these populations indicated that the δ^+ cells contained a 4-5 fold higher frequency of precursors for the hapten fluorescein when it was presented as a T independent antigen Flu-POL. When cells which had been separated in the above fashion were cloned in agar,

$\mu^+\delta^-$ cells were found to be 2-3 fold less efficient in cloning than either the starting population or the δ^+ population (183). These authors point out that the presence of IgD on the cell does not necessarily correlate with the ability of B cells to be cloned since fetal liver cells have comparable cloning efficiencies to those of adult spleen cells even though the former cells lack sIgD (72).

Other studies have indicated that it may be not only the qualitative differences in surface immunoglobulin isotypes but also quantitative differences which define different B cell populations since sIgM and sIgD are expressed in different proportions on B cells at various ages (129). B lymphocytes from neonatal mice show almost exclusively sIgM, by about 3 weeks of age sIgM = sIgD and by adulthood sIgM < sIgD (72). Although the ratios of δ/μ represent an averaging of all B cell sIg, the fact that $\mu^+\delta^+$ represents the dominant phenotype in adult splenic B cells (70%) does indicate that these cells must show an increase in their δ/μ ratio. When the responses of neonatal cells and adult cells were compared by challenging with various TIA's, it was found that two broad categories TIA's existed, those which both neonatal and adult lympocytes would respond to, the TI-1 antigens (LPS, TNP-BA) and those which could activate only adult spleen cells, the TI-2 antigens (TNP-Ficoll, TNP-Dextran) (230). Evidence that the cells responding to the two categories of TIA's were of two distinct populations was obtained using anti Lyb-5 sera(3). This antiserum was prepared by immunizing C57B1/6 mice with DBA/2 spleen cells and absorbing the antiserum with (CBA/N X DBA/2)F_1 male spleen cells from animals showing the X-linked CBA/N defect. CBA/N mice have numerous X-linked immunological defects, one of which is their inability to respond to T1-2

antigens suggesting that the cell population responsive to these antigens is either absent or defective. When normal adult spleen cells were treated with anti-Lyb-5 plus C' it was possible to eliminate the response to TI-2 antigens while leaving the TI-1 response unaffected. Furthermore, the sIg phenotype of the remaining B cells was changed from $sIgM^+/sIgD^+$ to $sIgM^+/sIgD^-$. The results of the studies on sIg phenotype and functional capacities of B cells demonstrate that the age of the B lymphocytes, the sIg phenotype and the ratios of the sIg isotypes all contribute to the immune status of the B cell.

Ia antigens which are expressed predominantly on B cells have also been employed for the examination of B cell heterogeneity although to a much lesser extent than sIg isotypes and mitogen responsiveness. The B cell Ia antigens are generally coded for in the I-A and I-C subregions of the MHC complex of the mouse (115). It has been suggested by several authors that the Ia antigens are critically involved in the IgM/IgG class switching of B cells as treatment of primed B cells with anti-Ia sera and complement results in the abrogation of the IgG antigen specific response (152). The role of Ia antigens in signal generation for class switching is not clear and evidence that the polyclonal B cell activator, LPS, can induce class shifts within a clone of B cells suggest that these antigens may not be the only means of inducing changes in the class of Ig secreted by a cell or its progeny (10).

This discussion out of necessity has avoided some of the most pressing problems of B cell physiology, such as sIg isotypes of IgG, IgA and IgE memory and secreting cells, the conditions for the generation of B cell memory and the events involved in class switching. These areas in B cell biology have proven to be some of the most difficult subjects to

study and consequently have not as yet been explored in depth.

Cellular Collaboration in The Immune Response

All immune reactions depend on the collaboration between several cell types for the generation of immunocompetent cells. The induction of antibody production requires at least three distinct cell types, the T cell, the B cell and the macrophage (35). Initial observations that allogeneic B and T cells were unable to co-operate in the generation of an antibody response were interpreted as evidence for the existence of cellular interaction gene products which were necessary for the effective collaboration of T and B cells (96). However, it has been recently demonstrated by several groups (219,220), using radiation chimeras that allogeneic T and B cells can mount an antibody response provided, that the T cells are primed in an environment containing cells of the same allogeneic genotype as those on which the T cells will subsequently be presented with antigen. Thus, when CBA T helper cells prepared from irradiated (CBA x DBA) F_1 hosts, which had been reconstituted with a mixture of bone marrow cells from both parents, were tested for their ability to co-operate with B cells of CBA or DBA origin, both allogeneic and syngeneic T/B cell combinations gave PFC responses. One of the explanations put forward for these differences in results was that the T cells might recognize antigen in association with an MHC gene product on another cell such as a macrophage. In allogeneic combinations the T cells would not "see" antigen in association with the MHC products with which the T cells were originally primed (219). However, in the chimeric animals antigen could be associated with MHC gene products of either donor strain and it would thus be possible to generate T_H cells which

were primed to antigen present on cells originating from the other partner of the chimera. Support for this concept of the recognition of two distinct determinants for T cell activation derives from experiments in which the specificities of cytotoxic T cells were examined (229). In this latter system virus infected macrophages were used to generate cytotoxic T cells which showed specificity for the virus and for the K or D region gene products of the MHC complex. That is, if T cells were induced with virus infected cells of an $\underline{H\text{-}2}^{K}$ phenotype, the cytotoxic cells would lyse cells with $\underline{H\text{-}2}^{K}$, $\underline{H\text{-}2K}^{K}$ or $\underline{H\text{-}2D}^{K}$ genotypes but not cells which expressed non-$\underline{H\text{-}2}^{K}$ phenotype at both the K and D loci. It therefore seems likely that the genetic restrictions which were originally thought to exist for T/B cell collaboration are not absolute but rather that they reflect the restriction placed upon T cell memory induction and the recall of this memory by the need for T cells to recognize at least two determinants on an antigen presenting cell. Whether the antigenic determinants are in direct physical association or merely present on the surface of the same cell as different entities is still a point of considerable discussion (205).

It has become increasingly apparent that macrophages play a key role in antigen presentation and that Ia antigens appear to be intimately involved in this process (214,148,193). Shevach et al ($_{193}$) observed that when Strain 13 T cells derived from peritoneal cells were depleted of alloreactivity to Strain 2 macrophages and subsequently cultured for 7 days with TNP conjugated Strain 2 macrophages a subset of Strain 13 T cells was induced which would only respond to TNP coupled Strain 2 macrophages. It appeared that there was no requirement for Ia identity between T cells and macrophages for successful priming once alloreactive T cells had been removed. When the role of Ia antigens in the response was examined

by the addition of anti-Ia sera directed towards either the Strain 13 T cells or the Strain 2 macrophages, it was found that only the anti-sera with specificity towards the macrophage Ia antigens were capable of inhibiting the proliferative response. Thus, the macrophage Ia antigens can be different from those of the responding T cell, however, macrophage Ia antigens must be accessible for T cell activation to occur. Perhaps the most direct evidence as to the importance of Ia antigens in T cell macrophage interactions comes from Erb and Feldmann (56). These authors developed a system for the in vitro induction of T_H cells which consisted of incubating T cells and macrophages with antigen for four days. The helper cell activity of these mixtures was assayed by combining the T_H with mixtures of B cells, macrophages and antigen, and culturing these cells for an additional four days after which the number of antigen specific plaque forming cells was determined. The presence of macrophages or macrophage derived factors was found to be obligatory for the development of T_H activity. The responses to soluble antigens required identity of the I-A subregions of the macrophages and the T cells used in the cultures to generate T_H cells. Recently this group has also provided preliminary evidence that there may be genetic restrictions of the level of the macrophage B cell interaction providing further support for the key role of the macrophage in immune induction (87).

In those cases where the response to an antigen is under the control of Ir genes it has been possible to demonstrate the association of a given Ia antigen specificity with the ability to respond to a given antigen (193, 181). Although these findings do not prove that the Ir gene product responsible for reactivity to a given antigen is an Ia antigen they do suggest a close

association between the two both genetically and physically.

The requirement for T/B cell collaboration in the induction of antibody production was the first example of cell cooperation in an immune response, however the potential for elucidation of the cellular and molecular events involved in this process was only fully realized with the demonstration of soluble T cell derived factors which could replace the requirement for the presence of T cells. The initial T cell replacing factors were not antigen specific (NS-TRF), but subsequently numerous antigen specific T cell replacing factors (AS-TRF) were reported.

The NS-TRF are generally derived from cultures containing T cells undergoing extensive stimulation i.e. MLR (53 ,168) or mitogenic challenge (130). There is considerable controversy as to the nature of these factors in terms of the antigenic determinants which they express and their biochemical properties (85). Armerding et al ,in characterizing an allogeneic effect factor(AEF) derived from the supernatants of mixtures of allogeneic cells, observed a single factor with a molecular weight of 30,000-40,000 Daltons which carried Ia and β_2 microglobulin antigenic determinants (6 , 7 , 8). In contrast,another group working mainly with a Con A induced factor found evidence for three biologically active fractions with molecular weights of 25,000, 30,000-35,000 and 45,000 Daltons, none of which possessed any H-2 coded antigenic determinants (88,130). The nature of the NS-TRF is further complicated by the fact that macrophages have been demonstrated to be capable of producing factors which can replace T cell function under certain circumstances (173). It would therefore appear that TRF is strictly a functional definition probably describing the action of several distinct factors each of unknown physiological significance.

Antigen specific factors are usually obtained from cultures which have contained the test antigen for extended periods of time in order to

generate an enriched T_H pool for the test antigen (60 , 116). Another approach has been to inject irradiated hosts with T cells together with antigen and to use the spleens of these animals 4-7 days after immunization as a source of "educated T_H cells" for factor production (210,197). Using the above methods of T_H generation the AS-TRF, which have been characterized, show some consistency in their properties namely they have a molecular weight of approximately 50,000 Daltons, they carry Ia antigenic determinants usually coded for in the I-A or I-C subregions and they are cytophilic for non-antigen specific cells such as macrophages. These properties are by no means descriptive of all of the AS-TRF which have been reported, in fact a point of considerable contention is whether or not at least one factor bears immunoglobulin determinants (60). Most factors have however been rather poorly characterized and perhaps with the introduction of cell fusion techniques it will be possible to obtain sufficient quantities of these factors to permit conventional biochemical analysis of them.

This section was intended to provide an outline of the various levels of cell collaboration which are required for the induction of a humoral response either by direct interaction of the cells or via the action of soluble mediators derived from these cells. Suppressive factors were not discussed, not because they may not play an important role in regulation of the immune response, for their significance appears to be rather well established (208,213) but rather the action of these cells appears to be at the level of the T cell. Since this discussion involves B cell induction it was not felt to be advantageous to discuss these factors further.

In the next section I should like to present a brief summary of the current status of models for B cell activation.

B Cell Activation - An Overview

One of the key questions in B lymphocyte activation relates to the role of sIg antigen interactions in the generation of an activating signal to the cell. The sIg clearly plays a critical function in the maintenance of the high levels of specificity which are observed in the humoral response. However the status of sIg as a trigger for lymphocyte stimulation has not been clearly demonstrated. A further complication to the above problem is the fact that several additional cell types may be necessary for most if not all antigen specific responses, thus making it difficult to assess the nature as well as the number of signal(s) which are required for lymphocyte activation. As a consequence of this general uncertainty as to the minimal requirements for B cell induction several models have arisen which explain the data from selected types of antibody responses in a satisfactory fashion but fail to provide a complete description of other types of immune phenomena. The models may be divided into two classes, those involving a single signal and those involving multiple signals for stimulation. Within the one signal models a controversy exists as to whether or not the sIg antigen interaction is indeed the signal for induction of antibody formation. It is apparent from the above discussion that sIg function in B cell activation is not well understood and that polyclonal systems might be highly advantageous in the treatment of this problem.

An important consideration in any model of B cell induction is the mechanisms by which clones of self reactive B cells are dealt with. Obviously an organism can not tolerate conditions under which a continuous response is being generated against self components if only from a consid-

eration of the economics of the metabolic expenditure required. Furthermore, the potential self induced damages which can result under conditions of uncontrolled self reactivity are clearly demonstrated in conditions of autoimmunity.

Several approaches have been taken in an effort to deal with this problem of the regulation of self reactive B cells.

1) B cells pass through a phase in their development where interaction with an antigen can lead only to paralysis or deletion. As self reactive B cells arise they are in contact with a continuous supply of autologous antigens which leads to the deletion of these clones.

2) The capacity for self-nonself discrimination is totally a T cell function thus allowing for the existence of self reactive B cells. The potentially self reactive B cells are not activated as in some undefined fashion self reactive T cells have been deleted. Since B cell activation normally requires T cell collaboration the lack of self reactive T cells ensures that the B cells will not be activated by normal physiological mechanisms.

3) The effect of antigen on B cells can be either stimulatory or inhibitory depending upon the availability of a T cell derived second signal. Self reactive T cells are envisaged as being deleted by interaction with self antigens in the absence of adequate T help by much the same mechanism which B cell deletion is postulated. Antigen is required for the activation of B and T cells however antigen in the absence of sufficient T cell help results in the deletion of the cell. As self reactive B cells arise they are paralyzed by autologous antigens since self reactive T cells are not present to provide the second signal.

These models clearly lead to quite different predictions as to the

presence of active self reactive B cells and this point will be discussed below in the context of each of the categories of models for lymphocyte activation.

Antigen Presentation and Pattern of Presentation Hypotheses

Certain antigens have the ability to induce an immune response in the absence of detectable T cell function and have therefore been called T independent antigens (TIA). The majority of these molecules are extended polymers of protein or carbohydrate with repeating subunits or degenerate conformations (62), thus, providing a matrix of antigenic determinants. On the basis of this similarity between the various TIA's, it was suggested that the mode of antigen presentation was critical in determining the outcome of an interaction between antigen and B lymphocytes (65). In the case of T dependent antigens which are mainly monomeric, globular proteins the macrophage is envisaged as the site of antigen presentation. Antigen through combination with a T cell derived factor is bound to the macrophage surface and displayed in the proper orientation for lymphocyte activation(46,119). The model invokes the pattern of presentation and the quantity of stimulus supplied as being the critical factors in determining whether a cell will be activated or tolerized.

A prediction of this model is that a T dependent antigen can be converted into a T independent form by immobilizing it to create an appropriate antigen matrix. As will be discussed below several groups have designed experiments to test this point and obtained conflicting results. Moller et al (124) suggest that the reason for their inability to induce a TI response with immobilized antigen was their choice of antigens i.e.

those which lacked any PBA activity. They point out that the antigen which Greaves et al (79 , 64) used KLH has weak PBA properties (97) and that by immobilizing this antigen on beads it was possible to concentrate sufficient PBA stimulus on an individual cell to activate it. However, one of the antigens which was employed by Greaves et al was produced by coupling TNP directly to polyacrylamide beads. This antigen preparation was found to elicit a response in the absence of apparent PBA activity. It could still be argued that the particulate antigens lead to the nonspecific activation of macrophages which in turn stimulate the response to a specific antigen by virtue of a close association between B cells reactive to the antigen and macrophages displaying antigen. It had originally been reported that macrophages were not necessary for TI responses (48), however, more recently there has been data indicating a requirement for macrophages in these responses (110), thus making the above suggestion somewhat more feasible. The results of experiments using TI antigens do suggest that the mode of antigen presentation can play a critical role in determining the subsequent fate of antigen binding B cells. However, they do not supply unequivocal proof of an active role for sIg in B cell activation. The role of antigen structure in B cell activation may be the critical parameter in the activation of a selected TI B cell population, whereas other factors may be the prime determinants in TD responses, in which case it becomes essential to define what B cell subpopulation is being activated since recently stimulated cells, i.e. those with high backgrounds, may have different requirements than virgin B cells.

Multiple Signal Models

Bretscher and Cohn(27,37)proposed a two signal model of B lymphocyte activation based on theoretical considerations in an effort to explain self-nonself discrimination at the B cell level. The simplest form of the Bretscher/Cohn model consists of a requirement for two signals for induction of antibody production. The initial stimulus (signal (1)) is delivered via the antigen receptor upon binding of the corresponding antigen to it. Signal (1) was proposed to be either immunogenic or tolerogenic, depending upon the events subsequent to its generation. If a second different signal (2) was not encountered by the antigen bearing cell within a certain time interval, the cell was tolerized and no longer capable of responding to antigenic stimulation. However, if signal (2) was received, the cells were activated to become fully competent immunoglobulin secreting cells. The signal (2) was postulated to derive from the antigen specific T cells which produced this "associative antibody" thus the capacity of self discrimination was a function of the T cell pool.

A prediction of the Bretscher - Cohn model is that TD antigens, in the absence of T help specific for the antigen, will result in the paralysis and/or deletion of the antigen specific B cell. Evidence in support of this comes from the findings that B cells from nude mice immunized with FGG will not subsequently respond to FGG in the presence of added signal (2). The argument being that the B cells first encountered FGG in an environment which was devoid of T help consequently the cells were inactivated by contact with antigen and were unable to respond upon secondary challenge with antigen and a source of signal 2. This is a very important observation as it forms the basis for the mechanism of the deletion of self

reactive clones since self reactive T cells are not present to supply signal 2 to the above B cells (174). This last point also focuses on one of the major points of difference between the one nonspecific signal and the two signal hypothesis i.e. the former does not accept that B cells can be tolerized but rather only blocked by antigen while the latter does allow for B cell tolerance.

One of the major shortcomings of the two signal model has been its inability to adequately explain the modes of action of thymus independent antigens and mitogens. It was originally proposed that TI antigens were not totally free of a requirement of T help but that they required much lower levels of signal 2 than other conventional thymus dependent antigens (25). It has more recently been suggested that PBA's may merely be a class of unique molecules capable of delivering the equivalent of signal 2 to cells which have recently been exposed to antigen in vivo thus explaining the apparent lack of requirement of antigen to induce a PFC response to a given antigen. (26).

Various models involving two or more signals have been proposed (170, 52, 105) in which the second signal may be non-antigen specific. In these cases, the antigens were essential for activation as was demonstrated by the requirement of both antigen and the nonspecific factors. However, these models obviously require a different means than that described above for deleting self reactive B lymphocyte clones since such B cells would be continuously exposed to autologous antigens and activated by the nonspecific factors generated during other ongoing responses. None of the above systems deny the existence of antigen specific factors but they use nonspecific factors in their experimental systems. It is quite conceivable

that a "specific" factor may show "nonspecific" properties if used at sufficiently high enough concentrations and this may indeed be the case in some systems so that there may in reality be little difference between some (170, 52) of these models and the Bretscher - Cohn model. Nossal and Pike have proposed an alternative mechanism of regulating self-reactive B cells namely clonal abortion (134). They propose that all B cells pass through a differentiation stage during which contact with antigen can lead only to the inactivation of that clone. Consequently as autoreactive clones are generated from stem cells they are eliminated by contact with the appropriate antigen before they can pass through that stage of development which leads to deletion. There is evidence for increased susceptibility to inactivation by antigen of bone marrow cells and fetal liver cells relative to spleen cells when exposed to antigen in culture, lending support to the above scheme. However immunocompetent self reactive B cells have been demonstrated in vivo and in vitro suggesting that there are means by which this type of paralysis can be and is avoided.

The One Non-specific Signal Model

This model is based upon the assumption that all thymus independent antigens are polyclonal B cell activators (41). At sufficiently high concentrations, the proliferation and differentiation to antibody secretion is induced in clones which do not produce antibody with specificities against the inducing agent. It is, therefore, postulated that certain subsets of B cells have non-clonally restricted receptors for PBA's which are involved in the induction of these cells to high rate antibody producers. In the case of TIA's, the haptenic determinants are responsible for the

passive focusing of the antigen non-specific PBA stimuli onto the surface of the hapten reactive B cell via surface Ig receptor (121). Evidence for the model derives mainly from experiments done with (4-hydroxy-3-5-dinitrophenyl) acetyl lipopolysaccharide (NNP-LPS). When spleen cells are cultured with low concentrations of NNP-LPS, only high affinity anti-NNP plaque forming cells are observed, but as the concentration of NNP-LPS is increased the high affinity response disappears, and is replaced by one of much lower affinity accompanied by low levels of ^{3}H-thymidine incorporation. At still higher concentrations, NNP-LPS displays PBA properties, there is an increase in the number of antigenically unrelated PFC and in the amount of ^{3}H-thymidine incorporation while the anti-NNP PFC response is largely inhibited. These experiments also demonstrate another important aspect of the model namely that the quantity of stimulus received by the B cell can determine its subsequent reactivity (122, 40).

There are two critical PBA concentrations which totally control B cell responses to these molecules: 1) a lower limit below which insufficient stimuli are supplied to activate the cells but above which the cells become fully committed to proliferation and/or differentiation and 2) an upper threshold above which excess signal results in the temporary "paralysis" of the cell. The results of the NNP-LPS experiments can be explained on the basis of these concepts, at low concentrations of NNP-LPS only those clones of B cells bearing high affinity surface receptors for NNP will be able to concentrate sufficient LPS on their surface to generate an activating signal, therefore large scale proliferation and polyclonal plaque formation are not observed. However, as the concentration of NNP-LPS is further increased the high affinity anti NNP B cells bind PBA at concentrations above the upper threshold of stimulation and become para-

lyzed. While the low affinity NNP reactive cells are not receiving sufficient stimuli to generate AFC. This is assumed to occur at concentrations which are still insufficient to fully activate non-NNP reactive clones so that low levels of nonspecific plaque formation and proliferation are observed. At those concentrations which are high enough to demonstrate the PBA activity of NNP-LPS even the low avidity anti-NNP clones are partially paralyzed and mostly non-NNP reactive PFC are demonstrated in the presence of the strong mitogenic responses which are induced induced by the NNP-LPS.

The main arguments which have been used by Moller et al against an active role for surface Ig in B cell activation derive from observations that pretreatment of splenic B lymphocytes with soluble, aggregated or immobilized antigens fails to effect the response to TDA's (124). Furthermore these results have been interpreted as evidence against those models ascribing a critical role to the pattern of antigen presentation in B cell activation. However another group has successfully induced a response to DNP on polyacrylamide beads thus making this last statement less credible (64). It has also been pointed out that multiple signal models fail to adequately explain the activation mechanisms involved for polyclonal activators.

Although the one signal hypothesis had originally been proposed to explain the activities of TIA's or PBA's, it has been adapted to account for the responses to thymus dependent antigens. It is postulated that there are qualitative differences in the triggering requirements for B cells which are responsive to TDA's as compared to those for TIA reactive cells. The TDA's interact with T cells, either directly or indirectly through the macrophage, to generate a nonspecific factor (NSF). This

factor is capable of activating B cells in a nonspecific fashion and the antigen receptor on the B cell serves only as a means of bringing antigen bearing macrophages or T cells into close enough association with the B cell to expose it to activating levels of PBA. B cell tolerance to PBA's is not permitted by the hypothesis, as surface receptors fail to deliver any signal to the B cell and the antigens themselves do not possess any activating capacity. Therefore those cases where specific B cell tolerance has been reported are rationalized by the authors as being likely due to blockage of the surface receptor, thus, preventing collaboration with NSF secreting cells or possibly blockage of the NSF receptor on B cell surface preventing the receiving of activating stimuli.

Some of the most difficult results for this model to explain are those in which synergism between antigens and PBA's or factors are observed (174,221). In these systems, the numbers of PFC observed in the presence of antigen and PBA are greater than the sum of the numbers of PFC observed with either component alone. In one case it has been demonstrated that extended exposure to a TDA was capable of inducing antigen specific paralyses of B cell function in nude spleen cell cultures. However, if PBA's were also present an antibody response was generated to the TDA. The one signal hypothesis would have predicted that antigen alone would not have any effect whatsoever on the subsequent ability to induce a response to a TDA using a PBA as a stimulus. Proponents of the model argue that the data on positive synergism can be explained if the PBA has an affinity for the antigen thus serving to focus it onto the appropriate B cell. Alternatively, they propose that the TDA may have very low levels of PBA activity such that the addition of PBA raises the level of stimu-

lation to an activating level thus what one is observing is an additive effect of the PBA and the TDA to give a positive response. This explanation is not completely satisfactory as neither the TDA or the PBA alone leads to the generation of PFC to the antigen. Only when both are present in the culture is the response observed. Also there is no explanation proposed for the paralyses induction by TDA alone.

The problem of self-nonself discrimination by B cells is totally bypassed in this model by stating that the recognition of self is strictly a T cell function. Therefore even though self reactive B cells exist they will not become activated as the appropriate T cell is absent. This type of handling of the problem is one of the weaker points of the hypothesis as it does allow for the possibility of nonspecific activation of self reactive clones and one might predict at an alarmingly high rate as numerous self reactive clones would be expected to exist. However, at least one of the original proponents of this scheme has accepted this prediction and dismissed such antibody as having little biological significance (122). Despite the apparent inability of this model to adequately explain self-nonself discrimination, it does offer a very plausible explanation of the mode of activation by PBA's.

A. A Proposed Polyclonal Model of Antigen Dependent B Lymphocyte Activation

The previous section represents a relatively brief survey of several models of B lymphocyte activation with emphasis being placed on the three major hypotheses. One objective of the discussion has been to emphasize that the currect knowledge of B lymphocyte triggering mechanisms is far from complete. The potential of a polyclonal model of T dependent B cell activation in examining this problem is clear and I should like to present a system which I believe could be useful in this context.

Although roles for antigen and T cell factors in the stimulation of B lymphocytes are not unanimously supported it is generally accepted that antigen does play an active or passive part in the activation of B cells. If one accepts that two distinct interactions with the B cell (not necessarily two signals) lead to an optimal response it would clearly be desirable to have a system which would allow for the temporal dissociation of these events 1) Ag-sIg interaction and 2) the supplying of a T cell or macrophage derived signal. Several such systems for the *in vitro* induction of antibody synthesis in which synergism between antigen and factors or mitogens exist and they provide a useful basis for a polyclonal model. Schrader (179) has demonstrated that the addition of FGG to cultures of spleen cells from nude mice can lead to the selective inactivation of the FGG reactive B cells, however, the mitogens LPS or POL can prevent this inactivation if they are added to cultures within 24 hours after the addition of antigen. The need for synergism between the FGG and the mitogens is clearly demonstrated as neither of the two stimuli will lead alone to the generation of anti-FGG plaque forming cells (174). Dutton (52) and

Schimpl and Wecker (170) have independently developed similar systems in which synergy between antigens (SRBC or BRBC) and T cells or their products have been observed. The basic experimental design is to incubate spleen cells from nude mice with the antigen for 24-40 hours during which time proliferation of the antigen reactive clones occurs. Con A activated T cells or T cell factors are then added and the cells cultured for an additional 72 hours at which time the numbers of PFC generated are determined. The results of these three groups of investigations suggest that there may be two seperate events in B cell activation 1) antigen dependent induction of proliferation and/or B cell inactivation and 2) a T cell dependent differentiation step leading to the generation of AFC.

If one were to employ antibodies directed against mouse immunoglobulin as the polyclonal antigen it should be possible to supply the appropriate nonspecific signal to induce a polyclonal antibody response. A basic assumption of such a model is that the interaction of anti-immunoglobulin and surface immunoglobulin is similiar in effect to those which occur when antigen and surface immunoglobulin interact. This assumption is not totally unwarranted, as there is considerable evidence that anti-immunoglobulins in species other than mouse can induce a limited degree of proliferation in the lymphocyte population (188, 69, 222). In no case to date has it been possible to attain antibody formation by treating cells with anti-immunoglobulins alone, in the absence of T cells or added PBA's.

Indeed, in at least two species it has been possible to elicit antibody production using heterologous anti-immunoglobulins and a source of T cell activity. Kishimoto and Ishizaka (105) were able to obtain elevated levels of IgG synthesis in cultures of rabbit lymph node cells which had

been treated with anti-IgG for 24 hours, subsequently washed and cultured in the presence of a nonspecific amplifying factor or T cells primed to the rabbit IgG molecules. When the cells were derived from DNP primed rabbits it was possible to elicit a 'secondary' anti DNP response. Friedman et al (67) observed a response in purified human B cells which had been isolated on affinity columns of anti-Fab antibody and then cultured with PWM or supernatants from tetanus toxoid challenged cultures of T cells. Treatment of the cells with either anti-Fab or PWM failed to elicit either a proliferative response or a PFC response. The failure to observe proliferation is more likely to be a result of the experimental conditions as it has recently been demonstrated that anti-IgG will induce a significant response in human B cells (69).

There are other lines of evidence which also suggest that B cells can respond to antigen in a manner other than simply differentiating to AFC. Roelants and Askonas (164) found that ATXBM mice were incapable of generating a primary or secondary IgG response to hemocyanin unless T cells were added back to the animal. However those animals which had been previously immunized with the hemocyanin,reconstituted with T cells and rechallenged gave an antigen specific IgG response while unprimed ATXBM mice generated a much smaller IgG response. The inference was that B cells required little if any T cell participation for the induction of IgG memory. Similar results were obtained by Davie and Paul (45),however, these authors explored the system in somewhat greater detail. ATXBM mice were primed then reconstituted with T cells, rechallenged and the frequency of ABC's as well as the frequency of IgM bearing ABC's determined. Those ATXBM mice which were immunized but not reconstituted showed comparable or greater numbers of ABC than those which had been reconstituted.

The proportion of IgM bearing ABC's was approximately 4x that observed in the reconstituted mice which showed predominantly no IgM bearing ABC. It was argued by the authors that the conditions were such that B ABC's were being inumerated and the increase in ABC's was not a consequence of T cells being counted. Schrader (176) also presented data which indicated that memory cells could be induced in nude mice, however the mitogen POL was required for the generation of the memory and it is not clear what the potential of the B cells in the absence of mitogen was.

These results suggest that B cells can respond to antigen by proliferating and/or differentiating in the relative absence of T cells. The main point to be drawn from this discussion is that it does appear feasible to dissociate the proliferative and the differentiation events to PFC in a fashion which may be more amenable to biochemical study.

Anti-Immunoglobulin Effects on B Lymphocyte Functions

Immature B Cells

It has been observed by several groups that cells from lymphoid tissues of fetal or neonatal origin and bone marrow are highly susceptible to inactivation by antigen at concentrations which have no effect on the responses of lymphocytes obtained from other adult lymphoid tissues (197,156). These results led to the proposal of the clonal abortion theory to describe how self reactive B cells were deleted(134). The suggestion was that B cells normally pass through a stage in their development during which they are obligatorily tolerized by interaction with antigen, thus, as the self reactive clones developed, they would be inactivated before reaching the antibody secreting stage.

In those cases where the effects of anti-μ on lymphocyte development have been studied there is a very good correlation between the in vitro effects observed with antigen and those seen with anti-immunoglobulin antibody (118). Extended treatment (18-48 hours) of adult bone marrow cells with anti-μ results in the irreversible loss of sIg from these cells and of their capacity to proliferate in response to LPS challenge (197). Adult spleen or lymph node cells are not effected by similar treatments as up to 1000x higher concentrations of anti-μ fail to inhibit the re-expression of sIg once the antibody has been removed (197, 98).

The effects of the antibody do not appear to be mediated by the F_c region of the molecule as both $F(ab')_2$ and Fab are effective in stripping sIg from fetal B cells. It should be appreciated that the ability of the Fab to inactivate these cells does not in itself constitute proof that cross-linking of sIg is not a mandatory part of the inactivation sequence since Fab are very prone to aggregate in solution. Evidence

that this process involved direct anti-μ/sIgM interaction derives from the observations that "Pre-B" cells which lack detectable sIgM are not affected by exposure to antibodies for 2-3 days, so long as the antibody is removed prior to time which sIgM normally appears on these developing B cells (155).

Melchers et al (117) have presented evidence that "Pre-B" cells in 12-14 day fetal liver do express low levels of sIgM which is rapidly turned over ($t_{\frac{1}{2}}$=45 minutes), however anti-immunoglobulin inhibition of LPS induced PFC is not observed until day 18-19 at which time the mitogen reactivity of normal fetal liver cells appears. The data was interpreted as evidence that anti-μ/sIgm intereaction was not sufficient to cause inactivation of the maturing cells. Melchers et al (117) have evidence which suggests that sIgM clearance does not necessarily lead to inactivation of fetal cells if such cells have not reached a certain undefined stage of differentiation. These authors demonstrated the presence of rapidly turning over sIgM on cells from the livers of 12-14d fetuses. The level of sIgM was very low and was not demonstrable by immunofluorescence however evidence was presented to support the notion that the IgM being observed was of membrane origin rather than being derived from the cytosol of damaged cells. The fact that sIgM is present on these "Pre-B" cells but that these cells are not sensitive to anti-Ig inactivation until day 18-19 of fetal life suggests that simple modulation of the sIgM is not sufficient for inactivation of these cells. It appears that sIg clearance is not inhibitory to the developing B cells until they have reached a certain stage of differention and that this period of increased susceptibility to inactivation is transient as peripheral B lymphocytes are much less sensitive to anti-Ig mediated inactivation.

Although the studies on the effects of antigens or anti-Ig's on immature B cells are not extensive it does appear that these cells are much more sensitive to inactivation by receptor/ligand interaction than mature B cell populations. This interpretation should be viewed with some caution as several groups have provided evidence of nonspecific stimuli affecting the range of the B cell repetoire and the differentiation state of B cells. It is therefore quite conceivable that in the secondary lymphoid organs the levels of nonspecific stimulants being produced by the numerous on-going responses are sufficient to shift virgin B cells into a physiological state where they are less susceptible to inactivation by receptor modulation. Thus the cellular environment of the B cells may contribute significantly to the ultimate fate of these cells in a totally nonspecific fashion. In the case of fetal organs and possibly bone marrow the levels of nonspecific stimulation would be expected to be much lower than in the adult organs either as a consequence of a lower level of antigen load or of fewer cells mature enough to produce such factors (136).

Regardless of these points the encouraging fact is that anti-Ig and antigen give comparable results suggesting a similarity in their modes of action.

Mitogen Responsiveness

Polyclonal B cell activators are thought to stimulate B lymphocytes by interacting with mitogen specific receptor molecules on the plasma membranes of the responding cells (139). The binding of the mitogen results in variable levels of proliferation and differentiation which depend upon the PBA used and on the subset(s) of B cells which respond to the activator. The generation of the activating signal by PBA's does not require their binding to sIg as the enzymatic removal of sIg does not effect the subsequent responsiveness of the cells to the PBA's (178). Since mitogens provide a means of assessing the proliferation and differentiation capacities of a large proportion of the B cell population, these agents have been used to monitor the effects of anti-Ig's on B cell functioning.

Elson et al (55) reported that the pretreatment of splenic B cells with anti-Ig's resulted in a marked inhibition of the mitogenic and PFC responses of these cells to LPS. Others have observed that pretreatment with anti-Ig's leads to a reduction in the proliferative responses to LPS, Pol, PPD, FCS, NWSM and trypsin (175, 9 ,158,199). There are however discrepancies as to the conditions which are necessary for demonstrating the inhibition and as to which mitogenic responses are inhibitable by anti-Ig's. Schrader found that pretreatment of B cells for 1 hour with anti IgG before the addition of LPS resulted in reduced thymidine incorporation by these cells. If both anti-IgG and LPS were present during the preincubation step the inhibition was much greater suggesting that the exposure to LPS led to an increased susceptability to anti-Ig mediated inhibition (175). Sidman and Unanue demonstrated inhibition of the proliferation induced by LPS, NWSM, PPD or trypsin

in mitogen pulsed cultures. Cells were treated first with anti-Ig for 1 hour, washed, cultured with mitogen for 1 hour and then washed and recultured for 48-72 hours in absence of added mitogen. Using this protocol, the authors found that the inactivation lasted for at least one week following the treatment and that the Fc portion of the anti-Ig antibody played a key role in the inactivation process as $F(ab')_2$ and Fab were ineffective in causing suppression. This unresponsive state was only demonstrable if a pulse exposure to LPS was employed. Mitogen continuously present in the final culture media resulted in the generation of responses comparable to those of control cultures which had not been exposed to anti-Ig's (199).

In contrast to the above results, several groups have reported that anti-Ig's are either noninhibitory or slightly stimulatory to LPS induced mitogenisis (9 , 99). Anderson et al found that exposure of cells to the $F(ab')_2$ or IgG of anti-μ, K, antibody for 1 hour at 4^oC followed by washing and subsequent culture for 1 hour at 37^oC before LPS lead to a marked inhibition of PFC production without affecting the proliferative responses. At concentrations of antibody 10-100x higher than those needed for inhibition of PFC there was a 1.5 - 2 fold increase in thymidine uptake relative to cultures exposed to LPS alone. The inhibitory effects of the anti-Ig's on PFC production were only observed if the cells were exposed to antibody immediately before culturing them with LPS or if the cells were pulsed with antibody within six hours of exposing the cells to LPS (9). It appeared that cells exposed to LPS for 6 hours lost their sensitivity to anti-Ig inhibition of PFC induction. The cells were not apparently permanently inactivated by the anti-Ig treatment since removal of the anti-Ig-sIg complexes by pepsin or by culturing the cells for 12 hours to allow for

the clearing of the complexes resulted in a population of cells which were fully competent to respond to LPS. If, however, cells were exposed to both LPS and anti-Ig's for one hour, cultured for 12 hours in the absence of mitogen or antibody and restimulated with LPS the PFC response was inhibited. These results were interpreted as evidence for the requirement of a simaltaneous undefined LPS generated signal which in conjunction with anti-Ig binding leads to the inactivation of the B cells.

Kearney et al have also found a dissociation between anti-μ mediated inhibition of LPS induced immunoglobulin production and the synergism with LPS induced proliferation (98 , 99). Continuous exposure to high concentrations of anti-μ antibody and mitogenic concentrations of LPS lead to a two fold increase in the number of cells recovered relative cultures receiving LPS alone; however there was a greater than 95% reduction in the number of Ig producing cells relative to control cultures which received LPS alone. The stimulation required the antibody to be present for at least the final 96 hours of culture. Exposure of the cells to antibody alone for 1 hour at 37^{o}C did not lead to the inhibition of LPS induced immunoglobulin nor did the antibody alone result in an increase in proliferation in these culture.

The results collectively support the notion that anti-Ig/sIg interaction leads to the inhibition of AFC induction. However the effects of the antibodies on the proliferative capacities of B cells are much less consistant and it does not seem justified to make any generalizations about the consequences of anti-Ig treatment on this aspect of B cell function. A point which must be considered when comparing results in this area is the isotype(s) of the surface immunoglobulin which are being bound by the antibody. Especially in light of the observations (see next

section) that cells capable of proliferating or proliferating and differentiating to antibody forming cells in response to LPS may bear different sIg isotypes. The effects of anti-Ig's on proliferation and on differentiation may in reality be on different pools of responding B cells rather than on a single B cell pool. It is consequently much more difficult to assess what effects the antibodies are actually having as in both of these responses (Antibody formation and Proliferation) one is only observing net effect i.e. the contributions of all responding cell pools. Thus very different distributions to responding B cell subsets can lead to the same observed effect.

Selective Isotype Modulation

The demonstration of IgD on the surface of B lymphocytes of several different species has led to a re-evaluation of the roles of sIgM & sIgD functions in B cell activation . In ontogenesis IgM is the first detectable surface immunoglobulin appearing at day 15 of fetal life on liver cells; IgD is not detectable on splenic B cells until several days after birth and does not achieve adult levels until the animals are ~7 weeks of age, at which time it represents the predominant isotype of surface immunoglobulin on spleen and lymph node B cells. This sequence of appearance of sIg isotypes, combined with the knowledge that immature B cells are more susceptible to inactivation by treatment with antigen or anti-immunoglobulins, led to the suggestion that sIgD might be important in preventing the tolerance induction of mature B cells (217). However until very recently the study of IgD function in B cell activation has been hampered by the unavailability of specific anti-δ serum and indirect approaches were employed.

Similar to its human counterpart, murine IgD is extremely sensitive to proteolytic digestion to Fab δ and Fcδ, whereas sIgM remains virtually untouched under similar conditions (22 ,203). Cambier et al treated B lymphocytes with papain under conditions which selectively removed IgD from the plasma membrane thus producing cells with an sIg phenotype similar to that of immature B cells (32). These cells were cultured for 24 hours with various concentrations of TNP-HGG, washed and challenged with either TNP-BA or TNP-SRBC to assess the TI and TD anti-hapten responses respectively. The enzyme treated cells showed a higher degree of susceptability to inactivation than normal adult B cells did for a TD response while that of the TI reactive cells was unaffected. These results were interpreted as

indicating that TD B cells bore IgM and IgD on their surfaces while TI B cells had only IgM, furthermore the sIgM was suggested to have a tolerizing function in that binding of antigen to sIgM in the absence of sIgD would lead to inactivation of the cells. A criticism of this approach was that papain is nonspecific in its action and even though none of the five other surface molecules which were examined showed any reduction following exposure to the enzyme it was still possible that another surface molecule was being effected by the treatment and that sIgD removal was not the causative event in the increased susceptibility to inactivation. More recently both allogeneic and xenogeneic anti-δ antisera have become available and a more comprehensive examination of IgD function has been undertaken.

Vitetta et al (215) repeated the above experiments, using a rabbit anti-δ serum to modulate off the sIgD in the presence of a TD antigen, and then examined the ability of the cells to subsequently respond to TI and TD forms of the same hapten. The results were identical to those obtained using papain to remove the sIgD. Scott et al (184) reported on a system very similar to that of Vitetta et al (215) in which sIgM or sIgD was removed with specific antisera and the cells were cultured for 24-36 hours in the presence of the TD antigen Flu-HGG and the antisera. The cells were then washed and challenged with Flu-PoL. The responses of those cells which were treated with anti-μ, anti-δ or TD antigen in the first culture were not affected upon challenge with the TI antigen, however, cultures containing both anti-δ and antigen were markedly inhibited. The inhibition was hapten specific and not mediated by anti-μ thus indicating that the inhibition was isotype specific. These results seemed to be contradictory to those of Vitetta et al for in this case the response to

the TI antigen was inhibited by anti-δ plus antigen suggesting that the population of cells responsive to POL were δ^+ cells rather than the $\mu^+\delta^-$ phenotype proposed for TNP-BA reactive cells by Vitetta et al (215). Both groups had data which suggested that sIgD in some way prevented B cell inactivation but for different B cell populations.

The problem appears to have been partially resolved by the establishment of subpopulations of B cells reactive to different forms of T independent antigens (230). The TI-1 antigens are capable of eliciting responses in B cells from neonates or CBA/N mice which are both characterized by a high μ to δ ratio of sIg. The TI-2 antigens can activate a population of cells present only in adult spleens or lymph nodes of normal mice but absent from those of CBA/N mice. This more mature population is characterized by the presence of a high δ to μ sIg ratio. When normal spleen cells were challenged with a TI-1 antigen (TNP-BA) or a TI-2 antigen (TNP-AECM-Ficoll) in the presence of anti-δ alloantisera the TI-1 response was unaffected while the TI-2 response was strongly inhibited. These results were found to be true for several TI-2 antigens and were interpreted to mean that at least some TI-1 reactive cells lack functional sIgD while TI-2 cells appear to possess this isotype in an active form.

Cambier et al (31) extended the above observations by culturing cells continuously in the presence of anti-μ, anti-δ or anti-Ig sera together with either the TIA (TNP-BA) or TDA (TNP-SRBC). Anti-δ had no effect on the TNP-BA response but it fully inhibited the TD response. Anti-Ig which would fully modulate off both sIgM & IgD totally suppressed the TD and the TI responses as one might expect since the cells were presumably devoid of receptors for antigen. The anti-μ inhibited both TI and TD responses although the effects on the TI response at low antibody concen-

trations were somewhat variable.

Scott et al(109,183,182)have performed the most extensive examination of the reactivity of δ^+ bearing cells. The general approach has been to use affinity columns for the purification of δ^+ cells from the spleens of 3-4 week old animals. The spleens of these animals contain $\mu^+\delta^-$ and $\mu^+\delta^+$ bearing cells in approximately equal proportions. Various parameters of B cell function were then examined in the purified populations. When reactivity to Flu-POL was assessed following fractionation to bulk of the responding cells were in the $\mu^+\delta^+$ population. Interestingly pretreatment of the cells with antiδ for the fractionation resulted in a 2 - 5 fold reduction in Flu-POL precursor frequency which was revealed only when limited dilution analysis was performed. Similarly when the ability of μ^+ and δ^- cells to be cloned in agar was determined the δ bearing cells represented the majority of the colony forming cells in the adult spleen, furthermore the presence of anti-δ in the agar inhibited the formation of colonies by δ^+cells. These authors are,however, quite emphatic in pointing out that although IgD does appear to be diagnostic of a more fully immunocompetent cell type the presence of this isotype is not essential for B functioning as 1) fetal liver and neonatal B cells which lack sIgD can be readily cloned in agar, 2) although the δ^- populations contain lower frequencies of Flu-POL responsive cells they do respond,3) anti-δ suppressed mice are fully capable of generating a primary and secondary IgM response. Indeed,some of the most puzzling data as to the function of sIgD derives from experiments in which lymphocytes are stimulated with the mitogens LPS (23) or PWM (153) and then examined for the presence of sIgM and sIgD. There is a marked increase in the ratio of μ/δ by day 2 as determined by surface iodination (23) or immunofluorescence. This

increased ratio persists for the duration of the cultures (23, 153). Since the bulk of the LPS stimulated cells revert back to small lymphocytes under the conditions employed (13), it must be asked whether these small lymphocytes are more easily tolerizable because of their loss of sIgD, or whether a change in sIg phenotype with the loss of sIgD represents a further maturation of the cells as has been found for IgG memory B cells (19). A recent observation by Mosier et al that immunization of neonatal mice at a time 2 weeks before the animals are capable of mounting an immune response to the antigen results in an increase in the subsequent response by these animals to the antigen suggesting that memory induction may be generated by a TI antigen in a population of cells which can not be induced to differentiate to AFC. A further implication is that sIgD may not be required for the induction of cells to the memory stage or at least that TI-2 interaction with cells which can respond only to TI-1 antigens does not necessarily lead to inactivation of those cells (127).

The part played by IgD in B cell induction and regulation is far from clear at this point, however, the effects of modulating one sIg isotype off a B cell on subsequent responsiness are perhaps the most direct and compelling evidence for an active role of sIg-ligand interaction in B cell regulation.

Anti-immunoglobulin induced proliferation of lymphocytes

The observation by Sell et al(190)that anti-immunoglobulin reagents caused increased levels of blast transformation and thymidine incorporation in rabbit lymphocytes in vitro led to numerous attempts to elicit similar responses from lymphocytes of other species using anti-immunoglobulin reagents. The results of such studies have been quite variable and the responses of rabbit lymphocytes still remains as the prototype for anti-Ig induced stimulation with the lymphocytes from only a few other species showing similar properties.

Sell et al reported that antibodies directed against the rabbit a and b locus allotypes as well as antibodies to purified IgA, IgG, IgM and isolated L chains,led to very high levels of blast transformation in PBL's and splenic lymphocytes (187,188,190) after 72 hours in culture. The observation that heterologous as well as allogeneic antisera do induce blast transformation has been confirmed by several groups (43 , 57 ,29),however, there is considerable variation in opinion as to what are the optimal conditions for stimulation. Optimal exposure periods of the cells to antibody range from a few hours (187) up to a requirement for continuous exposure (43) to generate an optimal response. Cross linkage of sIg was originally reported not to be an obligatory requirement for lymphocyte activation (189) however others have found only $F(ab')_2$ or the intact IgG molecules of anti-Ig were capable of eliciting either a proliferative (57) or a differentiative response (106). There has however been general agreement that anti-immunoglobulin stimulation does not lead to the generation of AFC (106,180,190). Several points about the nature of the responding cell types in cultures of rabbit lymphoid tissues bear mentioning. Whereas it has been observed that

50% of spleen cells or PBLs stain with fluoresceinated anti-IgM and only about 10% these cells bear also sIgG (146) as high as 80% of the cells undergo blast transformation using anti-IgM or IgG reagents (187). Thus the apparent numbers of responding cells exceed the numbers of cells bearing the specific class of immunoglobulin. Such results may be interpreted as 1) evidence of the recruitment of non-Ig bearing cells by sIg^+ cells following anti-Ig treatment,2) an indication that the activation of some of the cells does not involve sIg/anti-Ig interactions but possibly Ig/anti-Ig complexes,or 3) a suggestion that the extent of blast transformation is an overestimate possibly due to the selective recovery of blasts after 3 days in culture. The thymidine incorporation data lends some support to the last point since stimulation indices of 5 - 10 are routinely observed with anti-Ig, in other systems involving mitogen induced proliferation,in which only 15-20% of cells undergo blast transformation,stimulation indices as high as 15-25 are observed (43). Rabbit lymphocytes can be divided into subsets of 'T' and 'B' cells on the basis of sensitivity to mitogens (29 ,159,191) the presence of thymocyte surface markers (159) or adherence to nylon wool (160). Using such procedures it was found that purified B cells respond to anti-Ig treatment (29 ,159). However, it was pointed out that there was some anti-Ig responsiveness also in the T cell fraction (160). When PBL are examined for the presence of sIg using anti allotypic antisera in a mixed antiglobulin rosetting assay as high as 80% of the cells can be shown to bear IgG allotypic markers (191) in low quantities. The bulk of the PBL are responsive to PHA, PWM, Con A and anti-Ig but not to LPS; if an analogy is to be drawn between the mitogen reactivities of B and T lymphocytes in other species a portion of the anti-Ig responsive cells would

be classified as T cells (93). This suggestion gains credibility with the recent observations that some rabbit thymocytes carry immunoglobulin V_H allotypic markers (108). The data collectively suggests that B cells are the predominant responding cell type to anti-Ig in the spleen but that in certain lymphocyte pools e.g. blood a significant proportion of the responding cells may be T cells. The question as to a requirement of T cells for anti-Ig induced proliferation remains open at this point although some reports indicate that the requirement for T cells, if any, is rather low (34).

Chicken lymphocytes also have been shown to undergo blast transformation and to incorporate thymidine following treatment with anti-Igs (104). Alm and Petterson (5) reported that rabbit anti-chicken IgG antisera caused an increase in thymidine incorporation by chicken lymphocytes after 48-72 hrs. in culture. The response was not radiation sensitive, but bursectomy and irradiation of the lymphocyte donors markedly inhibited the response suggesting that the target cells were B cells. Skamene and Ivanyi (204) found somewhat contradictory results in that anti-IgG antibody did not induce proliferation or inhibit the subsequent responsiveness of the cells to challenge with anti-IgM. The stimulation indices which they reported were considerably lower than those of the other groups which was consistent with the observation that only about 10% of the cells were undergoing blast transformation. Weber (222) has recently presented evidence that there is clearly a B cell component in the response elicited by rabbit anti-chicken Igs but points out that he has no data to rule out a T cell requirement for or a T cell contribution to the increase in thymidine incorporation.

Pig lymph node cells respond to challenge with anti-IgG by increasing

thymidine incorporation and the rate of phosphatidyl-inositol turnover (113). Bivalency of the antibody was required as only the intact molecule or the $F(ab')_2$ induced the above changes. No data werepresented as to the responding cell type however only a small proportion of the population responded i.e. approximately 10% of the lymph node cells underwent blast transformation (personal communication M. J. Crumpton).

The responses of human lymphocytes to anti-Ig treatment have proven to be very variable with results ranging from no effect (86 ,77) to marked increases in thymidine incorporation (69). Holt et al (86) failed to induce blast transformation with several rabbit antisera against purified IgG or IgM. A modest increase in blast transformation has been observed on day 6 using anti-IgG but not with anti-IgM antibody (2), in another case anti-IgM, IgG and IgA were all found to be stimulatory causing the induction of 8-10% blasts (138). Greaves (77) reported that consistant stimulation with anti-immunoglobulins was not observed using either anti-IgM or anti-IgD but that anti L chain antisera produced the most consistant stimulation. Froland and Natvig (68) prepared antisera to the $F(ab')_2$ of normal human IgG and reported a mean stimulation index of 4.1 in 41 separate experiments. That all antisera do not posses this stimulatory activity was demonstrated by Oppenheim et al (138) when observed that only $\sim$ 50% of over forty monkey antisera to human immunoglobulins were mitogenic.

Recently Gausett et al (69) reported that rabbit antibodies to highly purified human IgG, IgM and IgA induced significant levels of ^3H-thymidine incorporation in lymphocytes from various lymphoid organs. They were able to show an absolute requirement for both T and B cells for the motogenesis suggesting that collaboration between the two cell types was

necessary for the stimulation to occur. However, these workers also found that T cells in the presence of mitomycin treated B cells could give rather a strong response to challenge with anti-Ig, and this was interpreted as evidence for a T cell component in the proliferative response.

The pooled data on anti-Ig induced mitogenesis would appear to support the notion that such antisera can in some cases cause blast transformation, but with the observed variability of different antibodies one must consider carefully what are the critical determinants which these antisera are recognizing. Does the mitogenesis depend upon reaction with selected regions of the Ig molecule or possibly upon cross reactions with determinants present on other molecular species on the cell surface.

The lack of activation of murine lymphocytes with anti-Ig has been a point of considerable discussion and speculation as to whether sIg could be critically involved in the triggering mechanism (79). However within the last two years there have been several reports of anti-Ig induced mitogenises of murine lymphocytes. Parker (144) was the first to obtain evidence of anti-Ig mediated activation using rabbit anti-K antibodies immobilized on polyacrylamide beads. An increase in I-UdR incorporation was observed 48-72 hours after exposure to the antibody which was comparable to that induced by LPS. However no evidence of polyclonal antibody formation was found.

It was subsequently reported by Weiner et al (225) that soluble rabbit anti-IgM antibody caused a 10-15 fold increase in thymidine incorporation in lymphocytes obtained from mice greater than seven months of age. The mitogenesis required bivalent binding but not the Fc and occured in all fracitons enriched for B lymphocytes (226). In an extensive series of experiments this group has demonstrated that with NNTX or ATXBM cell

donors the responsiveness to anti-Ig occured somewhat earlier than in control animals (227). The same authors went on to point out that even though the anti-Ig induced proliferation appears to be T cell independent the development of the ability to respond appears to be under the regulation of T cells. When a comparison was made between the responses of spleen cells from aged mice and young mice to the IgG and $F(ab')_2$ fractions of the antibody it was found that whereas old mice responded to both of the preparations those cells from young mice were activable only by the $F(ab')_2$ of the anti-IgM. The majority of responding cells were B cells as demonstrated by the presence of Ia and FcR, however these cells did not have detectable sIg. If however the cells were washed free of the anti-Ig and cultured for 24 hours in fresh medium at least 80% of the blasts could be shown to posses sIgM (228).

In an attempt to elucidate the role of T cells in the response to the intact IgG of anti-immunoglobulin mixtures of T cells from young mice and B cells from old mice were examined to determine if suppressor T cells were present in the young animals. The B cells of the old mice were fully reactive to the antibody. In fact, the addition of T cells resulted in a significant increase in the levels of incorporation in these cultures relative to those containing B cells alone. Furthermore, T cells from old mice did not support a proliferative response in B cells from young mice, thus, suggesting that the inability to respond to the antibody is a function of the B cells of young mice (186).

The results have been incorporated into a hypothesis suggesting that anti-immunoglobulin reagents have two effects, one positive due to the binding to the surface Ig and the other negative mediated via the binding of the Fc to the FcR of B cells (186). In aging mice it would appear that

the inhibitory capacity of the FcR has some how been lost so that only a positive signal is generated following binding of the anti-Ig. It will be of considerable interest to know if B cells from aging mice bear FcR and to determine what role T cells might play in either the acquisition or loss of FcR on B cells. Although this model is an attractive one for explaining the failure of earlier groups to find anti-Ig induced mitogenesis of mouse lymphocytes several reports indicate that Fc bearing anti-Ig antibodies can activate murine lymphocytes.

Sidman and Unanue (201) observed that rabbit anti-IgM, IgG/L and all antisera to IgD failed to induce lymphocyte proliferation. If, however, the reducing agent 2-mercaptoethanol (2-Me) or a component derived from FCS treated with 2-Me was added to the medium with anti-IgM, a modest increase in thymidine incorporation was observed after 72 hours in culture. The anti-IgM also induced a response in spleen cells treated with anti-Thy 1 and C' suggesting that the responding cell type was a B cell and that little if any T cell participation was required in generating the response. Cells from neonatal mice (9d of age) were suppressed by the anti-IgM. An anti-IgG serum containing antibodies to both γ and L chains had no demonstrable effect on the proliferative response of anti-IgM treated cells suggesting that this specific antiserum lacked reactivity against the sIg determinants required for activation.

Sieckman et al (199) have also reported anti-Ig induced mitogenesis in murine cells using goat anti μ, μ/K and rabbit anti mouse K chain antiserum in media containing 2-Me. The antiμ reagent was the most stimulatory inducing peak responses on day 3. On a molar basis the $F(ab)_2$ and the IgG of the goat anti-μ were of equal potency ruling out Fc involvement in this

process. The response was apparently not a consequence of endotoxin contamination as the LPS non-responder strain C3H/HeJ was also activated using the same antibody preparations. Interestingly, there appeared to be some genetic restrictions in the ability to respond as CBA/N mice failed to respond. These authors did not examine the role of T cells in the response but reported that there was no detectable differentiation of cells to the antibody secretion stage.

In conclusion, it would appear that the evidence in favour of anti-Ig inducing mitogenesis of lymphocytes from several species is quite overwhelming. However, it is not clear at this point that the process is T independent or even whether it is totally a B cell phenomenon. I shall return to a discussion of these points and attempt to explain some of the discrepancies in the data in light of my own results.

Methods

Animals

Male $B_6D_2F_1$ mice (C57BL/6 X DBA/2)F_1 were used throughout the experiments. The animals were supplied initially by North American Laboratory Supplies, Gimli, Manitoba, and later by Jackson Laboratories, Bar Harbor, Maine. The mice were used at 6-12 weeks of age, unless otherwise indicated. New Zealand white rabbits of either sex were obtained through the Central Animal Care Services (CACS) of the University of Manitoba. All of the animals were maintained in the CACS facilities and allowed free access to food and water.

Sheep Erythrocytes

Sheep blood was collected from a single donor in sterile vacutainers containing EDTA and stored at $4^{o}C$ for at least one week before use. Prior to use as antigen or target cells the erythrocytes were washed 3x in at least 10 volumes of 0.85% NaCl.

$F(ab')_2$ of Normal Mouse IgG

Normal Mouse serum (30 mℓ) was heated at 56^{o} for 45 minutes then centrifuged to remove aggregated material (20,000 Xg, 20 min.). The immunoglobulins were precipitated in the cold by slowly adding an equal volume of saturated ammonium sulfate (SAS), pH 7.0, the resulting pellet was washed twice in cold 50% SAS, redissolved in distilled water and dialyzed exhaustively against PBS pH 7.2. The resulting crude immunoglobulin preparation was passed through a column containing 10 mℓ of Protein-A-Sepharose-Cl-4B (Pharmacia Fine Chemicals), the column was then washed with 50 mℓ of PBS pH7.2 and the bound IgG eluted with 0.1M acetic

acid -0.85% NaCl (71). The IgG fraction was dialyzed against PBS pH 7.2, concentrated by ultrafiltration through an Amicon XM50 membrane and applied to a Sephadex G-200 column (90x2.5 cm). The IgG fraction was pooled, concentrated to 15 mg/mℓ and dialyzed against 0.1M sodium acetate buffer pH 4.5.

The $F(ab')_2$ of this IgG preparation were prepared by digesting with pepsin (2% W/W) at 37°C with continuous stirring (131). The reaction was stopped after 20 hours by raising the pH to 8.0 with solid tris(hydroxymethyl)aminomethane (TRIS) and the precipitated material removed by centrifugation (20,000 x g for 20 min). The $F(ab')_2$ and the intact IgG were separated from the pFc and the peptide fragments on a Sephadex G-200 column (90 x 2.5 cm). The IgG and the $F(ab')_2$ were poorly resolved as the digestion appeared to be only about 50% efficient on the basis of the areas under the two peaks, therefore, the two peaks were pooled and passed through a Protein-A-Sepharose-Cl-4B column to remove the intact IgG. The unbound material was collected, dialyzed against saline, adjusted to a concentration of 500 μg/mℓ and stored frozen at -80°C. The yield of $F(ab')_2$ was 10 mg from a starting pool of 50 mg of IgG.

Antisera

Several polyspecific antisera (RMG) were raised against the 40% saturated ammonium sulfate precipitable proteins of normal $B_6D_2F_1$ serum. In addition to these, two antisera (RMF) were raised against the $F(ab')_2$ of normal mouse IgG prepared as described above. All antisera were prepared according to the following immunization scheme. Rabbits were injected subcutaneously at multiple sites with a total of 0.5-1.0 mg of antigen in complete Freund's adjuvant and boosted 4-6 weeks later with 0.5-1.0 mg

of antigen in incomplete Freund's adjuvant. Two weeks later the animals were bled and these sera tested for antibody activity by immunoelectrophoresis and immunodiffusion against normal mouse serum proteins. The rabbits were bled at two week intervals thereafter and boosted again if the activity of the antisera began to decrease.

All rabbit sera were heated at 50°C for 45 minutes and absorbed at 0°C with at least 1 mℓ of packed mouse red blood cells per 10 mℓ of serum for 60 minutes prior to purification. The IgG fraction was isolated from whole sera using a Protein A Sepharose-C1-4B column. Preliminary experiments using commercial DEAE purified rabbit IgG had shown that ~98% of the IgG was bound and recoverable from the Protein A Sepharose column.

The sera were centrifuged to remove aggregated material (58,000 x g, 20 min) before being applied to the column. Ten to twelve millilitres of serum were passed through a column containing 10 mℓ of Protein A Sepharose-C1-4B at a rate of 40-60 mℓ/hr. The sample was allowed to run almost completely into the gel bed before washing the column walls down with a volume of PBS,pH 7.2 equal to the starting sample volume. This was also allowed to run almost completely into the gel and the column was then washed with PBS until the effluent absorbance at 280 nm reached baseline levels. Any excess buffer was removed from above the gel and approximately 40 mℓ of 0.58% V/V glacial acetic in 0.85% NaCl was added for the elution of the IgG. Once the eluate OD_{280} was to baseline, the column was washed with PBS,pH 7.2 until the effluent was pH 7.2 and either reused immediately or stored at 4° in PBS pH 7.2 containing 0.1% NaN_3. The eluted IgG was neutralized with 1N NaOH, concentrated to 10-20 mg/mℓ by ultrafiltration (Amicon XM 50) and stored at -20°C. An extinction coefficient of 14.1 for a 1% IgG solution was used in all calculations

to determine IgG concentrations.

$F(ab')_2$ Preparation from Rabbit Sera

The concentrated rabbit IgG solutions (15-20 mg/mℓ) were dialyzed overnight at 4°C against 0.1M sodium acetate buffer, pH 4.5 and cleared of aggregated material by centrifugation (58,000 x g, 20 min). The IgG was stirred at 37°C for 18-20 hours with 1.5% W/W pepsin (Sigma Chemicals) after which sufficient solid Tris was added to raise the pH to 8.0. The mixture was either frozen and stored or fractionated immediately. During the digestion the pepsin IgG solution became turbid as the pepsin was added, however, the solution cleared almost completely once the pH was raised to 8.0.

Purification of Rabbit $F(ab')_2$

The peptic digest of the IgG (150-200 mg) was applied to a G-100 column (2.5 x 90 cm) and run at a flow rate of 20-25 mℓ/hr. The leading peak, which consisted of $F(ab')_2$ and undigested IgG, was collected and passed through a Protein A Sepharose column. This step removed any intact IgG and the unbound fraction consisted of $F(ab')_2$. The $F(ab')_2$ fraction was concentrated by ultrafiltration to 5 mg/mℓ, dialyzed against saline and sterilized by filtration.

This method proved to be much faster and to give purer products than using Sephadex G-200 for the separation of $F(ab')_2$ and IgG. It also had the advantage that the peaks did not have to be cut to exclude IgG thus reducing the losses of $F(ab')_2$ during the purification.

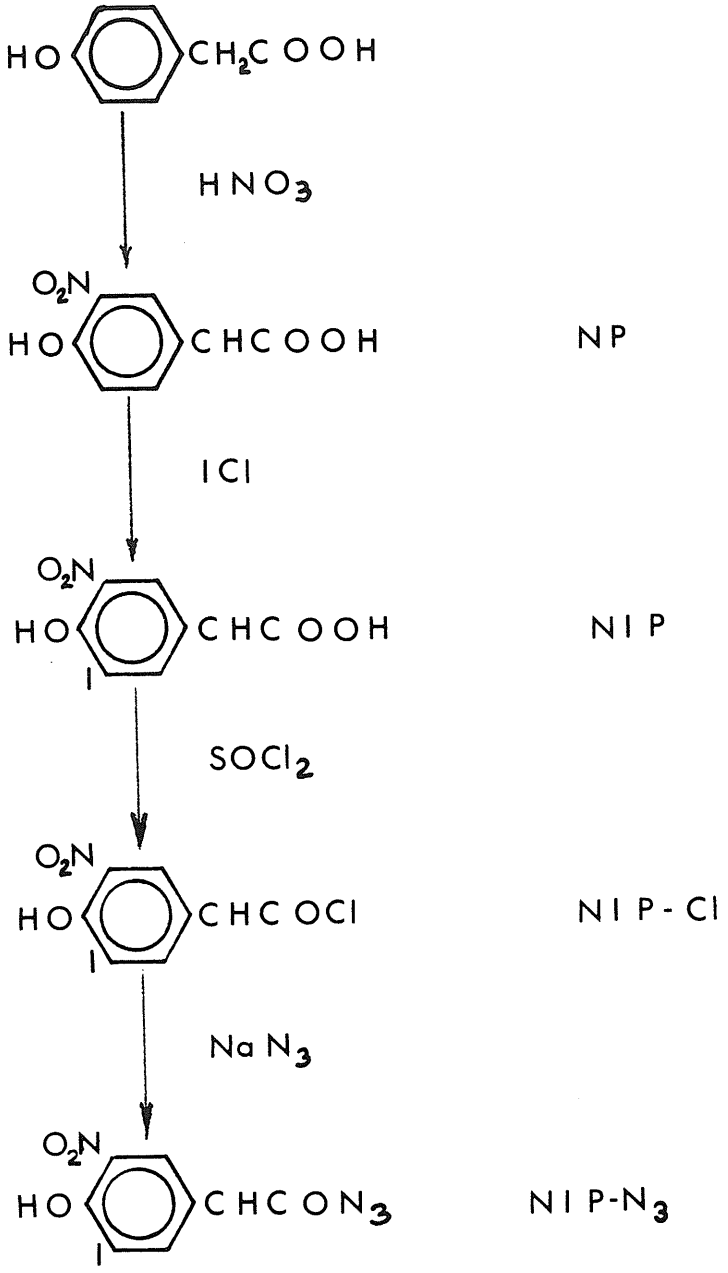
The Synthesis of 4-Hydroxy-5-Nitrophenylacetic Acid (NP)

The compounds 4-Hydroxy-3-nitrophenylacetic acid (NP) and 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) were prepared according to the method of Brownstone et al (28) and the NIP-azide was synthesized from its acid chloride as described by Hatcher and Mäkela (84). A flow chart is given (Fig.1) which outlines the steps in the synthesis of NIP-azide.

Twenty grams of 4-hydroxy-phenylacetic acid (Aldrich Chemicals) were dissolved in glacial acetic acid (110 mℓ) which had been cooled to just above the freezing point of the acetic acid (~12°C), a mixture of concentrated nitric acid an acetic acid (8 mℓ HNO_3 + 16 mℓ HOAc) was then added slowly with mixing so as to maintain the temperature at less than 15°C. Once all of the acid was added the mixture was left to stand for two hours at ~15°C, and the resulting crystals collected by filtration. The product was dissolved in boiling water and recrystallized twice at room temperature. The yield of NP was 8.5 g which corresponded to 33% of the theoretical yield.

4-Hydroxy-3-iodo-5-nitrophenylacetic acid (NIP)

Three grams of NP were dissolved in glacial acetic acid (150 mℓ) and iodine monochloride (BDH Chemicals), (3g), in acetic acid (30 mℓ) was added slowly with continuous stirring. The reaction mixture was heated to 60°C for 30 minutes after which 3 volumes of water were added slowly and the product allowed to cool to room temperature. A sufficient volume of a sodium metabisulfite solution (20% w/v) was then added to change the colour of the supernatant from a red-brown to a pale yellow. The crystal-


HO
CH2COOH
HNO3
O2N
HO
CHCOOH
NP
ICl
O2N
HO
CHCOOH
I
NIP
SOCl2
O2N
HO
CHCOCl
I
NIP-Cl
NaN3
O2N
HO
CHCON3
I
NIP-N3

lized product was collected by filtration, air dried and dissolved in a minimum volume of boiling ethanol (~100 mℓ). The NIP was recovered by adding sufficient water (9 mℓ) to just initiate crystallization from the hot ethanol and then allowing the solution to cool overnight to room temperature. The yield was 61% of the theoretical yield (3.90 g).

4-Hydroxy-3-iodo-5-nitrophenylacetyl chloride (NIP-Cl)

All apparatus were fitted with calcium chloride drying tubes and all joints were sealed with silicon grease as the acid chloride and azide are readily converted back to NIP upon contact with moisture. Small batches of the azide were prepared in sufficient quantities to be used within one to two weeks after synthesis. NIP (0.5 g) was added with stirring to 20 mℓ of purified thionyl chloride at 70°C, the solution was then refluxed for 30 minutes with stirring. The heat source was removed, 6 mℓ of dry benzene added and the flask fitted with a short condenser topped with a stopcock. During the reaction droplets of a thick brown oil formed and these coalesced to form a layer in the bottom of the flask once the heating and stirring were stopped. The benzene was removed under reduced pressure using a water aspirator and the residue washed twice more with dry benzene (6 mℓ) to remove any remaining thionyl chloride.

4-Hydroxy-3-iodo-5-nitrophenylacetyl azide (NIP-N_3)

The acid chloride from the previous step was assumed to give the theoretical yield and was not weighed because of its lability. The NIP-Cl was dissolved in 6 mℓ of acetone and chilled in an ice water bath. A cold

solution of sodium azide (105 mg in 0.375 mℓ H_2O) was added dropwise and the mixture stirred for 10 minutes in an ice water bath. The slow addition of ice-cold water to the mixture resulted in the formation of a precipitate which was collected by filtration, washed with ice water and lyophilized. The azide was stored desiccated at -20°C for short periods of time (1-2 weeks). The NIP-azide did not usually form discrete crystals even after lyophilization but formed a coating in the storage vessels. It had a melting point of 66-69°C with decomposition.

Both NIP and the N-hydroxysuccinimide ester of NIP (NIP-oSu) are now commercially available from Biosearch, 3095 V. Kerner Blvd., San Rafael, CA 94901. The NIP-oSu appears to be a much easier reagent to handle than the NIP-azide as the former has a much greater stability and better solubility in aqueous media. It would therefore be advisable in future work involving the hapten NIP to synthesize the NIP-oSu rather than the azide.

Ficoll and Derivatives

Several different antigens were synthesized utilizing Ficoll 400 (Pharmacia Fine Chemicals) as the matrix to which the haptens 4-hydroxy-3-iodo-5-nitrophenyl acetic acid (NIP) and 2,4-dinitrobenzene (DNP) were coupled.

2,4-Dinitrophenyl Lysyl Ficoll (DNP-Lys-Ficoll)

This compound was synthesized according to the method of Sharon et al (19). One gram of Ficoll was dissolved in 4 mℓ of 1N NaOH and 500 mg of $KHCO_3$ were added with continuous stirring. Two millilitres of a

solution of dimethyl formamide containing 300 mg of trichloro-S-triazine (cyanuric chloride) were added slowly to the alkaline Ficoll solution. It was important to have the Ficoll solution stirring while the triazine was being added as it tended to cause gelation of the Ficoll if the reactants were not adequately mixed. Once the triazine was in solution, the mixture was stirred for 4 minutes and a solution containing 1 gm of 2, 4-dinitrophenyl-L-lysine (DNP-Lysine) in water (adjusted to pH 9.0 with 1N NaOH) was added. The solubility of DNP-Lysine at room temperature was quite low at this pH and it was found that about 400 mℓ of water were required to dissolve one gram of DNP-lysine. The reaction mixture was stirred overnight at room temperature and dialyzed extensively and sequentially against distilled water and saline. The DNP-Lys-Ficoll was centrifuged to remove aggregated material, sterilized by passage through a millipore filter (0.22μ) and stored frozen at -20°C until required.

The degree of conjugation of DNP-lysine to Ficoll was calculated by determining the concentration of DNP-lysine in the sample, using a molar extinction coefficient of 1.74×10^4 at 365 nm; and the carbohydrate content of the sample utilizing a modification of the method of Dubois et al (50). Briefly, the method consisted of mixing 0.5 mℓ of a Ficoll solution containing 5-50 μg of carbohydrate with 0.1 mℓ of a freshly prepared 10 fold dilution of an 80% phenol stock in distilled water. Concentrated sulfuric acid (1.2 mℓ) was added directly to the surface of the Ficoll/phenol solution and stirred virgorously immediately. This step was found to be critical as any delay in mixing resulted in a reduction of the amount of colour developed. The most reproducible results were obtained by adding the

sulfuric acid with a Cornwall repeating syringe fitted with an 18 gauge stainless steel needle and stirring immediately with a vortex mixer. The reaction mixture was held at room temperature for 20 minutes, and incubated at 30°C for 20 minutes. The samples were allowed to cool to room temperature and the absorbance determined at 490 nm against a phenol-sulfuric acid blank. Dubois (50) had reported that it was necessary to distill the phenol before using it to prepare the stock phenol solution. However, it was observed that the use of an analytical grade phenol obviated the need for the distillation step.

N-(2-aminoethyl) carbamylmethylated-Ficoll (AECM-Ficoll)

AECM-Ficoll was prepared according to the method of Inman (90). A 1.35 M solution of sodium chloroacetate was prepared by dissolving 64.4 g of chloroacetic acid in 435 mℓ of an NaOH solution (300 mℓ H_2O plus 135 mℓ 5.0 N NaOH) and adjusting the pH of the solution to 7.0 at 25°C with either 5N NaOH or a 10% W/V chloracetic acid solution. The volume was adjusted to 500 mℓ with distilled water.

Carboxymethylated-Ficoll (CM-Ficoll)

Ficoll (Pharmacia Fine Chemicals) (13.3 g) was dissolved in 185 mℓ of 1.35 M sodium chloracetate solution and brought to 40°C by immersion in a water bath. The reaction was started by mixing 50 mℓ of 10 N NaOH at 40°C with the Ficoll solution followed by the addition of 15 mℓ of water (40°C). The solution was maintained at 40°C with stirring for 2 hours after which 2.76 g of NaH_2PO_4-H_2O in 12 mℓ of H_2O were added and the pH adjusted first to 9.0 by the addition of concentrated HCl and finally to pH 7.0 with

5 N HCL. The solution was adjusted to a volume of 500 mℓ with distilled water and dialyzed in the cold for 5 days against 6 changes each of 4 litres of distilled water. The CM Ficoll was recovered by lyophylization. The CM-Ficoll was cream coloured as compared to that of the starting material, Ficoll, which was white. The yield was 14.0 gm.

N-(2-aminoethyl) carbamyl methylated-Ficoll (AECM-Ficoll)

CM-Ficoll (6 g) was dissolved in 200 mℓ of distilled water and ethylene diamine dihydrochloride (28.6 g) was added with stirring. The pH of the solution was adjusted to 4.7 with 1N NaOH and ethyl dimethylamine propyl carbodiimide hydrochloride (3.5 g) was added over a 10 minute period with stirring. The pH was maintained at 4.7 during the addition of the carbodiimide and throughout the reaction with 1N NaOH or 1N HC1. After 3.5 hours at room temperature, the reaction mixture was dialyzed first against 0.5M NaC1 for 2 days and then distilled water for 4 days. The AECM-Ficoll was recovered by lyophylization and stored in a vacuum desiccator. The degrees of substitution of the Ficoll derivatives were determined by hydrogen ion binding measurements as outlined by Inman & Dintzis (91).

DNP-AECM-Ficoll

AECM Ficoll (1 gm) was dissolved in 16 mℓ of 1M $Na_2CO_3/NaHCO_3$ buffer pH 10.6 and 100 mg of 2,4 dinitrobenzene sulfonic acid, sodium salt was added slowly (90). Once all of the salt had dissolved, the reaction mixture was passed through a sterile 0.45μ millipore filter into a sterile stoppered vial and left at room temperature in the dark for 36 hours. The product was dialyzed extensively against distilled water and lyophilized.

The degree of DNP conjugation was determined by taking the absorbance at 365 nm of a solution of known concentration of DNP-AECM-Ficoll and correcting for the weight contribution of the DNP groups to the dry weight of DNP-AECM-Ficoll. No correction was made for the absorbance due to AECM-Ficoll at 360 nm since this was negligible in comparison to the DNP-Lysine molar extinction coefficient of 1.74×10^4.

NIP-AECM-Ficoll

AECM-Ficoll (500 mg) was dissolved in 5 mℓ of water and mixed with 7 mℓ of 0.2 M sodium bicarbonate. Freshly prepared NIP-azide (19 mg) was dissolved in 2 mℓ of dimethyl formamide and added slowly to the Ficoll solution. The reaction was allowed to proceed overnight at 4^oC with continuous stirring after which the product was dialyzed exhaustively against distilled water before being lyophylized. The degree of substitution was determined by taking the absorbance of a solution of known concentration of NIP-AECM-Ficoll at 430 nm, pH 8.4 and correcting for the weight contribution of the NIP groups to the dry weight of the NIP-AECM-Ficoll. No correction was made for the absorbance due to AECM-Ficoll at 430 nm since this was negligible in comparison to the 5×10^3 molar extinction coefficient of NIP-^{E}-amino-n-caproic acid.

Preparation of Protein Sepharose 4B

Packed Sepharose 4B beads were activated (15) by mixing with an equal volume of a cold aqueous solution of cyanogen bromide 50 mg/mℓ. The pH was maintained at 10.5 - 11.0 with 1N NaOH for 10-15 mins., completion of the reaction was indicated by a steady pH with no further addition of NaOH.

The gel was then sequentially washed on a sintered glass filter with ~200 volumes of cold distilled water and cold 0.1M sodium bicarbonate buffer pH 8.4. One volume of sample containing 10-15 mg/mℓ of the protein to be coupled to the Sepharose in 0.1M bicarbonate buffer was added to an equal volume of the packed activated Sepharose and the suspension stirred gently overnight at 4°C. The coupled Sepharose was washed by centrifugation 3X in 0.5M $NaHCO_3$ buffer pH 8.4, and once in 0.1M $NaHCO_3$ buffer, pH 8.4, containing 0.2M ethanolamine. The gel was resuspended in 5 volumes of the ethanolamine bicarbonate buffer and stirred overnight in the cold. The following day the Sepharose was washed by centrifugation 3X in 0.1M acetate buffer, once in 0.5M $NaHCO_3$ buffer, pH 8.4, and finally with the buffer in which it was to be used. The Sepharose protein conjugates were stored in buffers containing 0.1% sodium azide in the cold and washed extensively before being used.

Culture Media

The medium used in the initial experiments to characterize the in vitro response to DNP-Ficoll was Eagle's Minimal Essential Medium (MEM) containing L-Glutamine (1 mM), 10 mM Hepes, 0.22% sodium bicarbonate and 5% heat inactivated fetal calf serum. Penicillin (100 units/mℓ) and streptomycin (100 mcg/mℓ) were routinely added to the media just prior to use. In most of the experiments 2-mercaptoethanol (BDH Chemicals) (2-Me) was used at a final concentration of 10^{-4}M unless indicated otherwise. In subsequent experiments where the in vitro response to sheep red blood cells (SRBC) was examined a modified RPMI 1640 based medium as described by North and Askonas (132) was adopted and used for all cultures from that point on (Table 1). The medium routinely contained 5 x 10^{-5}M 2-Me and 5% fetal

Table 1 A) Composition of Culture Medium

B) Composition of Nutritional Cocktail

1A MEDIUM COMPOSITION*

	Amount per litre of Medium
RPMI 1640[a]	9.9 g
Glutamine 200 mM	10 mℓ
Non Essential Amino Acids 100 x	10 mℓ
Sodium Pyruvate 100 mM	10 mℓ
HEPES	10 mM

* final pH 7.2

[a] Gibco Cat. No. H-18

1B COMPOSITION OF NUTRITIONAL "COCKTAIL"*

RPMI without $NaHCO_3$ with 10 mM HEPES+ pH 7.2	35 mℓ
Essential Amino Acids 50 x	5 mℓ
Non Essential Amino Acids 100 x	2.5 mℓ
Glutamine 200 mM	2.5 mℓ
Dextrose	500 mg
Sodium Bicarbonate 75%	7.5 mℓ

* Complete cocktail contains 25% (V/V) FCS

\+ RPMI 1640 prepared according to the manufacturers method (GIBCO) with the $NaHCO_3$ ommitted and 10 mM HEPES added.

calf serum. A nutritional cocktail (Table 1B) was prepared as described by Mishell & Dutton with the exception that RPMI 1640 was substituted for the Eagles MEM as the medium base.

Hepes (25 mM) buffered RMPI 1640 without sodium bicarbonate was used for the preparation and the washing of cells and shall be referred to as H-25 in subsequent sections.

Preparation of Spleen Cells

Mice were killed by cervical dislocation and the spleens were transferred aseptically to 35 mm tissue culture dishes (Falcon 3001) containing 2 mℓ of H-25/5% FCS per spleen. The splenic capsule was cut at one end with a curved dissecting needle and the pulp of the spleen gently forced out with the dissecting needles. The capsule was discarded and the splenocytes suspended by gentle mixing with a pasteur pipette. The suspension was passed through a stainless steel screen (200 mesh) into a 50 mℓ conical centrifuge tube (Falcon 2070) and washed twice in cold H-25/5% FCS by centrifugation at 400 x g for 10 minutes. The cell pellet was resuspended in the complete culture medium without 2-Me and portions stained with trypan blue or Turk's solution. The proportion of viable cells and the total number of mononuclear cells was determined by counting on an improved Neubauer hemocytometer. The cell concentration was adjusted with culture medium and any desired additions of antigens made. Freshly diluted sterile 2-Me was prepared from a frozen 0.1M stock solution (0.7 mℓ 2-Me/100 mℓ H_2O) by diluting the stock in culture medium to 10^{-3}M and passage through a millipore filter.

For most experiments 200 µl of cell suspension containing 10^6 viable nucleated cells were cultured in the wells of Microtest II plates (Falcon

3001). However, in some cases 2 mℓ cultures containing 5 x 10^6 cells were grown in sterilin repliplates (Qualicum) (25 wells of 20 x 20 mm).

Cells were collected from the larger culture vessels, repliplates and 35 mm petri dishes, by gently scraping the cells loose with a plastic policeman and suspending them in 3-4 volumes of cold H-25/5% FCS. The cells were centrifuged (400 x g, 10 min, 4°C), washed once more in H-25/5% FCS and resuspended to their original volume in H-25/5% FCS. Several dilutions of the cells were assayed for PFC's and those giving 20-150 plaques per slide were counted.

Microcultures were processed in a manner similar to that described by Pike(149). Approximately 100 mℓ of the culture fluid was removed and replaced with 100 μl of H-25/5% FCS. The plates were sealed with self-adhesive transparent sheets and centrifuged (400 x g, 10 min, 4°C) in a PRJ centrifuge equipped with a yoke which would accommodate microtitre trays. Approximately 150 μl of the supernatant was removed and replaced by 100 μl of H-25/5% FCS. The entire volumes of the cultures were transferred to assay tubes with finely drawn pasteur pipettes or a pipetman pipettor. To ensure reproducibility of the assay great care was taken to suspend all of the cultured cells since many of the cells adhere tenaciously around the peripheries of the wells.

Measurement of ^{3}H-Thymidine Uptake

Quadruplicate samples were cultured in microtitre trays (Falcon 3001) at 0.5 - 1x10^6 cells in 200 μl of medium for varying lengths of time. The amount of thymidine incorporation was determined by pulse labelling the cultures with 0.1 μCi of ^{3}H-thymidine (Amersham/Searle 2.2 Ci/mMole) for 4-18 hours at 37°C after which the cultures were collected onto glass

filters using an automatic cell harvester (Skatron, Norway). The filters were dried overnight at room temperature, suspended in a toluene based scintillation cocktail (Omnifluor, New England Nuclear) and their radioactive contents determined using a Beckman LS-300 liquid scintillation counter.

Generation of Anti-Sheep Red Cell Response in vitro

$B_6D_2F_1$ mice were immunized i.v. with 0.3 mℓ of a 20% suspension of washed SRBC in saline and sacrificed 4 days later. The spleens were removed aseptically and prepared as described in a previous section. This procedure gave very large antigen dependent responses on day 3 and proved to be a useful means of circumventing the requirements for selected FCS batches in order to obtain an anti-SRC response in vitro.

Removal of T Cells from Spleen Cell Suspensions

The killing of Thy 1.2^+ cells in normal spleen cell populations was achieved with either AKR anti-C3H thymocyte antisera (161) or rabbit anti-mouse brain antisera (73) and complement. Both of the antisera were generous gifts of Dr. B.G. Carter.

Spleen cells were washed twice with H-25/5% FCS, suspended at $5x10^7$ cells/mℓ in either antiserum diluted 1:4 with medium and incubated on ice for 45 min. Control cells were suspended in H-25/20% FCS and treated in a similar fashion to the experimental cultures. The cells were collected by centrifugation (400 x g, 10 min, 4^oC), resuspended to their original volume in agarose absorbed normal guinea pig serum (1:3 with H-25) and incubated at 37^oC for 45 min. After two washes with H-25/5% FCS, the cells

were resuspended in culture medium and adjusted to the desired viable cell concentration for culturing. For the determination of the percentage of cells killed viability was determined using Trypan blue exclusion immediately after the 37°C incubation as during the washes dead cells were lost reducing the apparent percentage of killing by the antisera.

Production of T cell Replacing Factors (TRF)

A concanavalin A induced TRF (223) was prepared by incubating 5-7 x 10^7 normal $B_6D_2F_1$ spleen cells in 5 mℓ of serum free modified RPMI 1640 containing 2.5 mcg/mℓ of Con A (Miles Yeda). The cells were cultured for 24 hours at 37°C in 100 mm petri dishes (Falcon, Optilux) and the supernatants collected after centrifugation (800 x g, 20 min). The supernatants were adjusted to 5% FCS, sterilized by passage through a 0.22 μ millipore filter, and stored frozen at -20°C.

In some preliminary experiments supernatants from cultures containing a mixture of C57B1/60♂ and $B_6D_2F_1$ ♂ spleen cells (each at 5 x 10^6/mℓ) were used as a source of an allogeneic TRF_{allo} (169). However, the supernatants produced by this method were less active than those generated with Con A and $TRF_{Con\ A}$ was used in subsequent experiments.

IMMUNOFLUORESCENCE METHODS

Fluorescence Microscope

A Leitz Ortholux fluorescence microscope equipped with phase contrast and dark field optics, a 200 W mercury vapour lamp (Philips) and a Ploem epi-illumination system was used. Fluoresceinated antibody binding was

examined by using the excitation filter combination BG38, (4 mm), 2 x KP490 and a TK 510 dichroic mirror. For suppression, an S525 interference barrier filter was generally used. However, in cases where the fluorescence intensity was weak a K 510/530 barrier filter was substituted for the S525 filter. Photographs were taken with a Leitz automatic exposure photographic system (Combifort) with Kodak Tri-X Pan film. Although the Tri-X film is rated by the manufacturer as ASA 400, it was found to be more practical to use a meter setting of ASA 800, since bleaching of the fluorescein occurred on exposure to the excitation beam of light for a prolonged period and the intensity of the fluorescence dropped off quite rapidly with time so that little residual fluorescence remained after 30-45 seconds. By using an ASA rating of 800, the exposure system of the camera operated the shutter automatically, this was not the case with exposure times of 30-45 seconds.

Fluoresceinated Antibodies

Fluorescein conjugated goat anti-mouse IgM (Meloy), goat anti-mouse IgG (7s) and goat anti-mouse IgG_{2a} were used at 1:6 dilution in PBS. FITC sheep anti-rabbit IgG (Central Resources for Immunological Reagents) was used for indirect fluorescence methods at a dilution of 1:9 in PBS.

Immunofluorescent Staining of Cytoplasmic Immunoglobulin (cIg)

Lymphocytes to be stained for cIg were washed twice in ice cold phosphate buffered saline, pH 7.4, containing 1% fetal calf serum (PBS-FCS) and resuspended at $2\text{-}4\text{x}10^6$ cells per mℓ in PBS-FCS (100). Aliquots of cell suspensions, usually 100 μl, were concentrated by cytocentrifugation (1500 rpm, 10 min) onto microscope slides. The samples were air dried for 30-60

seconds, fixed for 15 minutes at -20°C in a solution of ethanol:glacial acetic acid (95:5) and washed 3 times in PBS-FCS (approximately 1 minute per wash) with agitation throughout the period. The slides were blotted dry with filter paper and stained in a moist chamber for 30 minutes at room temperature with the appropriately diluted class specific fluoresceinated antiserum. Each smear could be stained with as little as 50 µl of antisera if care was taken to dry the area around the cells before applying the antibodies. After staining the slides were washed 3 times with PBS-FCS, blotted dry, mounted in 50% glycerol PBS under a No. 1 coverslip and sealed around the edges with nail polish.

The frequencies of cIg positive cells were determined by examining 200-400 cells for fluorescence. The counting was always done across the diameter of the cytocentrifuge smear so as to assure consistency of sampling. The original point of examination was observed under phase contrast so as to prevent bias towards starting at an area with fluoresceinated cells. A continuous segment of cells across the smear was counted until the desired number of cells had been examined.

Immunofluorescent Staining of Surface Immunoglobulin (sIg)

Lymphocytes were washed twice in cold PBS-FCS containing 10 mM azide and resuspended in the same at 1-2 x 10^7 cells/mℓ. Samples which were stained using the direct method were incubated with the fluoresceinated anti-Ig (1:2 final dilution) for 1 hour on ice. The cells were washed twice in PBS-FCS and resuspended in the original sample volume in cold 50% glycerol PBS-azide. For indirect staining a sample of 100 µl containing 1-2 x 10^6 cells was incubated with the desired rabbit antisera (diluted 1:20 in PBS)

at 4°C for 45 minutes and then washed twice with 50 volumes of cold PBS-FCS. The cell pellets were resuspended in 100 µl of an FITC labelled goat anti-rabbit IgG serum for 30 minutes at 4°C, washed twice as above and resuspended in a volume of cold 50% glycerol PBS azide equal to the starting volume. Drops of the samples were placed on clean slides and sealed under No. 1 coverslips with nail polish. Although there was azide in the medium to prevent capping it was found that this concentration of azide did not totally inhibit capping so that after 5-10 minutes at room temperature on the slides the cells began to patch and cap, therefore, samples were not placed on the slides unless they were going to be examined immediately. The slides were screened as described for the detection of cIg.

The Preparation of TNP Coupled Sheep Red Cells

Moderately coupled sheep red cells were prepared according to the method of Rittenberg and Pratt (163). Crystalline 2,4,6-trinitrobenzene sulfonic acid (40 mg) was dissolved in 7 mℓ of 0.28 M cacodylate buffer pH 6.9 (Table 2). One millilitre of packed, freshly washed, 2-3 week old, sheep erythrocytes was added dropwise to the gently stirring TNBS solution and stirred in the dark for a further 10 minutes at room temperature. The reaction mixture was transferred to a 60 mℓ conical centrifuge tube containing cold phosphate buffered saline (PBS), pH 7.2 and centrifuged for 5 min. at 400 x g. The cells were resuspended in cold PBS containing glycyl-glycine (2.2 mg/mℓ), centrifuged and washed twice in PBS. For use in the plaque assay the haptenated cells were suspended at 14% V/V in H-25 / 5% FCS. The cells were used within 4 days of preparation and washed 2-3 times with PBS prior to use.

Table 2. The Composition of Buffers used for the Preparation of TNP-SRC.

CACODYLATE BUFFER

All solutions were stored at $4^{o}C$.

Method 1

Solution A 0.28M Sodium cacodylate

$Na(CH_3)_2AsO_2$ 44.79 g

Solution B 0.28M Hydrochloric Acid
Concentrated HCl 23.4 ml

A fresh working solution was prepared as required by mixing 25 ml of Solution A with 3.95 ml of Solution B.

Method 2

Solution A 0.28M Cacodylic Acid

$(C_2H_3)_2$ AsO_2H 36.65 g/1

Solution B IN Sodium hydroxide

The pH of Solution A was adjusted to 6.9 by the addition of Solution B.

The Preparation of NIP Coupled Sheep Red Cells

The N-hydroxysuccinimide ester of NIP, (NIP-OSu) was purchased from Biosearch, 3095 V Kerner Blvd., San Rafael, CA 94901, U.S.A. and coupled directly to red cells using a modification of the method described by Schlegel et al (172). One week old sheep red cells (SRC) were washed three times in 0.125M $NaHCO_3/Na_2CO_3$ buffer, pH 9.25 and resuspended at a final concentration of 10% in the above buffer. The NIP-OSu was dissolved at 2 mg/mℓ in the $NaHCO_3/Na_2CO_3$ buffer after which an equal volume of 10% SRC was added dropwise with gentle stirring and the coupling allowed to proceed for 1 hour at 4^oC. The cells were then washed in the cold with PBS, pH 7.2, then with PBS, pH 7.2 containing glycyl-glycine (2.2 mg/mℓ), twice with cold PBS pH 7.2 and resuspended in H-25/5% FCS at a final concentration of 14% V/V. The coupled red cells were prepared daily as required. The cells were then processed as described for the TNP-SRBC.

Assay for Plaque Forming Cells

The determination of plaque forming cells was done by a modification of the method of Fauci and Pratt (58).

A37 agarose (Industries Biologique), (0.8%W/V), was dissolved in water by boiling with continuous mixing on a stirrer hot plate. Once the agarose was fully dissolved, 0.2 mℓ aliquots were transferred to tubes containing 0.2 mℓ of 2X Hepes (10 mM) buffered Hanks in a 47^oC water bath. The agarose/Hanks solution was allowed to cool to 47^oC and 0.025 mℓ of target cells diluted 1:6 in media, 0.025 mℓ of normal guinea pig serum and 0.1 mℓ of lymphocytes in media were added to each tube. A 0.20 mℓ sample of assay mixture was placed on a plastic petri dish and immediately covered with a

22x30 mm coverslip. The plates were incubated for 2-3 hours at 37°C in a humidified CO_2 incubator and the number of plaques formed were determined by examining the gels under a dissecting microscope using indirect lighting.

Some batches of NGPS were found to contain levels of hemolytic anti-TNP activity which could be removed by absorbing 10 volumes of serum with 1 volume of packed TNP-SRBC for 1 hour on ice. These sera were not used as a complement source unless otherwise stated. Guinea pig serum for use in the anti Thy 1 mediated killing was absorbed with agarose (80 mg/mℓ) for 60 minutes at 0°C. The agarose was removed by passage through cheese cloth and the complement stored at -80°C until required.

Culturing of Staphylococcus aureus

A stock solution of Staph. aureus was obtained from Dr. Julia Levy (University of British Columbia) in a lyophilized form in sealed evacuated ampoules. The cultures were initiated by removing the contents of the ampoule in a few drops of sterile nutrient broth and culturing this suspension on a nutrient agar slant for 24 hours at 37°C. A sample of the culture was Gram stained and examined for morphology and staining properties while another portion was tested for coagulase activity. The bacteria were Gram positive, coagulase positive cocci and judged to be free of contamination indicating that the culture contained Staph. aureus. Gram stains were routinely performed on cultures before the initiation and harvesting of bulk cultures.

Stock cultures were maintained on nutrient agar slants at 4°C and subcultured every 3-4 weeks by transferring a sample to a fresh slant and culturing for 18-24 hours at 37°C. The new stock cultures were maintained

Table 3. Composition of the Culture Medium used for the Bulk Culturing of Staph aureus.

Modified Dolman-Wilson Medium

Part A: Dissolve 20 gm of Difco Proteose Peptone in one liter of distilled water. Adjust the pH to 7.8 with 4N NaOH, and autoclave at 15 lb. pressure for 15 min.

Part B: Add 14 mℓ of 0.1M NaH_2PO (13.8 gm/l) to 36 mℓ of 0.1M K_2HPO_4 (17.42 gm/l).

Part C: Dissolve the following salts in Part B and bring the volume to 100 mℓ with distilled water. Sterilize by filtration through a Millipore HA (0.45 μ) membrane. The pH of this solution should be about 5.8.

NaCl	5.0 gm
Ammonium lactate (80% syrupy solution)	6.0 mℓ
$CaCl_2$	0.11 gm
$MgSO_4 \cdot 7H_2O$	0.2 gm
(Tri) sodium citrate	0.24 gm

Complete medium: Add 5.0 mℓ of sterile Part C to 45 mℓ of sterile Part A. The final pH will be 7.2 - 7.3. This medium is ready to inoculate.

at 4°C until required.

I should like to thank Dr. G. Wiseman for his generous gifts of knowledge and facilities during the culturing of the bacteria.

Bulk Culturing of Staph aureus

The bacteria were cultured overnight on fresh nutrient agar slants and harvested the following day. The cells from several slants were collected by gently resuspending in 2 mℓ of nutrient broth. One litre flasks containing 300 mℓ of medium(Table 3) were innoculated with 0.2-0.4 mℓ of the above bacteria and cultured for 18-20 hr. at 37°C on a rotary shaker at 120 RPM. The cultures were harvested by centrifugation (5800xg, 10 min, 4°C), washed in PBS, pH 7.2 until the supernatant was clear and either frozen as a paste or formalized. For the preparation of the bacterial adsorbent (102) the washed bacteria were suspended at 10% (V/V) in PBS-azide (0.05%(W/V)) containing 1.5%(V/V) formalin for 1.5 hours at 23°C. Care was taken to totally disperse the bacteria in this solution as aggregates which remained after this step were difficult to disperse in subsequent steps and the bacteria were less efficiently killed. The cells were washed once in PBS-azide and resuspended to the original volume in PBS-azide. The suspension was placed in a large Erlenmeyer flask such that the depth of the suspension was less than 1.5 cm and heated in an 80°C water bath of 10 minutes with continuous swirling. The suspension was rapidly cooled to 4°C in an ice water bath and washed twice in cold PBS-azide. The bacteria were adjusted to 10% (V/V) in PBS-azide in a graduated centrifuge tube and stored at 4°C. A sample of this suspension was plated on a blood agar plate to ensure that no viable bacteria remained after this treatment.

Prior to use as an adsorbent the cells were collected by centrifugation (2,000xg, 20 minutes, 4°C) and resuspend to the original volume of suspension in 0.5% (V/V) Nonidet P-40 (Shell chemicals) in NET buffer (NaCl 150mM, EDTA 5mM, Tris 50mM, sodium azide 0.02%(W/V)), pH 7.4 for 15 minutes at 23°C. The cells were subsequently washed in 0.05% NET (2000xg, 20') and resuspended to original volume in 0.05%(V/V) NP-40 in NET containing ovalbumin (1mg/mℓ).

Preparation of Spleen Cells for Surface Iodination

Pooled spleen cells were prepared from at least three mice as described in a previous section with the modification that FCS was not present in the media at anytime prior to the surface iodination. Erythrocytes and dead cells were removed by centrifugation on a Ficoll-Isopaque mixture (F/I) p=1.09;12 parts 14%(W/V) Ficoll in distilled water and 5 parts 32.8%(W/V) sodium metrizoate (Isopaque Nyegaard & Co., Oslo, Norway) (44). The cells were isolated by layering up to 5 mℓ of cell suspension ($1X10^8$ white cells/mℓ) onto 4 mℓ of the F/I solution in a 16x100 mm polycarbonate centrifuge tube. Both solutions were at room temperature and contained 0.1%(W/V) sodium azide. The samples were centrifuged at 2000xg, 20 minutes, T>20°C, the relative centrifugal force was the calculated force at the F/I media interface. The layer at the interface as well as the F/I down to the pellet was collected, washed twice in large volumes of H-25 and resuspeded in PBS, pH 7.0 at $2x10^8$ cells/mℓ. This procedure resulted in a suspension of cells with greater than 98% viability and less than 1% contamination by erythrocytes.

Iodination and Isolation of Surface Immunoglobulin from Murine Lymphocytes

Radioiodination of cell membrane proteins was performed according to the method of Kessler (102 ,103). The cells were washed, 1x in FCS, 4x in PBS and the lysed in 0.5% NP-40-NET buffer ($2.5x10^7$ cells/mℓ) for 30 minutes at 4^oC. The insoluble material was removed by centrifugation (117,000xg, 20 minutes) and the supernatant collected. Aliquots of the solubilized membranes were were mixed with 10-15μℓ of undiluted antisera and incubated on ice for 30-60 minutes. The antigen-antibody complexes were recovered by adding 200μℓ of 10% (V/V) washed bacterial adsorbent and incubating for 15-20 minutes on ice. The pellets were washed 3x in 0.05% NP-40 NET by vortexing extensively and centrifugation 12,000xg, 5 minutes in an Eppendorf micro centrifuge. The immune precipitates were solubilized by heating 100^oC for 60-90 seconds in a solution of 0.0625M Tris, pH6.8, containing 2% (W/V) SDS and 5% (W/V) 2-mercaptoethanol. The bacteria were removed by centrifugation, 12,000xg, 20 minutes and the supernatant was mixed 1:1 with a solution of glycerol containing Bromophenol blue as the tracking dye. Electrophoresis was performed exactly as described by Conrad and Froese (38). The gels were cut into 2 mm fractions with a Gilson Model B-200 fractionater and the samples were counted in a Beckman Gamma Counter.

I would like to thank Dr.'s Dan Conrad and Ricki Helm for performing the SDS-PAGE analysis of the iodinated cell surface proteins.

Results

I wish to gratefully acknowledge the guidance and assistance given to me by Dr. Hubert Taube throughout the various syntheses.

Table 4 lists some of the observed properties of the various intermediates and compares them with the literature values (28). Both NIP and NIP-N_3 melted with decomposition making it difficult to attach a precise value to their melting points. However this property plus the similarities in melting point ranges agreed well with those cited for NIP and NIP-N_3. The NIP and NP were further characterized by Dr. Taube using mass spectrometry and nuclear magnetic resonance spectroscopy. The values obtained for the molecular weights of these two compounds agreed very well with those predicted from the formulae of NIP and NP serving as further evidence for the identity of these compounds. Elemental analysis (Table 4) (analysis performed by Microanalysis Laboratories Ltd., 329 St. George Street, Toronto, Ontario) of the NP and NIP compounds also demonstrated compositions comparable to those predicted by the formulae. These results collectively identify the compounds which were synthesized as NP and NIP. The NIP-N_3 was not similarly characterized because of its extreme lability.

Considerable difficulty was initially encountered in synthesizing the NIP-N_3 and it was found essential that the system was kept absolutely dry during the synthesis. Once the azide was prepared and lyophilized, it was extremely reactive and gave nearly quantitative coupling to proteins and AECM-Ficoll. However several attempts to prepare NIP coated SRBC were unsuccessful using a wide range of NIP-N_3 concentrations suggesting that the cells were not reacting with the NIP-N_3. One possible source of difficulty was the tendency for the azide to hydrolyze during storage thus

Table 4. Comparison of the Elemental Analysis and Physical Properties of Synthesized NP and NIP with the Literature Values

Elemental analysis was performed by Microanalysis Laboratories Ltd. 329 St. George St., Toronto. Mass Spectrometry was performed at the Chemistry Dept. of the University of Manitoba. MP values were obtained from Brownstone et al Immunology 10, 465 (1966).

		Composition				
	MP	C%	N%	H%	I%	MW+
NP Theoretical	145*	48.73	7.10	3.58	-	197.1
Observed	143-148	48.32	7.04	4.32	-	197
NIP Theoretical	213*	29.26	4.33	1.72	39.28	323.04
Observed	20-211	29.74	4.34	1.87	39.28	323
NIP-Azide	65-66*					
	60-69					

rendering the initial coupling mixture a combination of NIP and NIP-N_3. Since the azide is the reactive group the dilution with NIP would result in an underestimation of the azide being added to the reaction mixtures and insufficient levels of haptenation of the target red cells. This difficulty should be totally eliminated through the use of the N-hydroxy-succinimide ester of NIP which couples in aqueous bicarbonate medium. This ester appears to have a much greater stability and higher solubility than the azide.

The Cross-reactivity of Anti-NIP and Anti-DNP Antibodies

Since there were problems in performing a plaque assay using NIP-SRBC as target cells the possibility of a cross reaction between DNP and anti-NIP antibodies was examined with a view to using DNP coupled target cells. To this end IgE antibody against DNP or NIP was produced using the homologous ovalbumin hapten conjugates and the resulting anti-hapten activity was determined using heterologous PCA. Rats were sensitized and left for 24 hours before challenge with antigen. The titre of each antiserum was determined against NIP and DNP using NIP-Ficoll and DNP-Ficoll in this way an index of the cross reactivity was obtained. It was found that less than 4% cross reactivity of anti-NIP antibodies with DNP conjugates existed and less than 0.3% cross reactivity of anti-DNP antibodies NIP conjugates occurred (Fig. 2, 3). Similar results have subsequently been obtained when the IgM responses of normal mice to DNP and NIP were examined for crossreacting PFC (Table 5). The two haptens are obviously well suited for tolerance studies since there is such a low level of crossreactivity between these two haptens.

DNP_{66}-AECM-Ficoll has proven to be a useful means for challenge in

Table 5. The Cross Reactivity Between Anti-NIP and Anti-TNP PFC.

Groups of 5 female CBA/CaJ mice were immunized i.v. with 150 mcg DNP_{30}-AECM Ficoll or 200 mcg NIP_{45}-AECM Ficoll in saline - 4 days later the numbers of direct PFC per spleen were determined on pooled cell suspensions of optimally sensitized TNP-BRBC or NIP-BRBC. Background responses to the haptens were determined by pooling the spleens from 5 normal, age-matched mice.

Immunizing Antigen	Target Cells	
	TNP-BRC	NIP-BRC
	PFC/Spleen	
---	250	700
DNP-AECM-Ficoll	65,000	500
NIP-AECM-Ficoll	4,700	109,000

the determination of DNP specific PCA titres as the AECM-Ficoll matrix does not crossreact with the protein carriers used for induction of IgE synthesis. I would like to thank Ms. Suzanne Peeters for performing the IgE experiments and allowing me to use the results.

Figure 2. The Crossreactivity of IgE anti-DNP antibody with NIP-AECM-Ficoll.

Two groups of rats were sensitized with mouse anti-DNP serum and left for 24 hours. One group was challenged i.v. with DNP-AECM-Ficoll (●) while the other group received NIP-AECM-Ficoll (▲) Pooled sera from several different bleedings of immunized mice were compared in this fashion.

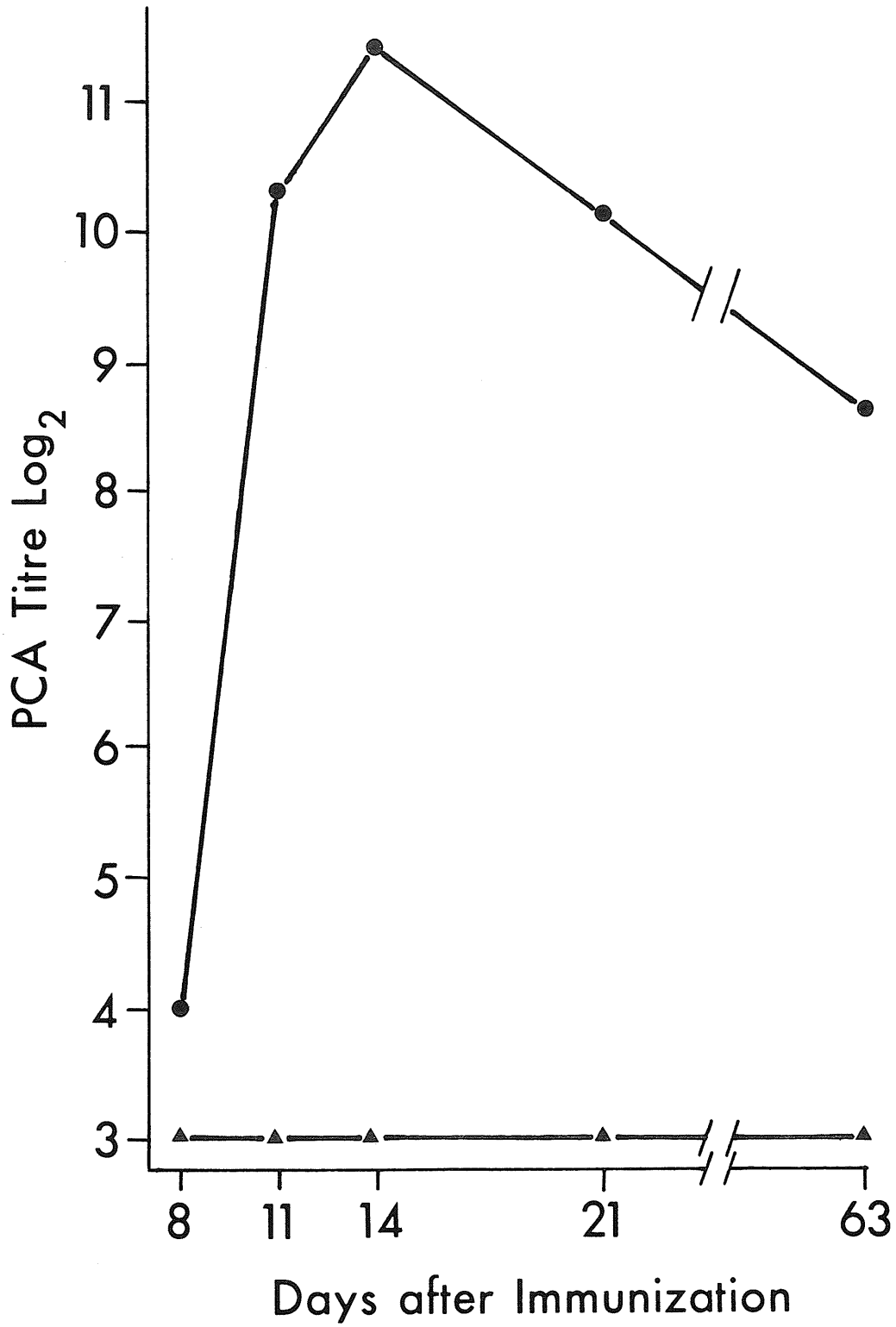
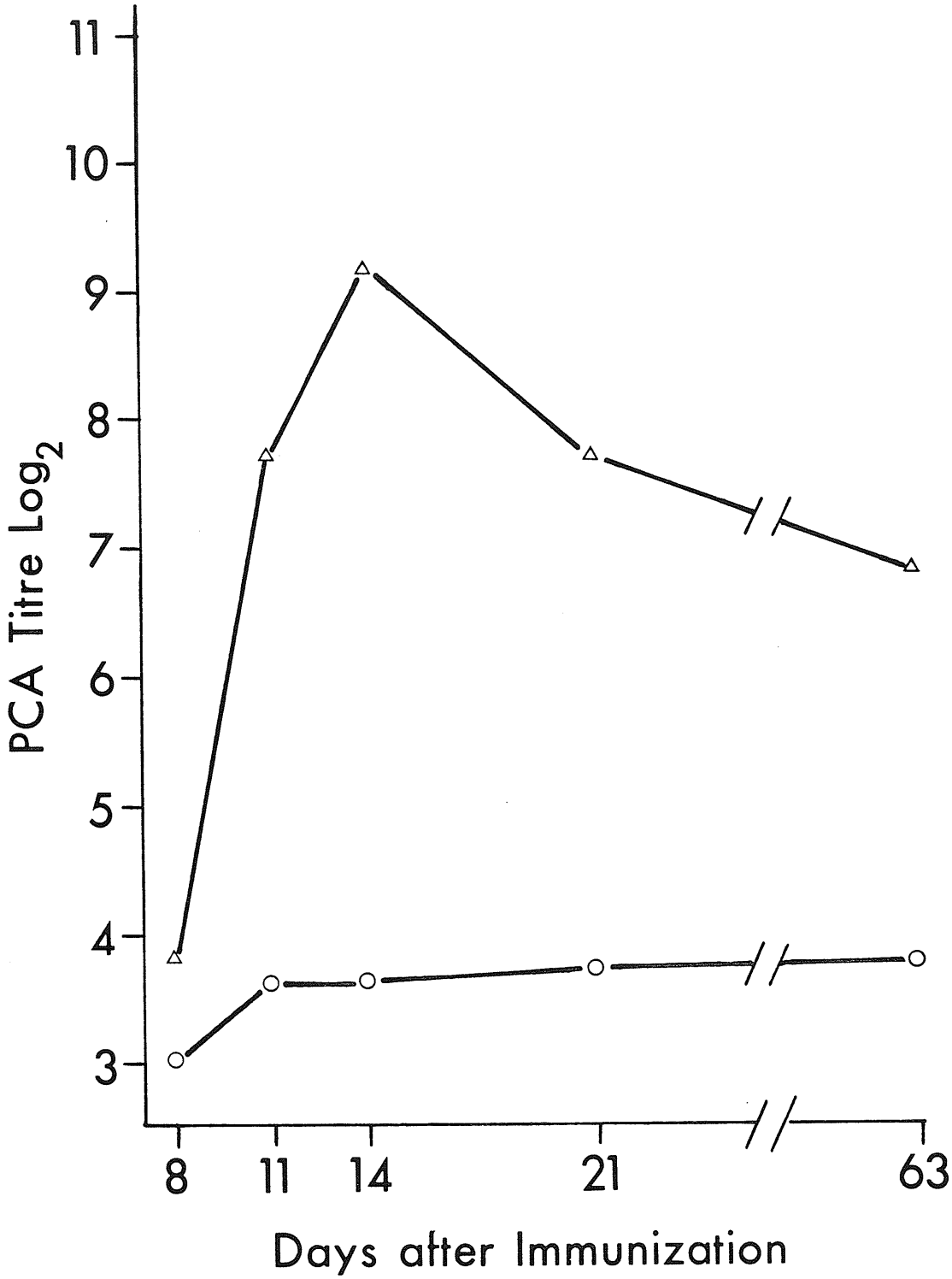

PCA Titre Log2
11
10
9
8
7
6
5
4
3
8
11
14
21
63
Days after Immunization

Figure 3. The Crossreactivity of IgE anti-NIP antibody with DNP-AECM-Ficoll.

Two groups of rats were sensitized with mouse anti-NIP serum and left for 24 hours. One group was challenged i.v. with NIP-AECM-Ficoll (▲) while the other group received DNP-AECM-Ficoll (●) Pooled sera from several different bleedings of immunized mice were compared in this fashion.


11
10
9
8
7
6
5
4
3
PCA Titre Log2
8
11
14
21
63
Days after Immunization

DNP-Lysyl-Ficoll

DNP-lysyl-Ficoll was prepared by the coupling ϵ-NH_2 DNP-lysine to the Ficoll matrix through the triazine ring of cyanuric chloride. The reaction is technically not a demanding one however it became apparent early on in the synthesis that the reaction products were not as predictable as was originally anticipated. There was a tendency for the Ficoll to become excessively crosslinked during the activation with the cyanuric chloride making the product unusable, also there was a considerable range in the numbers of DNP-lysyl groups coupled per mole of Ficoll under seemingly identical reaction conditions. These observations plus a discussion with Dr. W. E. Paul on the variability of the method led to the decision to synthesize AECM Ficoll.

A very good immunogenic preparation of DNP-lysyl-Ficoll was however synthesized and used for some preliminary in vivo and in vitro experiments. This compound contained 39 groups of DNP per mole of Ficoll and gave a signicant anti-TNP IgM and IgG in vivo response on day 6 (Table 6).

Table 6 . The Primary in vivo Response to DNP_{39}-lysyl-Ficoll

4 male BDF_1 mice were immunized intravenously with the indicated amount of DNP-lysyl-Ficoll dissolved in saline. The number of direct (IgM) and indirect (IgG) plaque forming cells per spleen was determined on the pooled spleen cells of each group six days after immunization.

Antigen Dose	PFC/spleen	
	IgM	IgG
1 mcg	44,500	18,300
10 mcg	80,000	32,400
100 mcg	108,000	45,000

AECM-Ficoll Derivatives

Inman's description of the synthesis proved to be an accurate and complete presentation of the steps involved. No difficulties were encountered in any of the synthesis. The functional group determination for the CM Ficoll was done using the pK value listed by Inman (90) as there was no clear endpoint in this titration (Fig.4).The calculated CM content of the CM-Ficoll was 190 groups/mole of Ficoll, while the amino content of the AECM Ficoll was 205 gps/mole of Ficoll. The latter value is thought to be more accurate as a clear endpoint was reached for the AECM-Ficoll. It would therefore appear that there was quantitative conversion of the CM groups to AECM groups.

Two preparations of DNP-AECM-Ficoll were synthesized containing 30.5 and 66 groups of DNP per mole of AECM Ficoll. The latter compound DNP - AECM-Ficoll proved to be nonimmunogenic in vitro and was not employed in the later experiments.

NIP_{45}- AECM-Ficoll was prepared by reaction of the AECM-Ficoll with $NIP\text{-}N_3$. As indicated earlier there was some difficulty in determining the immunogenicity of this antigen using the hemolytic plaque assay. However the problem was resolved and the NIP-AECM-Ficoll was shown to be immunogenic in vivo giving 40,000 direct plaques per spleen 7 days after i.v. immunization with 100 mcg per mouse. The immunogenicity of this compound has not been determined in vitro. The absorption spectra for AECM-Ficoll and the NIP and DNP derivatives are given in Fig. 5 and it is clear that if one wishes to synthesize AECM-Ficoll derivatives containing both NIP and DNP that it would be advisable to couple NIP first as it has a lower extinction coefficient than DNP and it does not contribute signicantly to the absorbance at 365 nm which is the wavelength at which DNP absorbs maximally.

Figure 4. Titration Curves of Ficoll, CM Ficoll and AECM Ficoll.

Sample was dissolved in water adjusted to a pH two units above the pKa of the functional groups being titrated and titrated with 0.1N HCl A) Ficoll B) AECM-Ficoll C) CM-Ficoll.

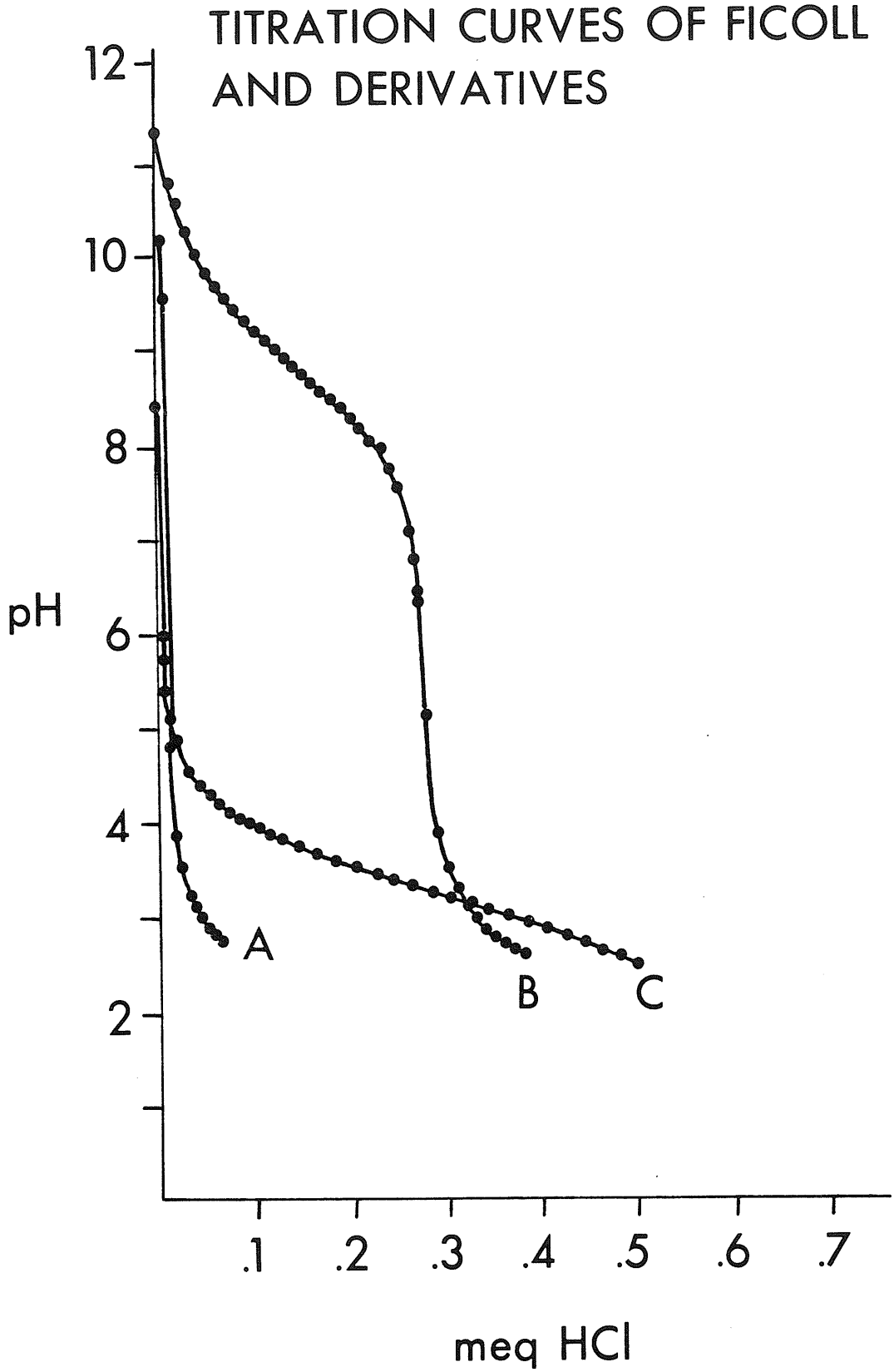
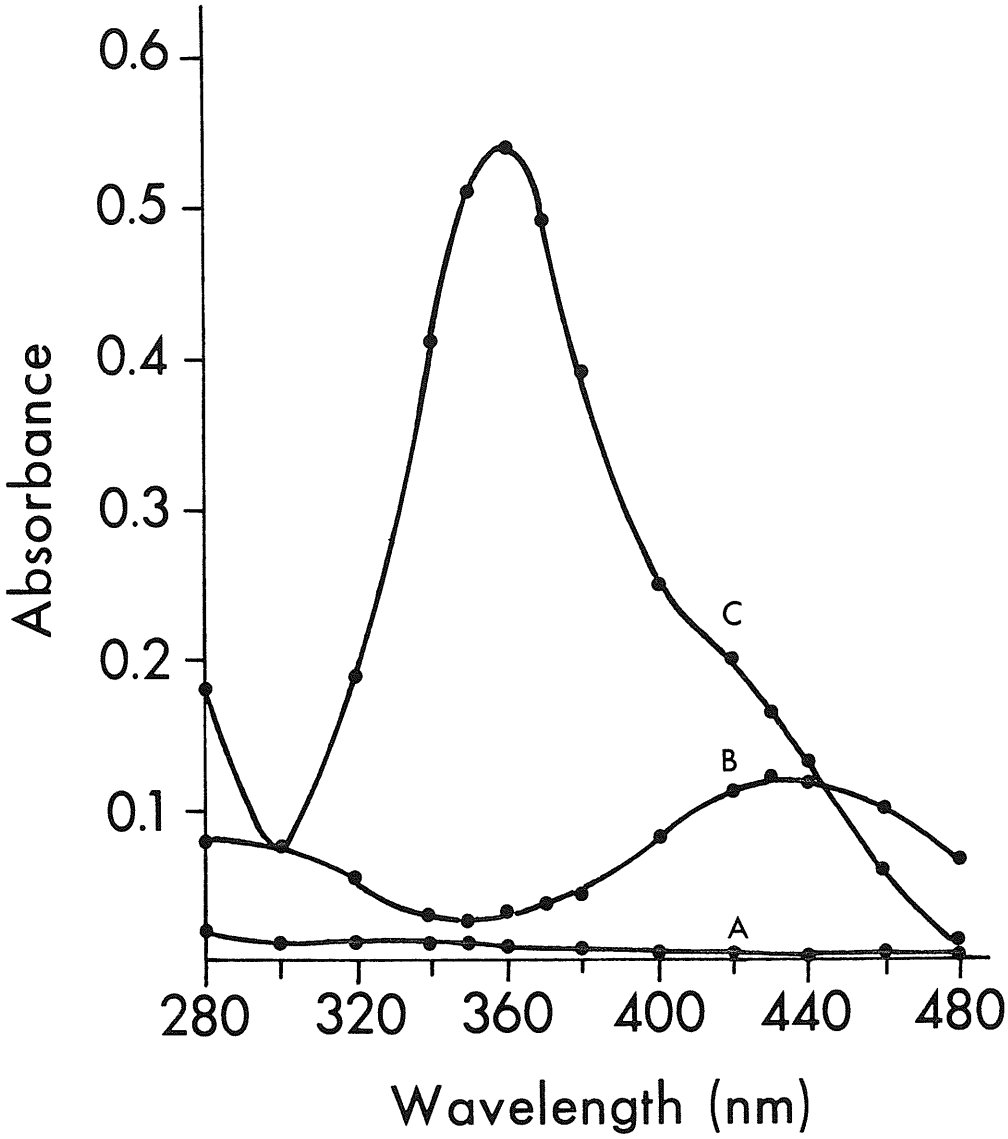

TITRATION CURVES OF FICOLL AND DERIVATIVES
12
10
8
6
4
2
pH
A
B
C
.1
.2
.3
.4
.5
.6
.7
meq HCl

Figure 5. Absorption Spectra of AECM-Ficoll, DNP-Ficoll and NIP Ficoll.

Samples were dissolved in PBS, pH7.2 and spectra recorded A) AECM Ficoll B) NIP-Ficoll C) DNP-Ficoll.


0.6
0.5
0.4
0.3
0.2
0.1
Absorbance
C
B
A
280
320
360
400
440
480
Wavelength (nm)

The AECM-Ficoll matrix offers considerable potential as a carrier for various haptens. It is theoretically possible to couple any hapten which will react with a free amino group and to generate the T independent form of that hapten. To date TNP, DNP, NIP and benzylpenicillin have been successfully coupled to AECM-Ficoll to produce immunogenic forms of the corresponding haptens.

Tissue Culture Results

The tissue culture systems employed evolved as a consequence of the changing requirements of the project. Initially a culture system was needed to characterize the antigens, DNP-lysyl Ficoll, DNP-AECM-Ficoll and the options were to use a Diener-Marbrook scheme or a Mishell-Dutton type system. It was anticipated that large numbers of cultures were going to be treated with numerous anti-metabolites and these considerations ruled out the Diener-Marbrook cultures. Initial trials with each new antigen usually involved culturing in larger dishes or trays as these more closely resembled the original Mishell-Dutton conditions. However once it was demonstrated that the test antigens were immunogenic in this system attempts were immediately made to adapt the microculture system to generate AFC in this latter system as it has the advantages of ease of handling, of requiring small quantities of reagents and of utilizing few cells.

Macrocultures

The response to DNP-lysyl-Ficoll was found initially to be quite variable ranging from a few hundred up to 2,000-3,000 PFC/culture. The reasons for this variability were not readily apparent so several parameters of the culture system were examined in an attempt to clarify the situation. An initial comparison of RPMI 1640, MEM and Mishell-Dutton modified MEM with daily feeding indicated that there were no differences between the various media in terms of the responses generated, MEM supplemented with $5X10^{-5}$M2-Me adopted as the routine culture medium. Cultures normally contained 2 ml/well as it was found that this volume gave higher yields of viable cells but not signicantly higher numbers of PFC/culture on day 4.

The effect of varying the DNP-lysyl-Ficoll concentration on the level of the response was examined on day 4, which preliminary experiments had indicated was the time at which PFC production peaked. As can be seen in Fig. 6 the anti-TNP response showed a sharp antigen concentration dependency. Antibody formation was observed over a range of 1-100 ng/ml but the peak numbers of PFC were observed between 1 and 10 ng/ml. In other experiments the responses to 0.01 and 0.1 ng/ml of antigen were shown to be marginal, thus eliminating the possibility that the cultures which had received 1 ng of antigen were still on the ascending portion of a dose response curve. A concentration of 10 ng/ml of DNP-lysyl-Ficoll was adopted for routine use.

The effect of varying cell concentration was also examined (Fig. 7). A similar number of plaques were observed over a relatively wide range of cell concentrations. This could have been due to regulation within the cultures or to variations in culture environments resulting in decreased viability. The latter possibility was examined by determining the number

Figure 6. Dose Response Curve for DNP_{39}-Lysyl-Ficoll

$5X10^6$ Normal BDF, spleen cells were cultured in 2 ml of MEM containing 5% FCS and 10^{-4}M 2-Me. The numbers of anti-TNP PFC per culture were determined on day 4 from pools of triplicate cultures. IgM PFC.

Fig. 6

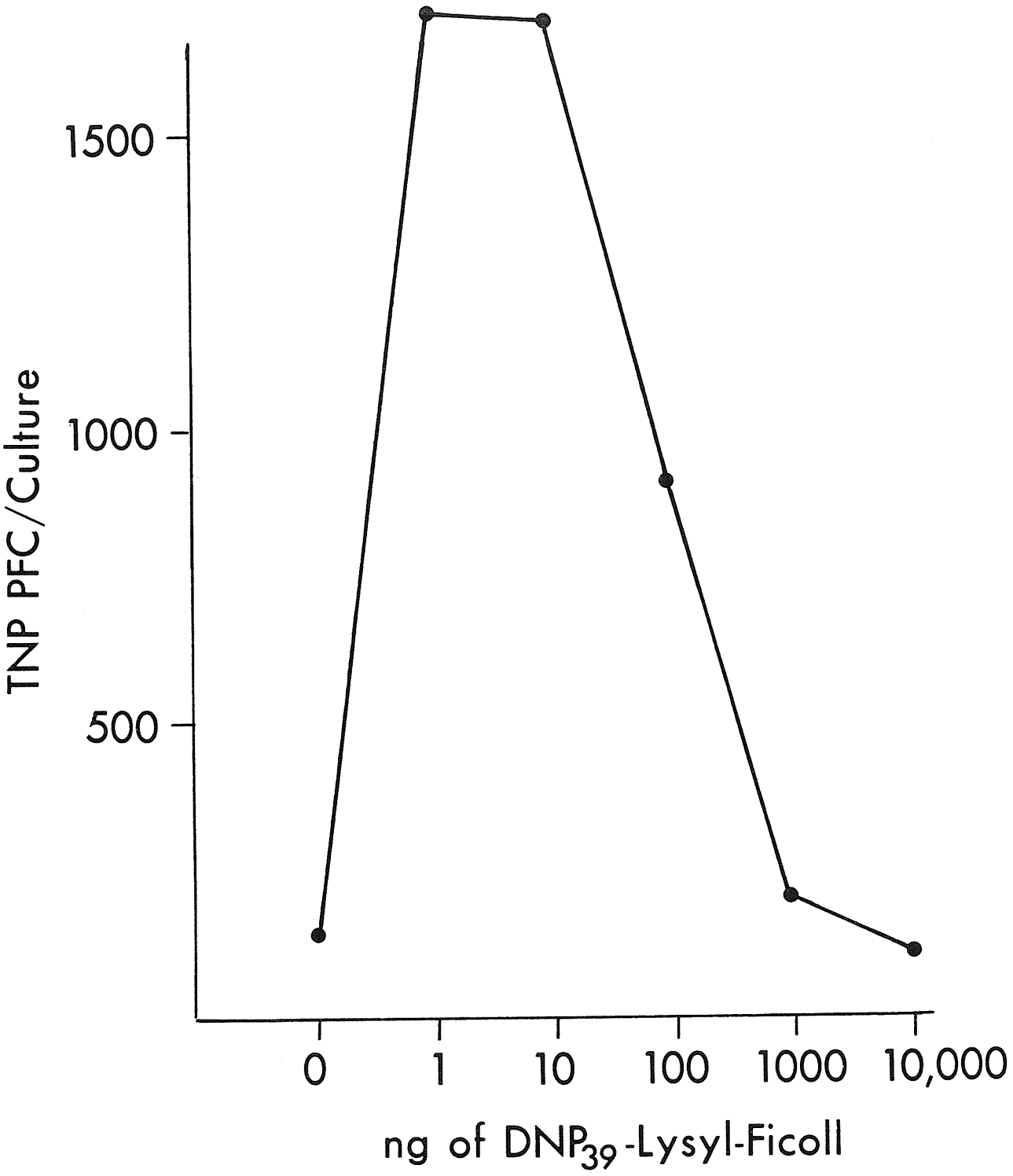
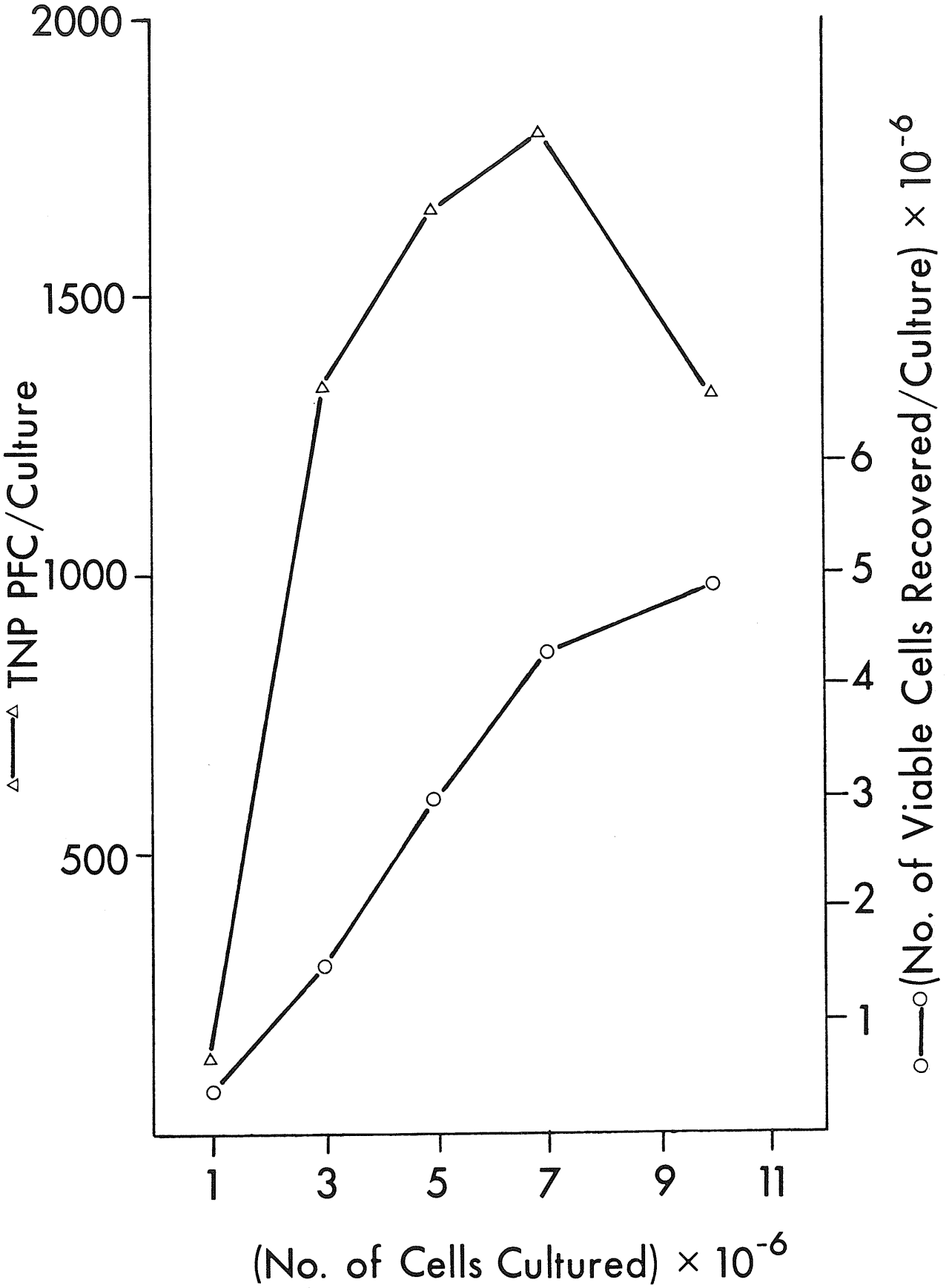

1500
1000
500
TNP PFC/Culture
0
1
10
100
1000
10,000
ng of DNP39-Lysyl-Ficoll

Figure 7. The Effect of the Number of Cells Cultured on The Anti-TNP Response to DNP_{39}-lysyl-Ficoll

The indicated numbers of cells were cultured in 2 ml of medium containing 10 ng/ml of DNP-lysyl-Ficoll. After 4 days in culture quadruplicate cultures were pooled and the number of viable cells and the number PFC recovered per culture determined. IgM PFC.

Fig. 7


2000
1500
1000
500
△—△ TNP PFC/Culture
6
5
4
3
2
1
○—○(No. of Viable Cells Recovered/Culture) × 10⁻⁶
1
3
5
7
9
11
(No. of Cells Cultured) × 10⁻⁶

of viable cells recovered per culture on the day of assay for PFC. The number of cells recovered was directly related to the number of cells placed in culture between 1 and 7 $X10^6$ cells per culture, however at cell concentrations of greater than $7X10^6$/culture the proportion of viable cells recovered decreased. The number of PFC generated per culture did not follow a similar pattern thus excluding differences in cell survival as being the only reason for the non-linear relationship between the number of cells cultured and the number of PFC generated per culture. Although $5X10^6$ cells/culture was slightly less efficient in terms of the numbers of PFC generated/No. of cells cultured, than $3X10^6$ cells/culture was, the former concentration was adopted for routine use as it represented a concentration on the plateau region of the concentration response curve, in this way more consistent responses would be expected despite small variations in the numbers of cells per culture.

Microcultures

The microcultures proved to be an excellent means of generating *in vitro* responses, however the requirements for obtaining these responses were much more stringent than for the macrocultures. Therefore considerable time and effort were expended upon the characterization of this system.

The Effect of 2-mercaptoethanol on the Anti-DNP-Ficoll Response

The effect of the reducing agent 2-mercaptoethanol on *in vitro* immune responses was first described by Click *et al.*(35a). This group found that there was an enhanced response to SRBC and that the overall viability of the cells in culture was increased in the presence of 5×10^{-5}M 2-Me.

The addition of 2-Me in the current microculture medium resulted in an increase in the number of PFC/culture by a factor of 2-3 at concentrations between 10^{-5} and 10^{-4}M (Fig. 8). Concentrations higher than 10^{-4}M resulted in a sharp decrease in the numbers of PFC to less than or equal to those observed in the absence of 2-Me. At lower concentrations (10^{-5}M) the response dropped rapidly down to those of the control cultures. There was often very little difference observed in the response obtained using 5×10^{-5}M and 10^{-4}M 2-Me in the media. However, on those occasions where there was a difference, concentrations of 10^{-4}M 2-Me more frequently gave a higher response and this concentration was routinely used in the media.

Effect of Cell Concentration on the *in vitro* Response

The conditions for obtaining a response to DNP-Ficoll were much more restricted in terms of cell numbers per culture than those observed with

Figure 8. The Effect of 2-Me on the Anti-TNP Response to DNP_{39}-lysyl-Ficoll.

$1X10^6$ Normal BDF_1 spleen cells were cultured in 200 μl of MEM containing 5% FCS, 10 ng of DNP-lysyl-Ficoll and varying concentrations of 2-Me. Cultures were assayed on day 4. Each point is the mean of 4 cultures assayed individually.

Fig. 8

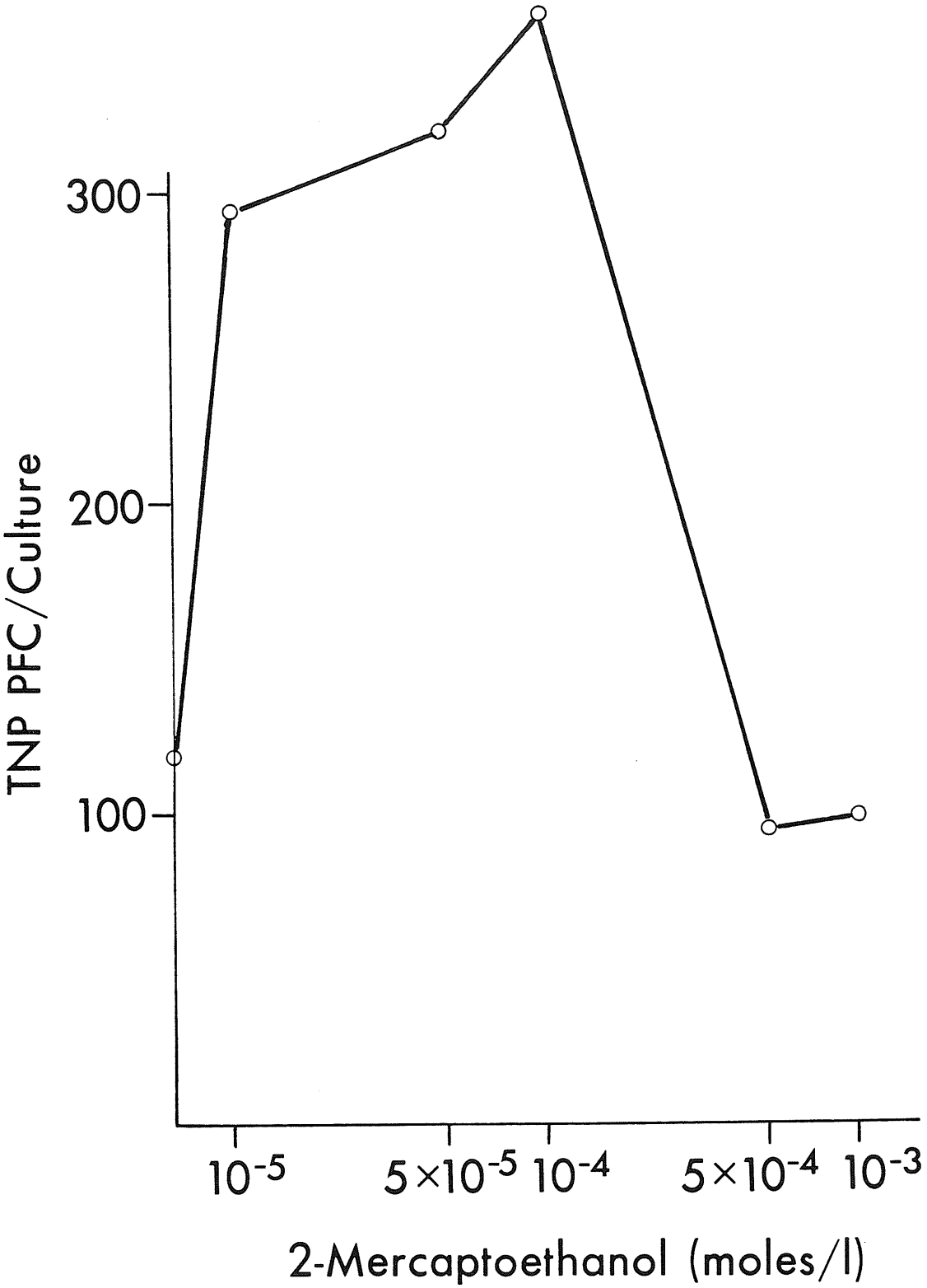
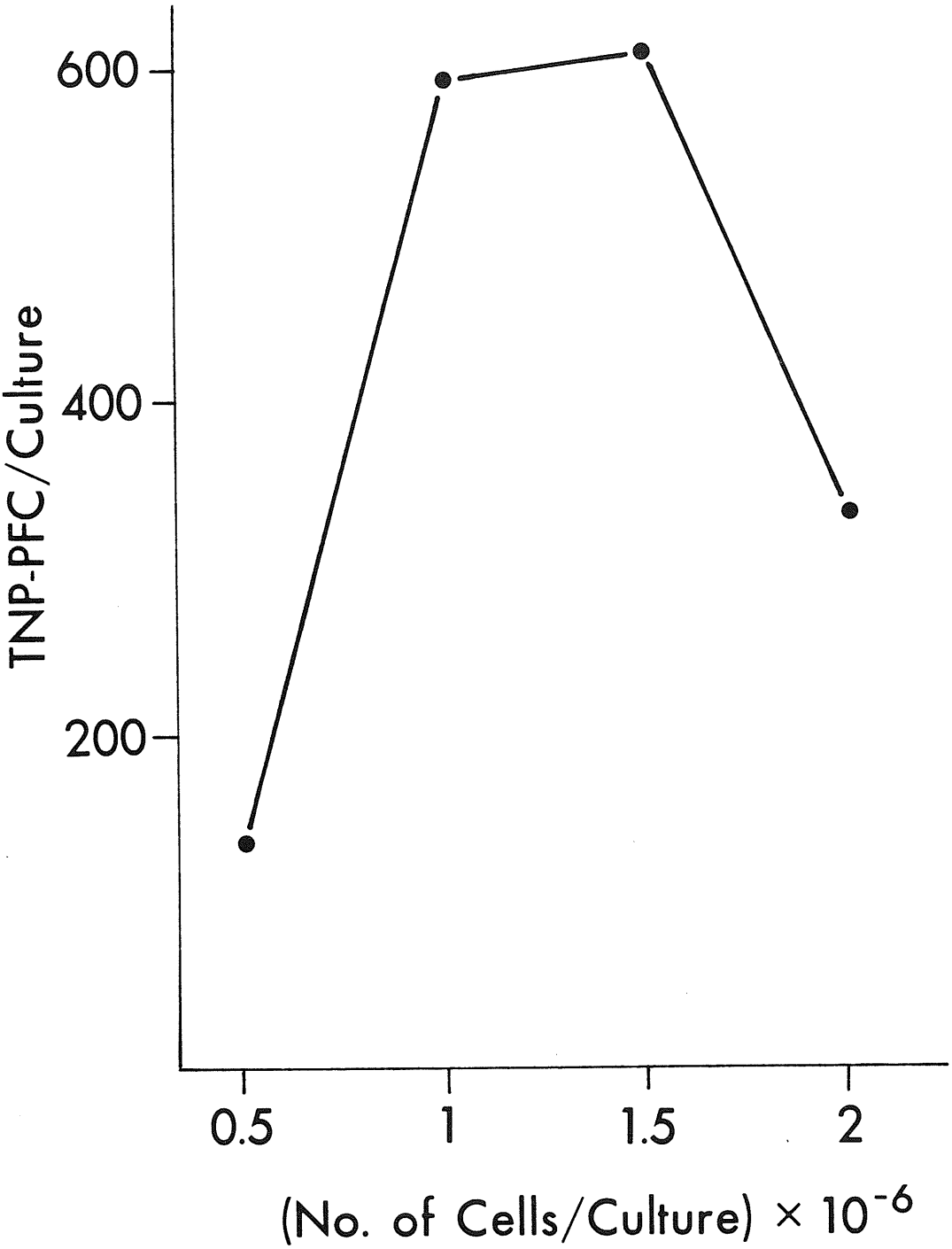

300
200
100
TNP PFC/Culture
10-5
5×10-5
10-4
5×10-4
10-3
2-Mercaptoethanol (moles/l)

Figure 9. The Effect of the Number of Cells Cultured on the Anti-TNP Response to DNP_{39}-lysyl-Ficoll

The indicated numbers of cells were cultured in 200 μl of medium containing 10 ng of DNP_{39}-lysyl-Ficoll. Cells were harvested after 3 days in culture and assayed for anti-TNP PFC. Each point is the mean of quadruplicate cultures.


600
400
200
TNP-PFC/Culture
0.5
1
1.5
2
(No. of Cells/Culture) × 10⁻⁶

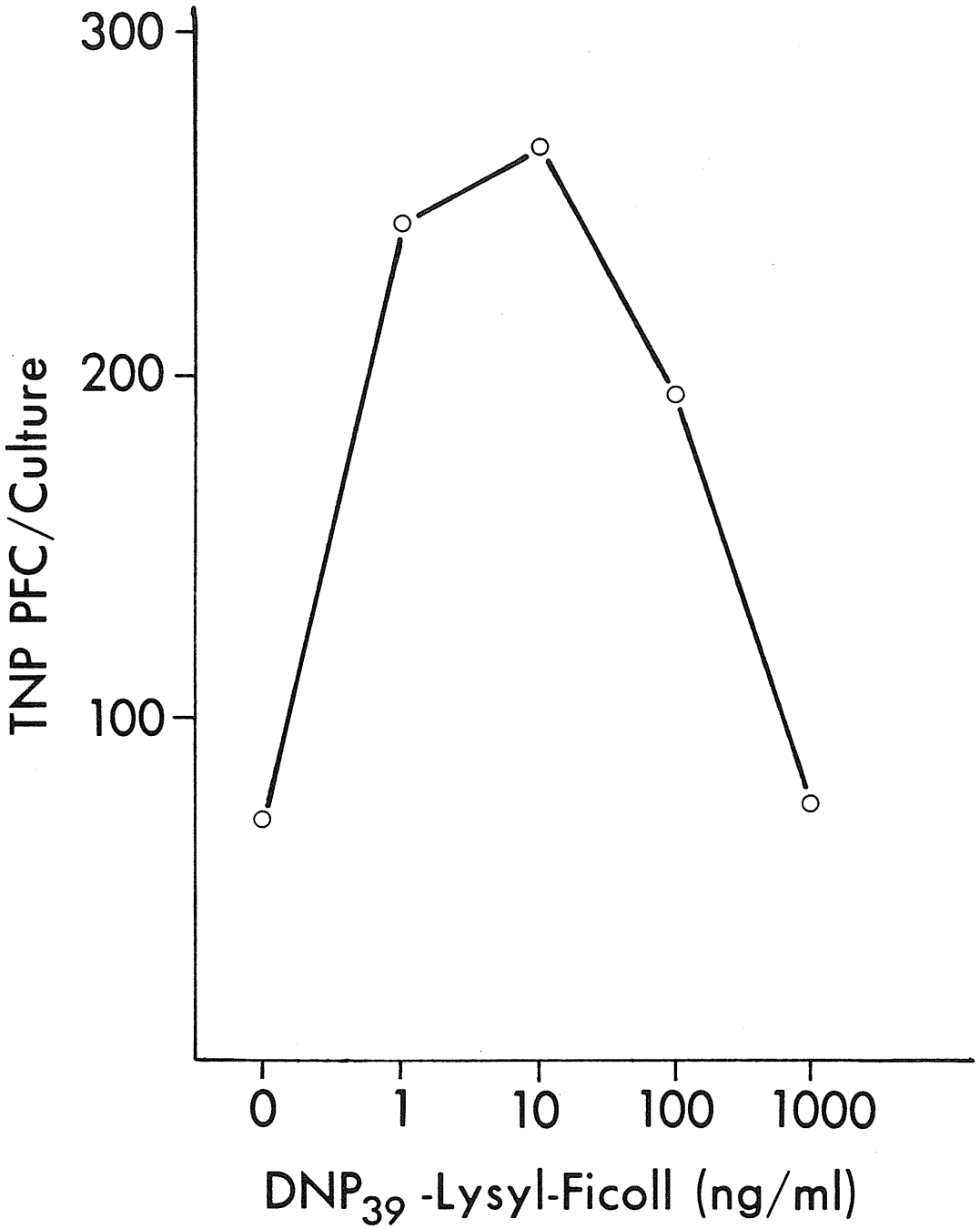
this antigen in the sterilin trays. (Fig. 9). Maximum numbers of PFC were observed between 1 and 1.5 x 10^6 cells per culture on day 3 of culture, and slight changes of cell numbers in either direction resulted in a very rapid fall off in the numbers of PFC recovered per culture. Routinely 8-10 x 10^5 cells/culture were used as this represented the region of maximum PFC formation while still keeping the cultures in a region of maximum sensitivity to factors which could effect the response, (i.e., on the inflection point of the curve).

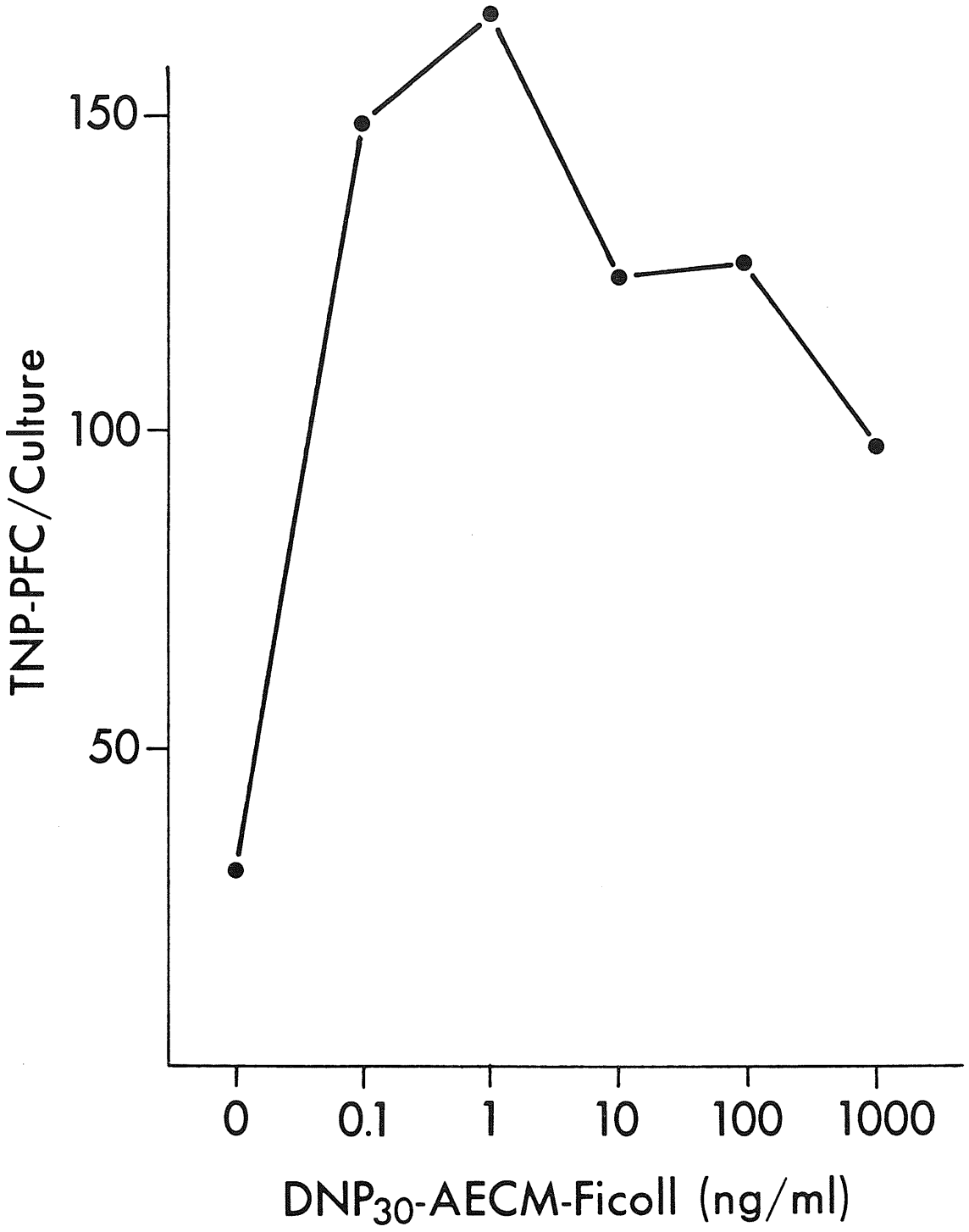
The Effect of Varying Antigen Concentration on the in vitro Response

The in vitro response to DNP_{39}-lysyl-Ficoll (Fig.10A) was much more sensitive to antigen concentration than that to DNP_{30}-AECM-Ficoll was (Fig.10B). The profile for DNP-lysyl-Ficoll was very similar to that observed for it in the macrocultures in that it showed a sharp peak at 1-10 ng/ml which dropped off rather rapidly at concentrations above or below the optimum. Furthermore, at concentrations of greater than 1 mcg/ml the response was less than or equal to that observed in cultures which had not received antigen. In contrast, the DNP_{30}-AECM-Ficoll showed a much wider range of immunogenicity and induced a response at concentrations up to 10 mcg/ml. The number of PFC observed in cultures containing 0.1 ng/ml was much more variable than that observed with either 1 or 10 ng/ml of this antigen, therefore, 1 ng/ml was routinely used as the antigen concentration. Another preparation of DNP-AECM-Ficoll containing 66 DNP groups per mole of Ficoll was not immunogenic at any concentration tested (0.1 ng - 10,000 ng/ml). It was a consistent observation that DNP-lysyl-Ficoll treated cultures gave ~25% higher numbers of plaques on day 3 than did DNP-AECM-Ficoll.

Figure 10. The Effect of Varying DNP-lysyl-Ficoll or DNP-AECM-Ficoll Concentrations on the Anti-TNP Response.

$1X10^6$ normal spleen cells were cultured for 3 days in microcultures with the indicated concentrations of A) DNP_{39}-lysyl-Ficoll B) DNP_{30}-AECM-Ficoll and assayed for anti-TNP PFC.


300
200
100
TNP PFC/Culture
0
1
10
100
1000
DNP39 -Lysyl-Ficoll (ng/ml)


150
100
50
TNP-PFC/Culture
0
0.1
1
10
100
1000
DNP30-AECM-Ficoll (ng/ml)

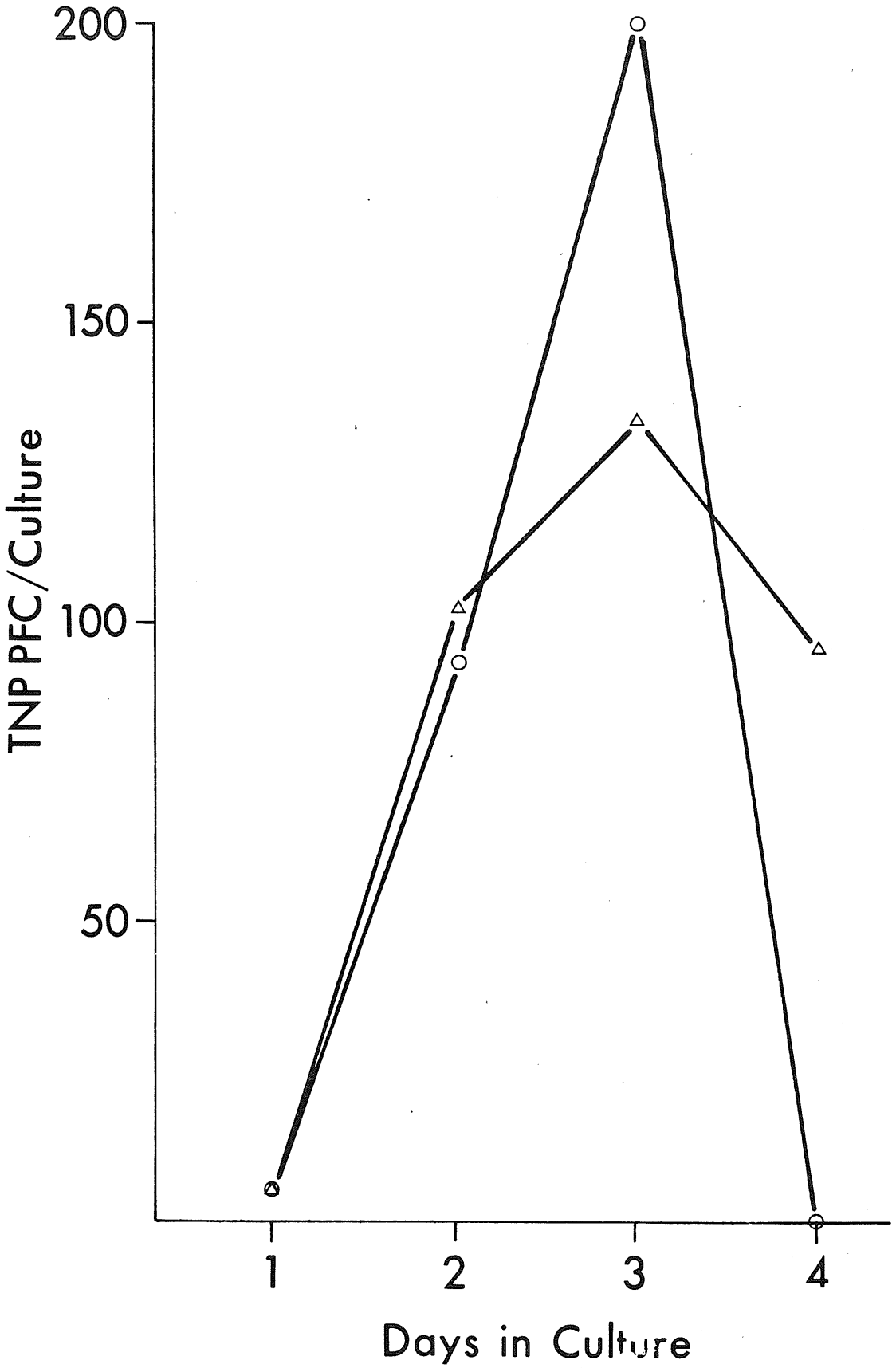
The Kinetics of the Anti-DNP response *in vitro*

The dose response curves for DNP-AECM-Ficoll had shown that there were comparable numbers of PFC generated by 1 and 10 ng/ml of this compound. It was of interest to determine if the kinetics of the antibody response to these two concentrations were different. The major difference between the responses induced by these doses was that the cultures receiving 10 ng/ml showed a more prolonged response than those receiving 1 ng but as was observed previously cultures receiving 1 ng/ml of antigen gave slightly higher responses on day 3. (Fig. 11). Attempts to detect an IgG anti-TNP response during the course of these experiments were unsuccessful, therefore, indirect anti-TNP plaques were not assayed for after this point.

Figure 11. The Kinetics of the Anti-TNP Responses to DNP-AECM-Ficoll

$1X10^6$ normal spleen cells were cultured with DNP_{30}-AECM-Ficoll, 1 ng/ml (O) or 10 ng/ml (Δ) and assayed on the indicated days for direct anti-TNP PFC.

Fig. 11



Anti-SRBC Responses *in vitro*

In some very early experiments it had been observed that none of the FCS which had been used for culturing would support a primary *in vitro* anti-SRBC response. It is possible to obtain from Gibco "Contract Approved Sera" which have been tested for their ability to support a primary *in vitro* anti-SRBC response. I was reluctant to use such a batch of calf serum as there had been reports in the literature (195,196) of such sera containing endotoxin-like activity and it seemed highly undesirable at the time to add another variable such as possible mitogen contamination into the culture system. Schreir and Nordin (179) had reported that priming animals with SRC 3-4 days prior to sacrifice resulted in a loss of dependency of the response on the batch of FCS employed, presumably as a result of the activation of antigen specific T cells. Furthermore, these authors had reported that RMPI 1640 was a better medium base than MEM for generating anti-SRC response *in vitro*. I, therefore, undertook to examine the anti-SRC response induced using primed cells.

Effect of Priming Mice upon subsequent Response to SRBC

When unprimed mice were used as spleen donors for *in vitro* culture the anti-SRC response observed was quite variable, even with a Mishell-Dutton tested FCS, giving a maximum number of PFC of $800/10^7$ cultured cells on day 4. (Table 7). This was not an acceptable response. When mice were primed with SRBC 4 days prior to sacrifice, a 20-40 fold increase in the number of PFC/culture was observed. The ratio of stimulation relative to the control cultures was often less than that obtained in unprimed cultures but the increase in the number of plaques recovered

Table 7 . The Effect of Priming with SRC on a Subsequent *in vitro* Anti-SRC Response.

Mice were primed 4 days prior to sacrifice with $1X10^8$ washed SRC given intraveinously. The cells were cultured $5X10^6$/2 ml medium in Sterilin repliplates and harvested on day 3 of culture.

Spleen Cell Source	SRC in Culture	
	-	+
Unprimed donor	11*	251*
" "	16	486
Primed donor	627	10,890
" "	249	13,735

* Direct PFC/culture

per culture more than compensated for the lower stimulation indices. As indicated earlier responses in macrocultures were observed more frequently than in microcultures and in some preliminary experiments to optimize the culture conditions in macrocultures a comparison was made between the responses given by primed cells in sterilin trays and in 35 mm petri dishes as originally described by Mishell and Dutton (178). The sterilin trays containing 8×10^6 cells gave a response 4-10X higher than cells cultured under standard Mishell-Dutton conditions (Table 8) when assayed on day 3. The difference in the responsiveness was not due to differences in the kinetics of the induction of anti-SRBC PFC as both culture systems showed a marked decline in PFC recovered/culture on day 4. The reason for this rapid decline is not known. It may be that a component is lacking in the FCS as this serum supported only a very weak anti-SRBC response in unprimed animals. It should be noted that the cultures contained 5×10^{-5}M 2-Me, this was found to be essential for obtaining an optimal response.

Requirement for T cells for Anti-SRBC Response by Primed Spleen Cells

The previous experiments had shown that the primed spleen cell population required in vitro exposure to SRBC in order to obtain anti-SRC PFC. It was possible, although unlikely, that the role of the antigen was merely to induce cells already undergoing proliferation and differentiation in the spleen to secrete immunoglobulin without any requirement for T cells. In order to determine if the response was T-cell dependent, the primed spleen cells were treated with rabbit anti-brain associated theta (RABT) and complement and cultured in the presence of SRBC. The response was reduced by 90-95% as compared with control cultures treated with NGPS only, indicating that the induction of antibody synthesis required T cells. (Table 9).

Table 8 . A Comparison of 2^{o} Cultures in Sterilin Trays and Falcon Petri Dishes.

Spleen cells from primed mice were cultured in Sterilin repliplates ($5X10^{6}$ cells/2 ml of medium) or in Falcon (Petri Dishes $1X10^{7}$ cells/2 ml of medium) under Mishell-Dutton conditions and assayed for anti-SRC response at the indicated times.

No. of Days in Culture		No. of SRC/culture			
		-	$5X10^5$	$1X10^6$	$5X10^6$
3	Sterilin Tray	660	21,600	21,400	13,760
	Falcon Dish	45	2,890	3,380	5,300
4	Sterilin Tray	0	3,140	3,440	1,620
	Falcon Dish	0	0	10	70

As an initial test for the efficiency of T cell depletion, treated spleen populations were cultured with Con A, a T cell mitogen or LPS, a B cell mitogen and their responsiveness compared to those of control cultures (Table 10). The RABT + C treatment resulted in a greater than 95% reduction in the Con A induced proliferation while showing no effect on the LPS induced ^{3}H-thymidine uptake.

Table 9 . The Effect of T-Cell Removal on a 2^{o} Anti-SRC Response.

Primed spleen cells ($5X10^{6}$/culture) were treated with either NGPS or Rabbit anti-mouse brain associated Thy 1 serum and then cultured at $5X10^{6}$ viable cells per culture in Sterilin repliplates for 3 days. The numbers of anti-SRC PFC/culture was determined on pools of cells from quadruplicate cultures.

Treatment	Antigen	A	B	C
			PFC/culture	
Normal Guinea Pig Serum		160	249	627
	$1X10^6$ SRC	9,920	13,735	10,890
Rabbit Anti-Mouse Brain Associated (θ)	-	20	-	-
+ Normal Guinea Pig Serum	$1X10^6$ SRC	30	178	142

Table 10 . The Effect of T Cell Depletion on the Mitogen Responsiveness of Normal BDF Spleen Cells.

Spleen cells were treated with either NGPS or R⊖B(⊖) + NGPS, washed and cultured at $8X10^5$ viable cells/culture in RPMI 1640, 5% FCS without $5X10^{-5}$M 2-Me, in the presence of the indicated mitogens. ^{3}H-Thymidine (0.2 Ci/culture) was added for the final 18 hours of a 72 hour culture, the cells were harvested and assayed for thymidine incorporation.

Addition to Culture	Treatment	
	NGPS	R B(θ)+NGPS
	Cpm/culture	
--	5,338	1,356
LPS 50 mcg/ml	61,369	74,967
Con A 5 mcg/ml	178,168	2,390
RMF $F(ab')_2$ 1:20	54,264	43,324

Reconstitution of the Anti-SRBC Response with TRF

Once the T cell dependency of the anti-SRC response of primed spleen cell populations had been established, it was possible to use this system to screen culture supernatants for TRF activity. This was an important point for although the characteristics of Con A induced TRF ($TRF_{con\ A}$)had been previously reported no information had been published on the concentrations of Con A used to induce $TRF_{con\ A}$. It was therefore necessary to use several different concentrations of Con A to generate culture supernatants and to test these supernatants for their biological activity in T depleted spleen cell populations.

The TRF activities of the supernatants showed considerable variability in their capacities to reconstitute the responses of primed or unprimed cells (Tables 11,12). The supernatants from cultures treated with 2.5 mcg/ml of Con A (5/5 preparations) gave the most consistant levels of TRF production. Concentrations of Con A either, less than 5 mcg/ml (2/6 preparations) or greater than 1.25 mcg/ml (1/2 preparations) resulted in the generation of supernatants with TRF activity. However these concentrations did not prove to be consistantly inductive for TRF activity. A concentration of 2.5 mcg/ml was adopted for the induction of TRF containing supernatants and was used in all subsequent experiments employing $TRF_{Con\ A}$ unless otherwise indicated.

Initially attempts had also been made to generate a TRF by producing a primary MLC reaction <u>in vitro</u> and collecting the supernatants (TRF_{Allo}). This had been the source of the originally reported TRF activities and it was thought to be worthwhile to compare the TRF_{Allo} and $TRF_{Con\ A}$ in terms of their biological activities. However considerable difficulty was encountered in generating supernatants with TRF_{Allo} activity and in those

preparations which possessed activity (2/5) the responses were markedly lower than those of $TRF_{Con\ A}$ (Table 14). The TRF_{Allo} was therefore not used in subsequent cultures. In an effort to optimize the generation of PFC in cultures of T cell depleted SRC primed spleen cells the effect of adding TRF to the cultures at different times was studied. Cultures were initiated in the presence of SRC and at various times a volume of TRF containing supernatant equal to the culture volume was added to the cultures. Three days after the start of the cultures the number of anti-SRC PFC/ culture was determined. The simaltaneous addition of $TRF_{Con\ A}$ with SRC on day 0 resulted in a response which was reduced relative to those receiving TRF on day 1. (Table 13). The addition of TRF on day 2 led to a lower and more variable response than did additions on day 0 or 1. In another series of experiments it was observed that the addition of TRF on both days 0 and 1 did not consistantly increase the number of PFC recovered per culture and TRF was therefore added on day 1 in all subsequent experiments.

The Antigen Dependency of $TRF_{Con\ A}$

An important consideration in the use of TRF's is whether their biologic activity replaces T cell function or they function as polyclonal activators and generate plaque forming cells in the absence of added antigen. The first examinations of the antigen dependency of TRF action indicated that TRF did indeed require antigen for its optimal effect. However subsequent experiments (Table 15) clearly demonstrated that the degree of antigen dependency of TRF activity was quite variable. Since the response facilitated by TRF was previously reported to be antigen dependent it seemed

Table 11 . Reconstitution of 1^{o} Anti-SRC response with $TRF_{Con\ A}$

Normal BDF, spleen cells were treated with either NGPS or R B(θ)+ NGPS, washed and cultured at $5X10^6$ viable cells/culture in either the presence or obsence of $5X10^6$ washed SRC/culture. After 48 hours in culture an equal volume of media on the 24 hour supernatants from cultures treated with 2.5 mcg of Con A ($TRF_{2.5}$) or with 5.0 mcg of Con A ($TRF_{5.0}$) was added and the cells harvested 48 hours later. The numbers of IgM anti-SRC PFC/culture was determined on pools of cells from quadruplicate cultures.

Reconstitution of 1^{o} Response with $TRF_{Con\ A}$

Treatment	Additions	PFC/culture	
		A	B
NGPS	-	11	16
NGPS	+	251	484
R B(Θ)+NGPS	+	61	-
	+ $TRF_{2.5}$	6,582	2,384
	+ $TRF_{5.0}$	89	2,673

Table 12 . Reconstitution of a 2^{o} Anti-SRC Response with $TRF_{Con\ A}$

Primed spleen cells were treated with either NGPS or R B(Θ) + NGPS, washed and cultured at $5X10^6$ cells/culture either in the presence or absence of $1X10^6$ washed SRC/culture. After 24 hours in culture an equal volume (1 ml) of media or 24 supernatants from cultures treated with 1.25, 2.5, 5.0 or 10.0 mcg/ml of Con A ($TRF_{1.25}$, $TRF_{2.5}$, $TRF_{5.0}$ and $TRF_{10.0}$ respectively) was added to the culture and the cells harvested 24 hours later. The numbers of IgM anti-SRC PFC/culture was determined on pools of cells from quadruplicate cultures.

Treatment	Addition	PFC/culture	
		A	B
NGPS	-	627	249
NGPS	SRC	10,890	13,735
R B(θ)+NGPS	SRC	142	178
R B(θ)+NGPS	$TRF_{2.5}$	208	-
R B(θ)+NGPS	$SRC+TRF_{10.0}$	-	100
R B(θ)+NGPS	$SRC+TRF_{5.0}$	285	5,259
R B(θ)+NGPS	$SRC+TRF_{2.5}$	1,440	5,348
R B(θ)+NGPS	$SRC+TRF_{1.25}$	1,033	-

Table 13 . The Effect of the Time of Addition of TRF on a 2^{o} Anti-SRC Response.

T depleted spleen cells were cultured in Sterilin Repliplates ($5X10^{6}$ cells in 1 ml of medium with $1X10^{6}$ washed SRC). After the indicated times an equal volume of $TRF_{2.5}$ was added to the cultures and the cells harvested after 72 hours in culture. The number of IgM anti-SRC PFC/culture was determined on pools of cells from quadruplicate cultures. Control cultures were treated with NGPS and did not receive $TRF_{2.5}$ but rather 1 ml of fresh medium at 24 hours.

Day of Addition	PFC/culture	
	A	B
0	813	3,439
1	1,440	5,259
2	351	2,546
No TRF Added	100	100
Control Response No T depletion, No TRF	10,890	13,735

Table 14 . A Comparison of TRF_{allo} and $TRF_{2.5}$ in their Ability to Reconstitute a 2° Anti-SRC Response.

Primed spleen cells were depleted of T cells and cultured in Sterilin repliplates $5X10^6$ viable cells in 1 ml of medium with $1X10^6$ washed SRC. After 24 hours in culture an equal volume of TRF_{allo} or $TRF_{Con\ A}$ was added and the cells were harvested 48 hours later. The number of IgM anti-SRC PFC/culture was determined on pools of cells from quadruplicate cultures.

Treatment	Additions	PFC/culture	
		A	B
NGPS		N.D.	249
NGPS+	SRC	9,920	13,735
R B( )+NGPS	SRC	20	178
"	$SRC+TRF_{allo}$	100	1,295
"	$SRC+TRF_{2.5}$	2,195	5,348

Table 15 . Lack of Antigen Dependency of TRF

Primed spleen cells were depleted of T-cells and challenged with SRC. After 24 hours $TRF_{2.5}$ was added and the cells were harvested 48 hours later. Control cultures were treated with NGPS and did not receive TRF. Experiment A $5X10^6$ cells/culture. Experiments B, C, D $1X10^6$ cells/ 200 µl in flat bottomed microtitre trays.

	SRC	$TRF_{2.5}$	PFC/culture			
			A	B	C	D
Control	-	-	627	82	32	80
	+	-	10,890	796	800	800
T-depleted	+	-	142	2	38	13
	-	+	208	106	380	87
	+	+	1,440	141	456	108

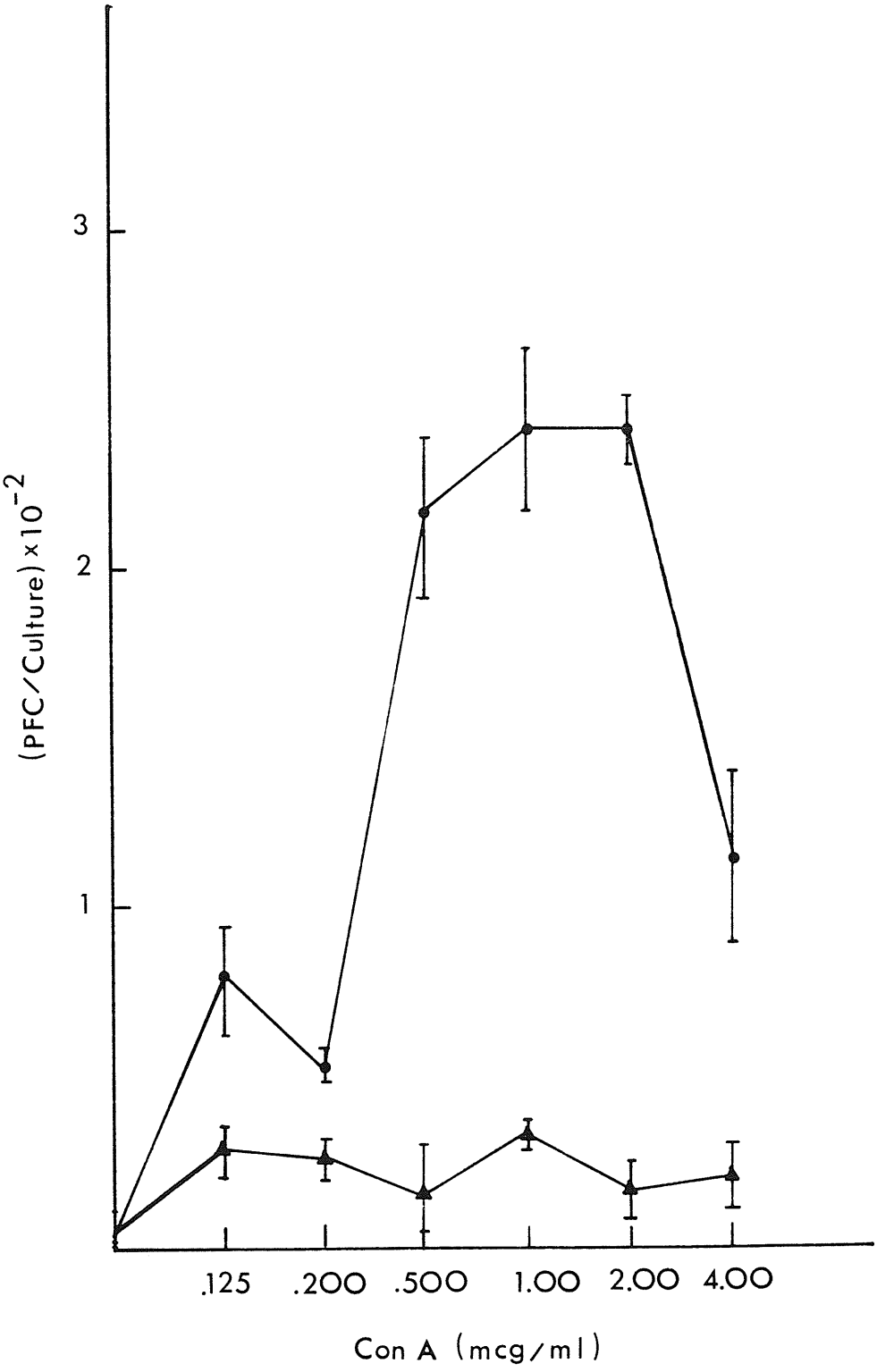
possible that the lack of antigen requirement observed in the present study was a result of the fact that the cells were obtained from primed animals. Since Schimpl and Wecker (89) had indicated that primed cells did respond to TRF in an antigen dependent fashion it was possible that the mode of immunization was where the problem lay. In the experiments described here the animals were immunized 4 days prior to sacrifice by an intravenous injection of SRC. It was therefore quite probable that the bulk of antigen would be accumulated in the spleens of these animals and that at the time of sacrifice sufficient residual antigen was present and synergized with the TRF. As a test these animals were immunized intraperitonealy, sacrificed 4 days later and the spleens used as a source of cells for culture. However the antigen dependency of the TRF assisted response was not increased by this immunization scheme. It seems somewhat less likely that antigen carryover was the explanation for these results and it may be that a proportion of the recently activated B cells show a reduced requirement for antigen but a strict requirement for T cell derived factors (30).

Con A Induced Helper Cells

An alternative approach to the generation of TRF containing supernatants was to induce nonspecific T helper cells in the cultures (51). Thus unprimed spleen cells were cultured for 24 hours in the presence of SRC at which time Con A was added to the cultures and the cells maintained for an additional 72 hours before assaying for anti-SRC PFC. The addition of Con A resulted in a 5-6 fold increase in the numbers of PFC observed relative to cultures which had received Con A alone, thus indicating that the effect was antigen dependent (Fig. 12). The degree of stimulation in

Figure 12. The Induction of Con A Helper Cells

$1X10^6$ Normal spleen cells were cultured in modified RPMI 1640 containing 5% FCS $5X10^{-5}$M 2-Me and $5X10^5$ SRC. Twenty-four hours after the initiation of the cultures the indicated amount of Con A was added and the cells cultured for an additional 72 hours before being assayed for anti-SRC PFC. (△) Con A (○) SRC & Con A.


3
2
1
(PFC/Culture) x 10^-2
.125
.200
.500
1.00
2.00
4.00
Con A (mcg/ml)

cultures receiving Con A and SRC relative to those cultures receiving only SRC was quite variable. Although these experiments were encouraging as to the feasibility of using Con A as a means of generating nonspecific help this system proved to be too variable of eight experiments, three showed an enhancing activity, two did not give any anti-SRC response and three showed a marked inhibition of the control anti-SRC responses in the presence of Con A. Such unpredictability in the response was obviously unacceptable and the use of Con A activated T helper cells was discontinued. During this period it was evident that animals drawn from the Immunology animal colony had gone through several periods where *in vivo* and *in vitro* reactivity to antigens and mitogens were reduced. It is possible that the failure to reproduce the initial results of Con A induced help may have been the result of an overall depressed reactivity of the cells as a consequence of infection of the donor animals.

Antisera

Six of the antisera were prepared against the 40% SAS precipitable serum proteins of a pool of normal $B_6D_2F_1$ serum (a gift from Dr. E. Rector) and two others were prepared against the $F(ab)_2$ of highly purified normal $B_6D_2F_1$ IgG. As an initial test of the specificities of the antisera immunoelectrophoresis was performed using normal $B_6D_2F_1$ serum as the antigen (Fig. 13). The lower slide A is a comparison of the precipitin patterns observed when RMF and RMG were used as the developing antisera. As was expected the RMG contained antibodies to immunoglobulins as well as non-immunoglobulin serum proteins. However the RMF gave a single precipitin bond corresponding to IgG. Slide B shows the precipitin patterns observed when NMS (upper well) and protein A purified normal mouse IgG (lower well) were co-electrophoresed and sheep anti-normal mouse serum (a gift from Dr. W. Y. Lee) in the central trough was used to develop the precipitin bands. One band is observed in the protein A purified IgG indicating that the preparation is relatively free from non-immunoglobulin contaminants. This IgG preparation was further purified on Sephadex G-200 (Fig. 14) and the IgG peak 2 was digested with pepsin. The digestion was only about 50% efficient as judged by the G-200 profile of the digested material therefore the digest was repassed through a protein A sepharose column. That the digestion had modified the IgG is evident in slide C where the purified IgG and $F(ab)_2$ were electrophoresed and the slide developed with a sheep anti-NMS serum.

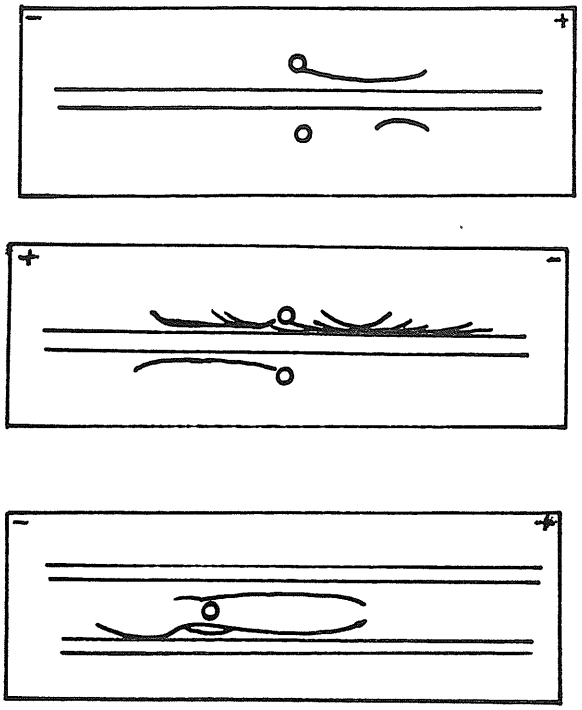
The cellular specificities of the antisera were examined by indirect immunofluorescent staining after the antisera had been absorbed with mouse erythrocytes. Both the RMF and the RMG antisera stained comparable proportions of spleen cells (57%) which were slightly higher than that stained

Figure 13. Immunoelectrophoresis Patterns of Various antisera and antigens.

A) Normal mouse serum was electrophoresed in the central well and either RMF (upper trough) or RMG (lower trough) were added to develop the precipitin pattern.

B) Normal mouse serum (upper well) or Protein A purified normal mouse IgG (lower well) were coelectrophoresed and a polyvalent sheep anti-normal mouse serum preparation was used to develop the precipitin pattern.

C) Protein A purified normal mouse IgG (upper well) or pepsin digested normal mouse IgG (lower well) were coelectrophoresed and a polyvalent sheep anti-normal mouse serum preparation was used to develop the precipitin pattern.


C
B
A

using fluoresceinated anti-IgM (~50%). However this difference (about 7%) may be attributable in part to the higher sensitivity of the indirect staining method used with the RMF and RMG sera. The values for the proportions of stained cells fall within the expected range for B cells in the spleen and the lack of staining of thymocytes is compatable with the suggestion that the antisera are detecting surface immunoglobulin.

The antisera most probably have specificities for determinants common to all immunoglobulin classes, either anti-light chains, anti Fab or both, since neither antisera had any detectable anti IgM activity yet the antisera reacted with a high proportion of spleen cells. It has been estimated that less than 1% of the total surface immunoglobulin is IgG and that this class is present on only a small proportion of splenic B cells (23). Therefore the antisera must recognize some determinants in addition to those specific for IgG heavy chains.

In order to establish what the cell surface target antigens of the antisera were, viable lymphocytes were surface radioiodinated, solubilized in a non-ionic detergent and aliquots of lysate were mixed with either RMG, RMF or NRS. The resulting antigen-antibody complexes were precipitated with either Protein-A Sepharose or Protein-A containing formalinized Staph aureus. The precipitated material was washed, eluted from the Protein A in an SDS containing buffer, reduced and finally electrophoresed in 10% polyacrylamide gels containing SDS. The gels were sliced and the fractions assayed for their radioactive content. (Fig. 15A & 15B.

The electrophoretic pattern revealed three major peaks of radioactivity corresponding in molecular weights to the heavy chains of IgM and IgD and the light chains of mouse immunoglobulins. These results suggest that the major determinants which the antisera react with on the surface of spleen

Figure 14. G-200 Elution Profile of Protein A Purified Normal Mouse IgG.

60 ml of Normal BDF serum were precipitated in the cold with 40% SAS (final concentration), washed 3X with 40% SAS and dialyzed extensively against distilled water and then PBS. The precipitated material was then passed through a Protein A Sepharose-4B column and the bound material eluted with 0.1M acetic acid in saline and dialyzed against PBS. This eluted material was passed through a Sephadex G-200 column (2.6X90 cm) and the fractions collected.

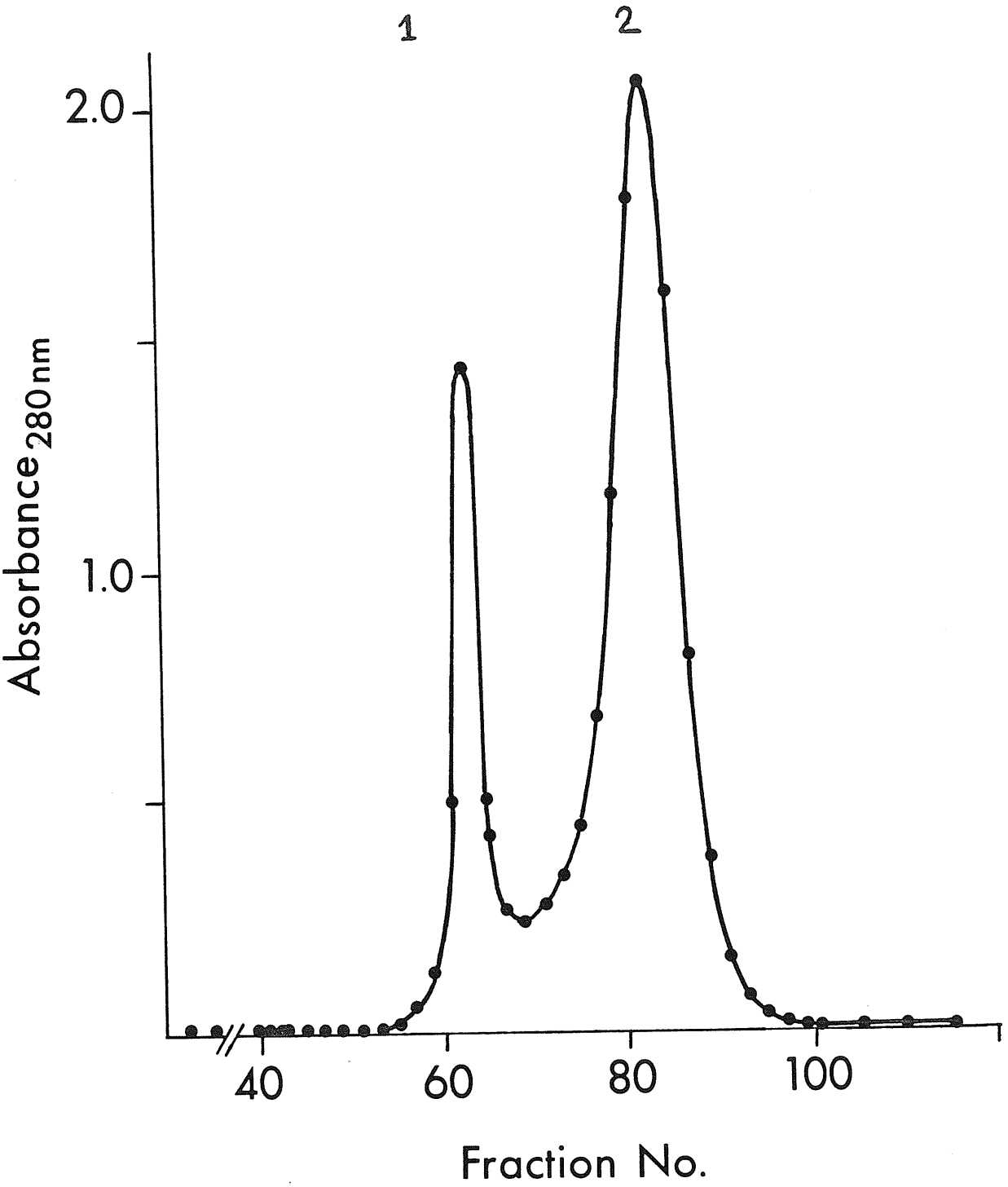

1
2
2.0
1.0
Absorbance 280 nm
40
60
80
100
Fraction No.

Figure 15. Electrophoretic profile of ^{125}I-labelled cell membrane proteins of spleen cells precipitated with RMG;RMF (solid line) or NRS (dashed line). 5X10^7 viable spleen cells were surface labelled with ^{125}I using the lactoperoxidase method. The washed cells were lysed in 1 ml of 0.5% NP-40 NET (150 mM NaCl, 5 mM EDTA, 50 mM Tris) pH 7.4, and the cellular debris removed by centrifugation at 20,000 x G for 20 mins. The resulting supernatants were mixed with 15μl of either NRS or A) RMF B)RMG for 20 minutes at room temperature after which 200μl of formalinized Staph. aureus (10% v/v in 0.05% NP-40 NET) or Protein A Sepharose were added to the mixture. The bacteria were collected by centrifugation at 2000 x G for 20 minutes at 4°C and washed twice with 0.05% NP-40 NET. The antigen-antibody complexes were eluted from the bacteria by heating for 1 min. at 100°C in 200μl of a 4% SDS,6M urea, 5% 2-Mercaptoethanol solution and cleared of bacteria by centrifugation at 12,000 x G for 15 minutes. A 100μl sample of the solubilized material was applied to a 10% polyacrylamide gel (140 mM running gel) containing 2% SDS and electrophoresed at 4mA/gel. The gels were cut into 2 mm slices and the radioactivity measured with a Beckman gamma counter. The arrows indicate the positions of the standards used to calibrate the gels; heavy chains of rat IgE (IR 162) (ϵ), bovine serum albumin (BSA), heavy chaings of mouse IgG_1 (MOPC 21A) (γ), ovalbumin (OA), light chains of mouse IgG_1 (MOPC 21A) (L).

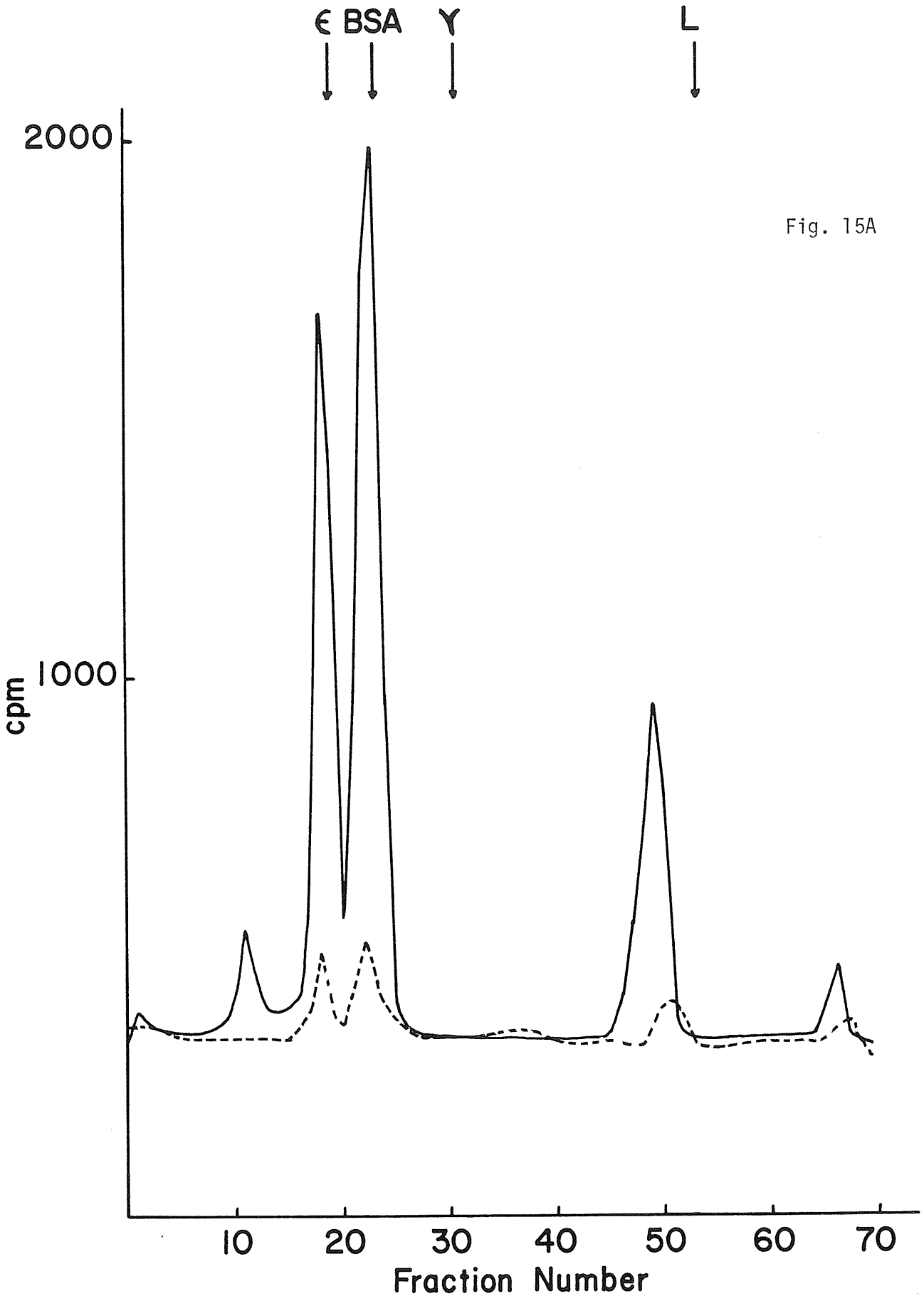


Fig. 15A

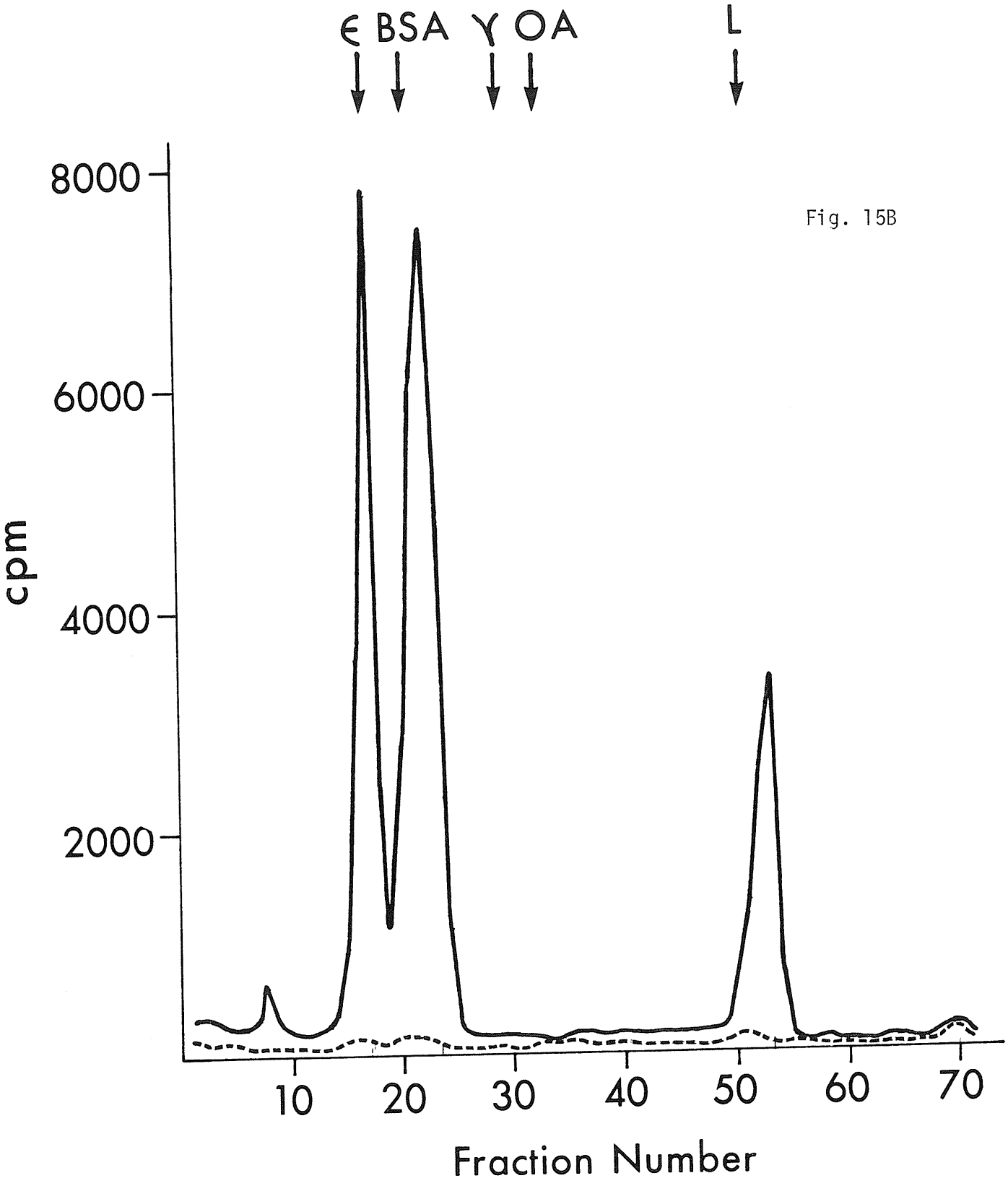

ϵ BSA
γ OA
L
8000
6000
4000
2000
cpm
10
20
30
40
50
60
70
Fraction Number

Fig. 15B

cells are sIgM and sIgD thus lending further support to the suggestion that the antisera contain activity towards either L chains, Fab or both.

I would like to acknowledge the assistance and advice of Dr.'s Ricki Helm and Dan Conrad in performing the SDS-PAGE analysis.

Leaf blank to correct

numbering

Anti-Immunoglobulin Effects in vitro

The Intact IgG of RMF and RMG

Initial attempts to induce proliferation in whole spleen cell populations with the IgG fractions of several different antisera consistently failed (Table 16). The IgG fraction of RMG was tested at concentrations ranging from 1 mg/ml to 100 ng/ml in doubling dilutions and no evidence of activation, as determined by increased ^{3}H-thymidine incorporation was observed on days 2, 3 or 4 relative to control cultures receiving normal rabbit IgG. However when RMG was added to cultures containing $5X10^{-5}$M 2-Me a modest but consistent increase in thymidine incorporation was observed on day 3. This stimulation was the result of two factors 1) on increase in DNA synthesis due to the antibody activity of RMG and 2) a suppression of the control levels of incorporation by the NRIgG (Table 17). This duality of the effects of anti-Ig's was further demonstrated when cultures were treated with either NRS or RMG. The NRS caused a 10-15 fold reduction in the levels of thymidine incorporation relative to those containing either FCS or RMG (Table 18). Another interesting point which is demonstrated in Table 18 is the difference in the effects of RMG and NRS on the LPS induced mitogenesis and plaque formation of spleen cells. The presence of NRS in the cultures containing LPS resulted in a marked reduction in the levels of proliferation relative to LPS treated control cultures,a proportionate reduction in the numbers of PFC recovered was also observed. In contrast RMG did not suppress the levels of thymidine incorporation to as great an extent as NRS yet the PFC response of the former cultures was almost totally eliminated.

Since the previous results indicated that the RMG might have two

Table 16. Failure of RMG IgG to Stimulate Spleen Cells.

$1X10^6$ Normal spleen cells were cultured in 200μl of medium in flat bottomed microtitre trays. The cells were pulsed with ^{3}H-Thymidine (0.2μCi/culture) for 6-8 hours prior to harvesting.

		Days in Culture (Cpm/culture ± S.E.M.)		
		2	3	4
Media		151 ± 29	151 ± 29	132 ± 18
NRG IgG	100 mcg.	348 ± 13	207 ± 13	235 ± 60
	50	392 ± 33	229 ± 12	179 ± 49
RMG IgG	100 mcg.	167 ± 21	180 ± 25	403 ± 200
	50 mcg.	150 ± 11	195 ± 10	166 ± 42
NRG $F(ab')_2$	50 mcg.	206 ± 11	113 ± 21	103 ± 28
RMG $F(ab')_2$	50 mcg.	6465 ± 591	7592 ± 1587	4455 ± 601

Table 17. The Effect of 2-Me on the Anti-Ig Induced Proliferation of Normal Spleen Cells.

Spleen cells were cultured in the presence of the indicated stimuli in the media lacking 2-Me or containing $5X10^{-5}M$ 2-Me. Cells were pulsed with ^{3}H-Thymidine for 4 hours before harvesting after 72 hours in culture.

	-2-Me	+2-Me
	cpm/culture ± S.E.M.	
Media	151 ± 29	3910 ± 323
NRG $F(ab')_2$	111 ± 2	1623 ± 272
RMG $F(ab')_2$	7592 ± 1587	9570 ± 560
NRG IgG	229 ± 12	1944 ± 179
RMG IgG	195 ± 10	4722 ± 437

Table 18 . The Effect of RMG sera or NRS on the LPS Induced Proliferation and Antibody Formation.

$1X10^6$ normal spleen cells were cultured in media containing 5% FCS and $5X10^{-5}$M 2-Me. The indicated additions were made to cultures in the presence or absence of 50 mcg of LPS. RMG 60 and RMG 61 are two different antisera. All sera were present in the cultures at a final dilution of 1:20. Thymidine incorporation was measured after 48 hours of culture and anti-TNP PFC after 72 hours of culture.

Additions	-LPS		+LPS	
	Cpm/culture	PFC/culture	Cpm/culture	PFC/culture
-	1867	1	24,460	203
NRS	209	0	9,172	105
RMG 60	3830	0	12,094	9
RMG 61	3111	0	15,320	11

different effects one of which also appeared to be produced by normal rabbit immunoglobulin it seemed that the F_c of the IgG molecule was a possible source of the observed inhibitory effects of NRIgG. Therefore the effects of $F(ab')_2$ of RMG and NRIgG were examined in vitro.

The effects of the $F(ab')_2$ of RMG in vitro

The initial preparations of the $F(ab')_2$ of RMG and NRIgG were prepared from the peptic digests of the 40% SAS precipitable proteins of the respective sera. When the conventional methods of purification of $F(ab')_2$ on Bio-Gel P-150 were employed the major peak containing the $F(ab')_2$ was found to be contaminated with intact IgG thus necessitating the use of samples from the descending portion of this peak. The latter fractions had been demonstrated to be free of intact IgG by Ouchterlony analysis.

Addition of the $F(ab')_2$ of RMG to cultures of normal spleen cells resulted in a 4-8 fold increase in thymidine incorporation relative to cultures receiving either NR $F(ab')_2$ or FCS. The increase in stimulation indices was therefore the result of an enhanced rate of thymidine incorporation relative to control cultures rather than due to the suppression of control responses by the $NRF(ab)_2$. None of the cultures used for the assessment of the mitogenicity of the $F(ab')_2$ had 2-Me in them and as shown in Table 17 the addition of the 2-Me had only marginal effects on the levels of ^{3}H-thymidine incorporation induced by RMF $F(ab')_2$.

In subsequent experiments on alternate approach for the purification of $F(ab')_2$ was employed. Rabbit IgG was purified on a Protein A sepharose column and then digested with pepsin. The resulting digest was fractionated on a Sephadex G-100 column and the voided material collected. This

step served to remove the pFc fragments and the pepsin from the voided $F(ab')_2$ and the undigested IgG molecules. The $F(ab')_2$ containing fraction was depleted of undigested IgG by repassing through a Protein A Sepharose column. The unbound fraction was concentrated and dialyzed against saline before being used in cultures. This procedure resulted in a considerable saving of time and provided higher yields of material with higher biological activity than those processed using the conventional Sephadex G-150 procedure consequently this method was routinely used for the preparation of $F(ab')_2$.

The mean stimulation index $\pm$ S.E.M. for 30 separate experiments was 43 ± 10 relative to control cultures receiving NRIgG $F(ab')_2$. The magnitude of the responses initially varied considerably however by including LPS in parallel sets of cultures it was possible to determine whether the variation was a result of lack of stimulation by the $F(ab)_2$ or rather a general unresponsiveness on the part of a particular spleen cell preparation. In greater than 90% of those cases where the LPS control was included the $F(ab')_2$ of anti-Ig induced thymidine incorporation $\sim$ 75% of the level observed in the LPS treated cultures (for example Table 10).

Stimulatory Activity of RMG $F(ab)_2$ is due to Anti-Ig Specificities of the Antibodies

The RMG sera had been partially characterized by immunoelectrophoresis, immunofluorescence and SDS - PAGE analysis of cell surface antigens detected by the antisera. However RMG also contained specificities against serum components other than immunoglobulins, therefore it was not possible by the above techniques to formally exclude a role for these antibodies in the RMG induced proliferation.

Table 19 The Effect of Absorbing RMG $F(ab')_2$ with Normal Mouse IgG - Sepharose 4B

A mitogenic preparation of RMG $F(ab')_2$ was passed through either an ovalbumen coupled Sepharose 4B column or a normal mouse IgG coupled Sepharose 4B column. The effluent was collected, concentrated, dialyzed against media and tested for mitogenicity. Cultures were labelled for the final 8 hours of a 72 hour culture period.

	cpm/culture ± S.E.M.					
	A			B		
NRG $F(ab')_2$	2,299	±	639	483	±	63
OA-Sepharose absorbed RMG $F(ab')_2$	10,070	±	1,167	14,368	±	881
NMIgG-Sepharose absorbed RMG $F(ab')_2$	4,961	±	468	929	±	297

Table 20 The Mitogenicity of Bio-Gel P-150 Purified RMG $F(ab')_2$

RMG IgG (40% SAS precipitate of RMG serum) was digested with pepsin and applied to a 2.6 x 90 cm column of Bio Gel P-150. The descending portion of the voided peak was collected, dialyzed and concentrated. The mitogenicity of this material was assessed in culture. Cultures were pulsed with ^3H-Thymidine for the final 8 hours of a 72 hour culture. The data is the mean $\pm$ SEM of 8 cultures.

Additions	Expt A	Expt B	Expt C	Expt D
	cpm/culture			
-	620 ± 48	222 ± 11	462 ± 24	271 ± 42
NRG $F(ab')_2$	432 ± 10	319 ± 12	525 ± 41	392 ± 8
RMG $F(ab')_2$	2,033 ± 387	4,254 ± 187	1,796 ± 46	3,914 ± 163

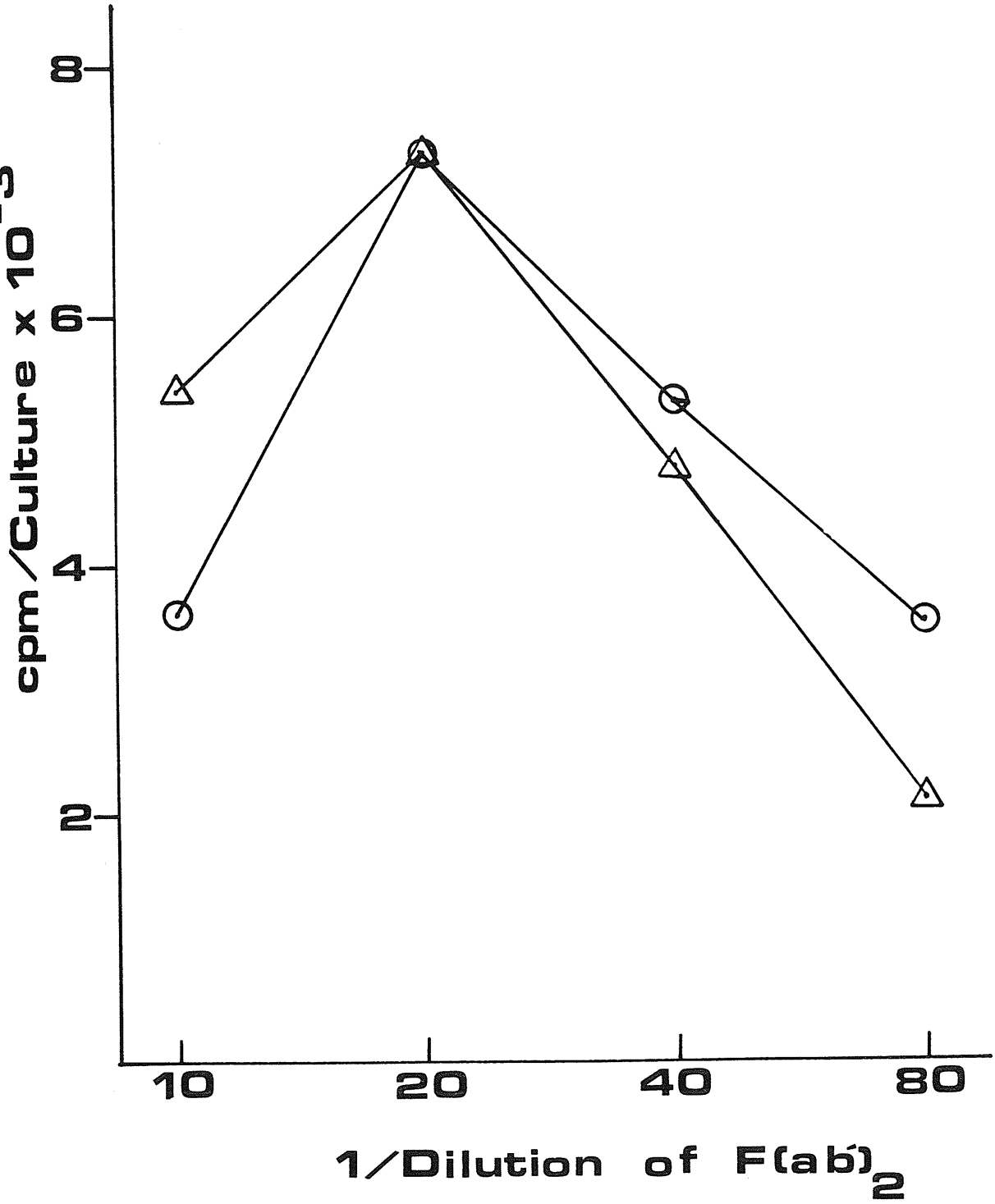
In an effort to exclude the above possibility portions of a mitogenic preparation of $F(ab')_2$ of RMG were passed through a column containing normal mouse IgG coupled to Sepharose-4B beads (a gift from Dr. S. Wie) or through an ovalbumin Sepharose 4B column, the mitogenic capacities of these two samples were then compared with those of $F(ab')_2$ of normal rabbit IgG. The passage of $F(ab')_2$ of RMG through the NMIgG - Sepharose column resulted in a marked reduction in the amounts of thymidine incorporated relative to the OA Sepharose passed $F(ab')_2$ of RMG thus indicating that the relevant antibody activity was directed against determinants present on normal mouse IgG. These results strengthen the previous suggestion that the relevant antibody specificities are directed towards determinants common to all immunoglobulin classes. (Table 19).

During the course of these experiments it was reported that Protein A was mitogenic for human and murine B lymphocytes. Although it seemed highly unlikely that Protein A was contributing to the observed proliferative response it was necessary to exclude this possibility.

Protein A purified normal rabbit IgG was coupled to Sepharose-4B and washed extensively under conditions used for the elution of IgG from Protein A Sepharose in an effort to remove any possible Protein A carried over from the Protein A Sepharose column. Samples of $F(ab')_2$ of RMF were passed through either the NRIgG Sepharose column or a control column of OA Sepharose and the mitogenic capacities of these two factors were compared. If Protein A were contributing to the $F(ab')_2$ induced proliferation it would be expected that the IgG Sepharose column would remove the Protein A as this is the conventional way of purifying this protein and that the unbound $F(ab')_2$ would no longer possess mitogenic activity. In contrast the OA Sepharose column does not bind Protein A and should conse-

Figure 16 . Effect of Absorbing RMG $F(ab')_2$ with NRG - Sepharose.

Protein A purified normal rabbit IgG or ovalbumen was coupled to Sepharose 4B (10 mg protein/ml packed Sepharose) and portions RMG $F(ab')_2$ was passed through each column. The effluent was collected, dialyzed against medium and tested for mitogenicity. Cultures were pulsed with ^{3}H-Thymidine for the final 18 hours of a 72 hour culture. LPS response was 14,300 cpm/culture. OA-Sepharose (Δ) IgG-Sepharose (O).


8
6
4
2
cpm/Culture x 10-3
10
20
40
80
1/Dilution of F(ab')2

quently not reduce the mitogenic properties of the $F(ab)_2$ of RMF.

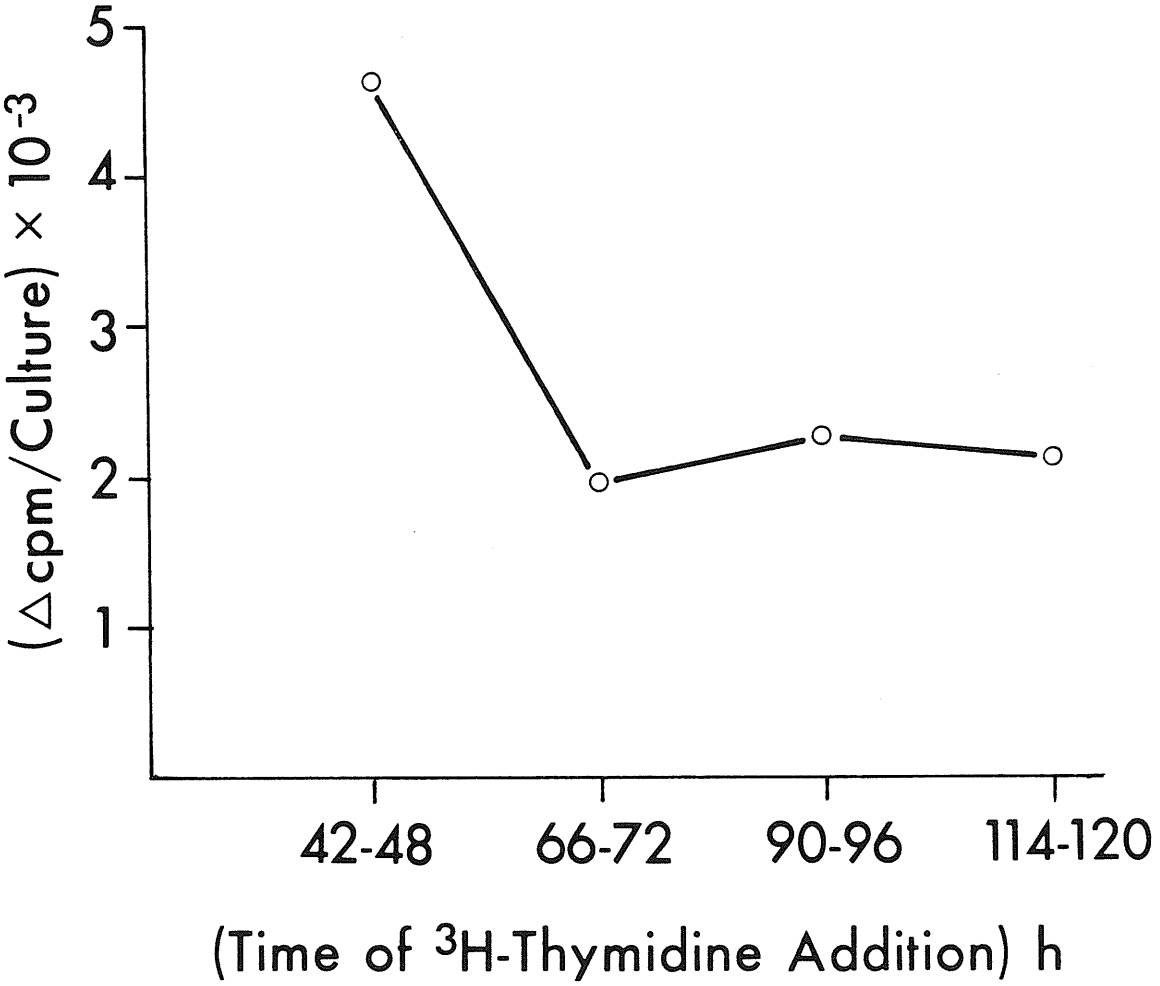
The results of such an experiment (Figure 16) show that there was not a differential effect on the $F(ab')_2$ mitogenicity by these two absorbtions making it highly unlikely that Protein A had contributed to the mitogenesis induced by this $F(ab)_2$. Several other points argue very strongly against Protein A contamination as the source of mitogenic activity. 1) Since both the NRIgG $F(ab')_2$ and the RMG $F(ab')_2$ were prepared in the same manner such that both preparations would be expected to be contaminated with similar quantities of Protein A. However NR $F(ab)'_2$ does not cause an increase in thymidine incorporation relative to control cultures, also the RMG $F(ab')_2$ induced proliferation is expressed as a stimulation relative to that induced by $F(ab')_2$ of NR IgG thus indicating that the RMF/RMG $F(ab')_2$ induces proliferation above any possible NR $F(ab')_2$ induced response. 2) The concentrations of Protein A which are required to achieve polyclonal activation are exceedingly high, to achieve such concentrations in the range required for Protein A mitogenesis 2-3 mg/ml of Protein A would have had to be present in the $F(ab')_2$ preparations. This represents approximately 1/3 - 1/4 of the total contents of the column used for purification however their was no detectable decrease in the capacity of the column to bind IgG. 3) The strongest argument against Protein A contributing to the mitogenic activity of the $F(ab')_2$ is the observation that $F(ab')_2$ prepared by a method not involving Protein A was mitogenic (Table 20).

Kinetics of the $F(ab')_2$ Induced Proliferation

Maximum levels of ^{3}H-thymidine incorporation were observed between 48 and 72 hours of culture (Figure 17). However the incorporation levels

Figure 17. Kinetics of $F(ab')_2$ of Anti-Ig Induced Proliferation.

$1X10^6$ Normal spleen cells were cultured in 200 µl of RPMI containing 5% FCS, 50 mcg of $F(ab')_2$ of RMG but not 2-Me. The cells were pulsed with ^3H-Thymidine (0.2 µCi, 2.2 Ci/mMole) for the times indicated and the levels of thymidine incorporation determined. The control cultures receiving the $F(ab')_2$ of NRG had less than 300 cpm/culture.


5
4
3
2
1
(Δcpm/Culture) × 10-3
42-48
66-72
90-96
114-120
(Time of 3H-Thymidine Addition) h

for the $F(ab')_2$ RMG treated cultures remained above control levels even after 5 days indicating that some proliferation was still occuring at this time. Cultures were normally pulsed with ^{3}H-thymidine after 48 hours in culture for an 18-24 hour period.

The Effect of Varying $F(ab')_2$ Concentration in Culture

Dose response curves for the $F(ab')_2$ of RMF and RMG showed peak proliferations at dilutions of 1:20 to 1:40 of the stock $F(ab')_2$ solutions(Figs. 18, 19). This corresponds to 25-50 mcg of protein per culture, however as this was not immunosorbent purified, the amount of $F(ab')_2$ with anti-Ig activity was likely considerably less than the total protein concentration. At protein concentrations of less than 10 mcg per culture the levels of incorporation observed are not significantly different from those observed with NRIgG $F(ab')_2$, thus the mitogenic activity appears to be effective over an 8 fold range making titration of the $F(ab')_2$ critical for examinations of the proliferative response.

The effects of high concentrations of $F(ab')_2$ were quite variable sometimes inducing levels of incorporation similar to those observed with 1:20 or 1:40 dilutions of $F(ab')_2$ and at other times causing a reduction in the levels of incorporation. These high concentrations were not often employed as they represented a considerable expenditure of $F(ab')_2$ plus the fact that the responses were too variable.

The data in Figure 19 indicate that both RMF $F(ab')_2$ and RMG $F(ab')_2$ induce comparable levels of mitogenesis over a similar range of concentrations. These results demonstrate that the mitogenic activity of the antibody correlates well with anti-immunoglobulin activity.

Figure 18. Effect of Varying $F(ab')_2$ of RMG concentration on the proliferative response of normal spleen cells.

$1X10^6$ spleen cells were cultured in the presence of either $F(ab')_2$ of RMG (●) or $F(ab')_2$ of NRG (■) for 72 hours. ^3H-Thymidine (0.2 μCi) was added to the cultures for the final 18 hours and the levels of thymidine incorporation determined.

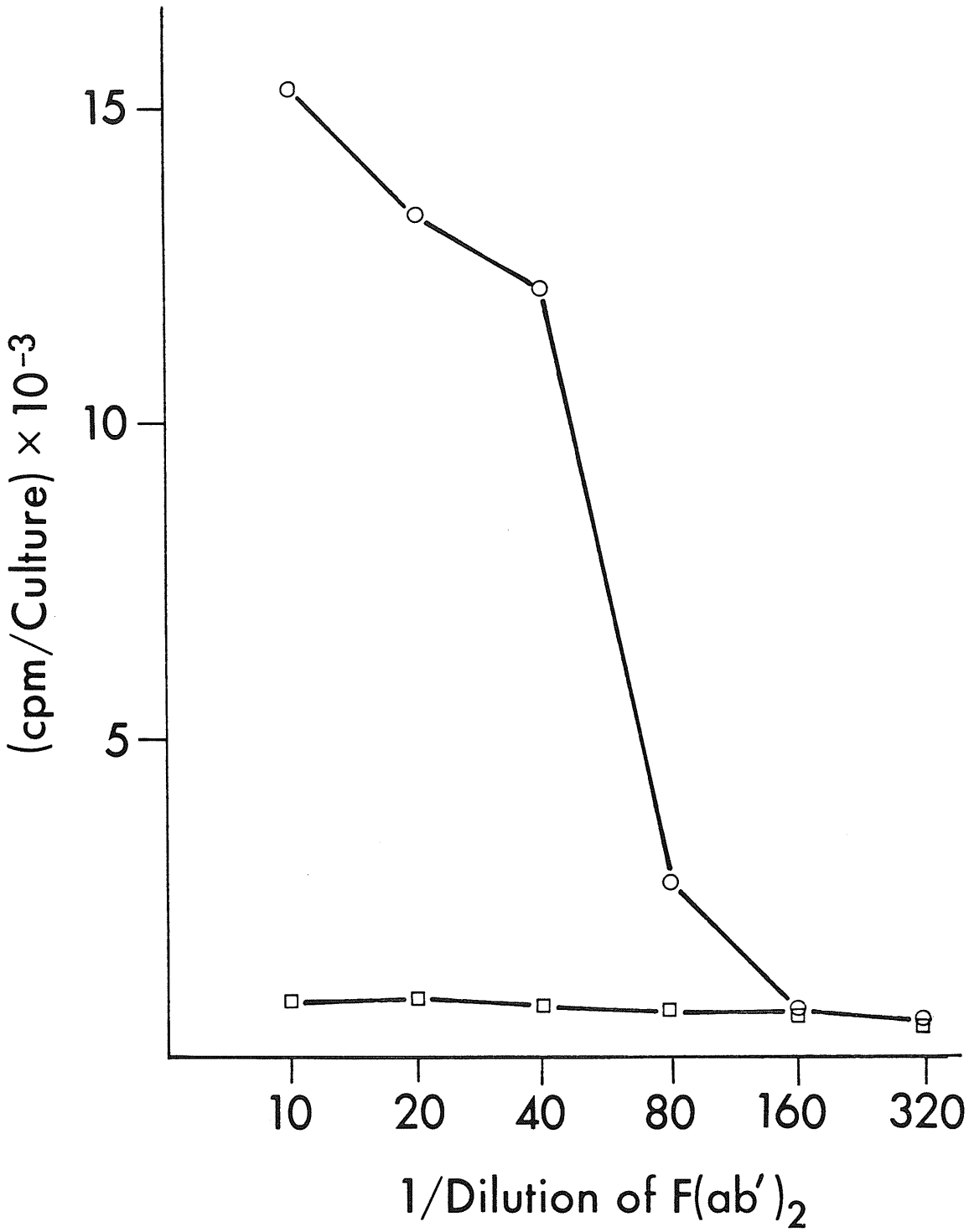
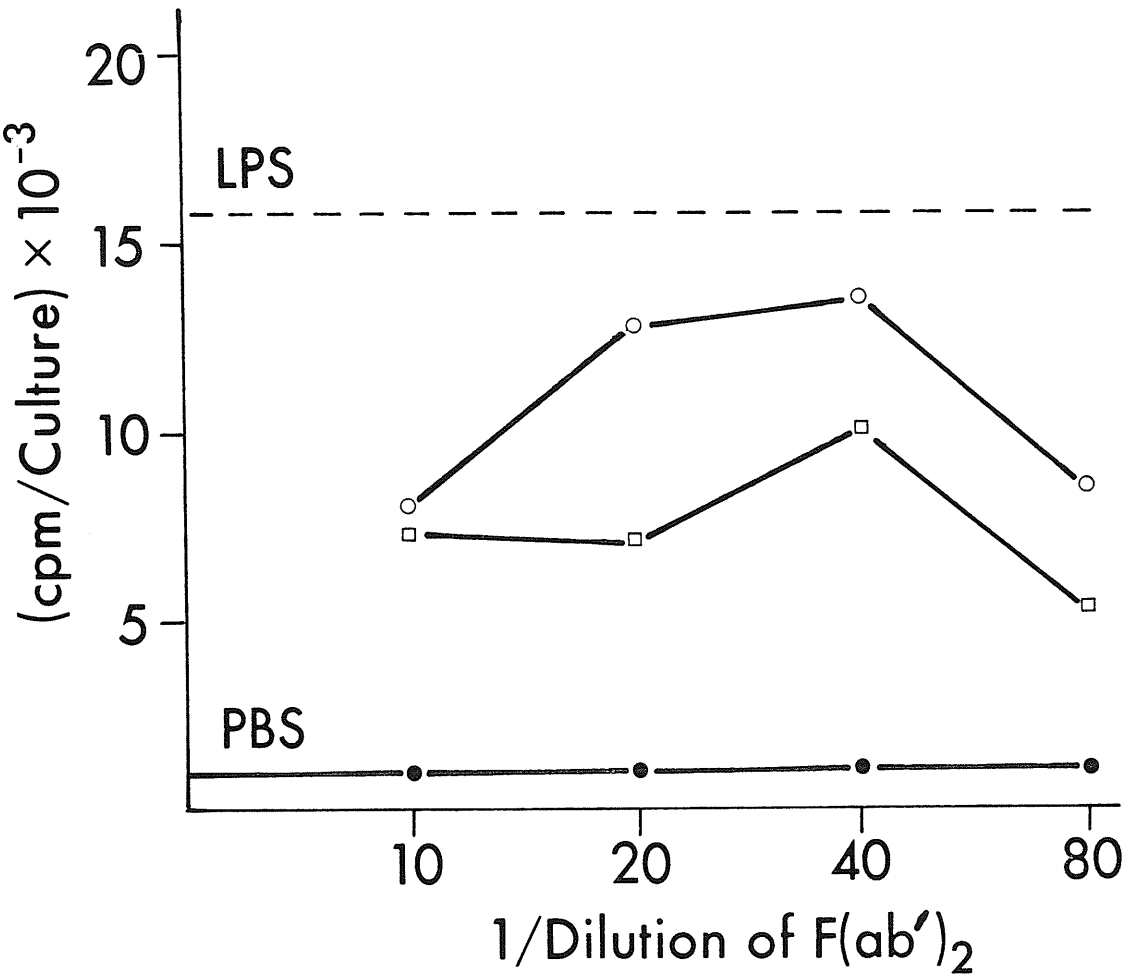

15
10
5
(cpm/Culture) × 10⁻³
10
20
40
80
160
320
1/Dilution of F(ab')2

Figure 19 . A comparison of the mitogenic properties of the $F(ab')_2$ of RMG and RMF.

$5X10^5$ Normal spleen cells were cultured in the presence of the indicated dilutions of PBS (●), RMF (O) or RMG (□) for 72 hours. As a positive control the response to 50 mcg of LPS is included (dashed line.)


20
15
10
5
LPS
PBS
(cpm/Culture) × 10⁻³
10
20
40
80
1/Dilution of F(ab′)₂

The Effect of Cell Density on $F(ab')_2$ Induced Proliferation

In some of the preliminary experiments there was a great deal of variability in the magnitudes of the responses to $F(ab')_2$ of RMF or RMG. Since the antibody responses in microcultures were so sensitive to cell density effects the responses to $F(ab')_2$ and LPS were examined to determine if this factor was contributing to the observed variability.

The LPS induced response consistently showed a maximal peak at $3\text{-}5X10^5$ cells/culture whereas the responses to $F(ab')_2$ were optimal over a range from $4\text{-}12X10^5$ cells per culture with the optimal varying somewhat from one experiment to the next (Figures 20A,B). A cell concentration of $8\text{-}10X10^5$/culture was eventually adopted as this represented the range for obtaining a consistant response.

Effect of Varying the Exposure Period of Cells to $F(ab')_2$ on the Proliferative Response

Most PBA's must be in contact with the lymphocytes for periods of 18-24 hours to obtain maximal stimulation, it was therefore of interest to determine if anti-Ig induced proliferation also required such an extended exposure period.

Spleen cells were incubated for varying times in the presence of $F(ab')_2$, washed and cultured in fresh medium. Forty-eight hours after the initiation of the cultures ^3H-thymidine was added and the following day the cells were harvested. The amounts of ^3H-thymidine incorporated into the cultures which had been washed at various times were then compared with those of the control culture which had had $F(ab')_2$ present throughout the culture period. Figure 21 is composed of the mean values of three experiments and the results are

Figures 20A,B The Effect of Varying Cell Concentration on the Anti-Ig Induced Proliferation.

The indicated numbers of normal spleen cells were cultured in 200 µl of RPMI containing 5% FCS and either 50 mcg/ml of LPS (△) or 50 mcg/ml of $F(ab')_2$ of RMF(●) ^3H-Thymidine incorporation was determined for the final 18 hour period of a 72 hour culture. Δ cpm = (Expt'l - Control) cpm/culture

Fig A

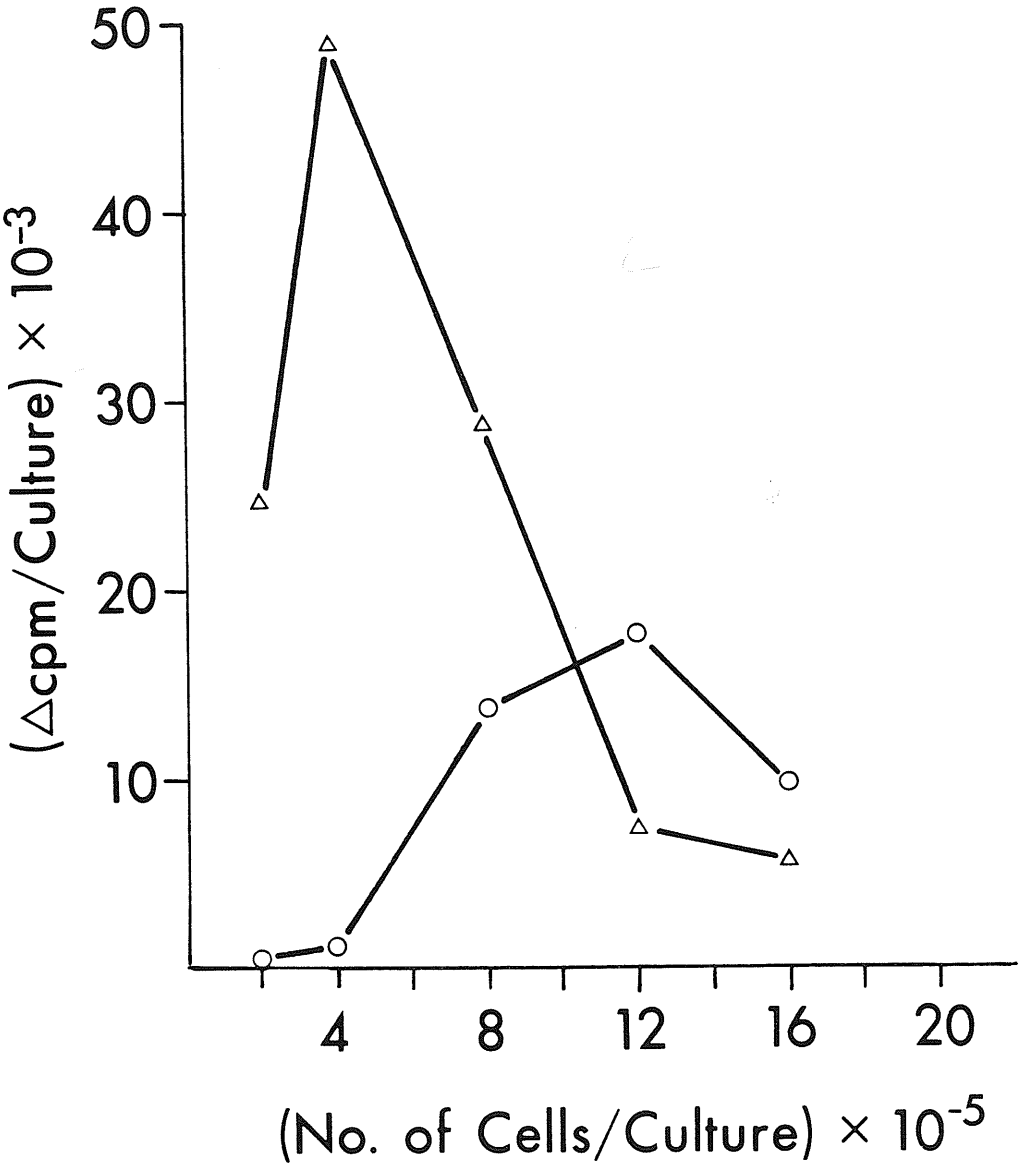
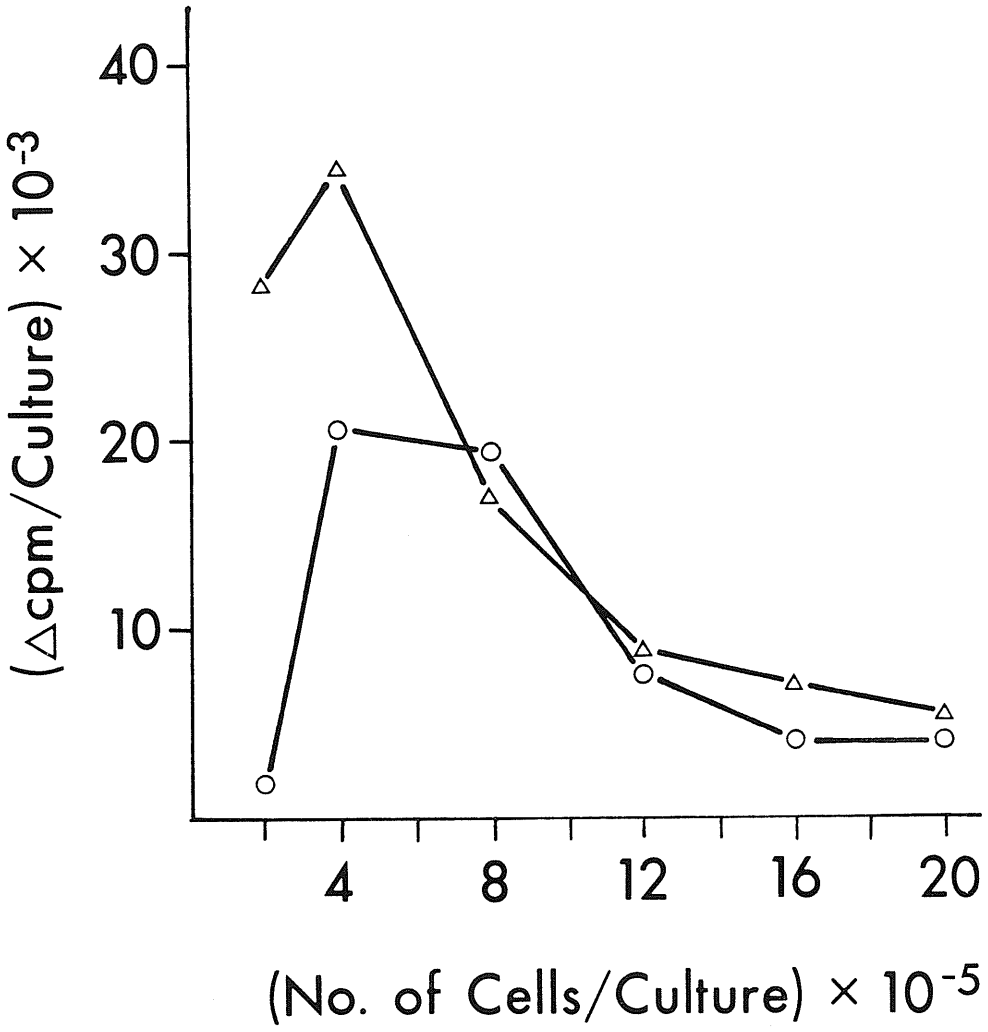

50
40
30
20
10
(Δcpm/Culture) × 10⁻³
4
8
12
16
20
(No. of Cells/Culture) × 10⁻⁵

Fig B



normalized by expressing them as percentage of the 72 hour response. Exposure of the cells to $F(ab')_2$ for 48 hours resulted in ~70% of the control response whereas a 24 hour exposure had no effect on thymidine incorporation. These results strongly contrasted with those observed by others with LPS and Con A where 24 hour exposure was sufficient for maximal stimulation.

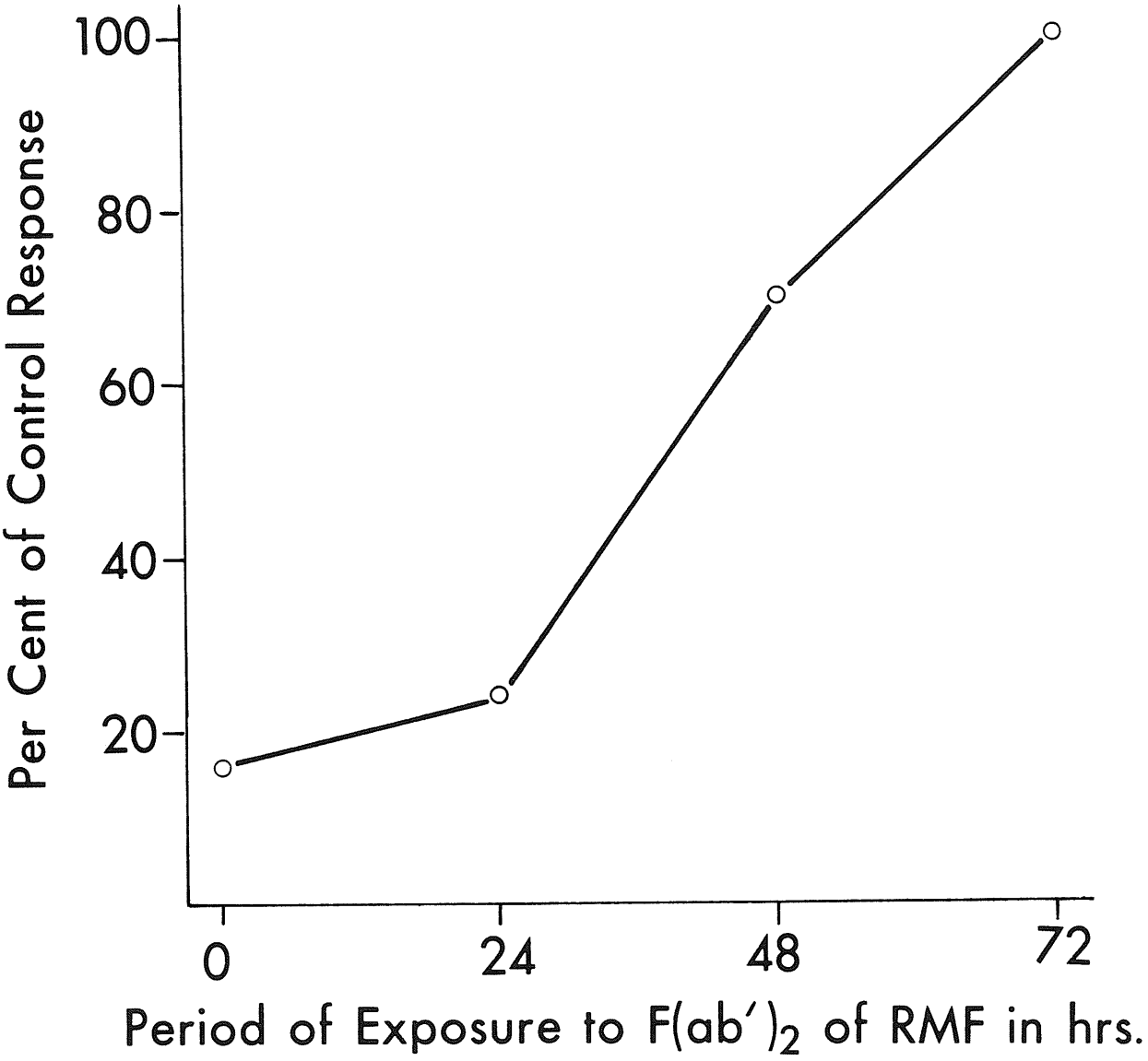
Fetal Calf Serum Requirements of the Proliferative Response

Although no attempt had been made to screen fetal calf sera (FCS) for their capacity to support the anti-Ig induced proliferation, it was possible that the batches employed had special properties and/or that the anti-Ig required some FCS mitogen for the activation of the splenocytes. In order to determine if these possibilities were contributing to the anti-Ig induced proliferation normal spleen cells or cells from ATXBM mice were isolated and cultured in serum free media in the presence of either NR $F(ab')_2$ or RAMG $F(ab')_2$. As was expected, culturing in serum free medium resulted in a significant reduction in the total amount of thymidine incorporated by both sets of cultures. However, when the stimulation indices of these cultures are compared to those of cells cultured with FCS and anti-Ig the magnitude of the responses are similar (Table 21).

In the course of these experiments five different batches of FCS have been used and only one batch failed to support the proliferative response to anti-Ig. This particular batch of serum was a contract approved lot from Gibco which was found to support a primary anti-SRBC response while the other lots failed to support such a response. There was no difference between the mitogenicity of this batch of FCS and that of several of the supportive FCS and it is not clear as to what property of this serum was

Figure 21. The Effect of Varying the Exposure Period of Cells to the $F(ab')_2$ of RMF.

$8X10^5$ normal spleen cells were cultured in the presence of $F(ab')_2$ of RMF (50 mcg/ml) for the indicated times, washed in fresh medium and recultured for a total of 72 hours. ^3H-Thymidine incorporation was determined for the final 18 hour period of the culture. The data represents the mean of three separate experiments background responses have been subtracted from the $F(ab')_2$ induced response.


100
80
60
40
20
Per Cent of Control Response
0
24
48
72
Period of Exposure to F(ab′)2 of RMF in hrs.

responsible for the inhibition of anti-Ig induced proliferation. These results suggest that the requirement for FCS is not obligatory.

T Cell Dependency of the Proliferative Response

The response to the $F(ab')_2$ was assumed to be a result of the activation of splenic B lymphocytes. However, it was conceivable that the responding cells required T cells for the response or even that T cells could have been activated by B cell products following the binding of anti-Ig to the latter. Therefore, the effect of the T depletion of the spleen cell populations on the subsequent anti-Ig induced proliferation was examined.

Treatment of normal spleen cells with AKR anti-C3H thymocyte serum and complement had no effect on the subsequent $F(ab')_2$ induced proliferation (Table 22). In previous experiments it had been shown that this treatment resulted in a ~95% reduction in the responsiveness to Con A. These results strongly implied that it was not a T cell population which was proliferating and that few, if any, T cells were required for the response.

As a further test of this assumption, spleen cells from ATXBM mice were cultured with $F(ab')_2$ and the subsequent proliferation examined. Once again there was a marked proliferation (Table 22) in response to $F(ab')_2$ adding further support to the conclusion that T cells were not the responding cell. It should, however, be noted that there was still some Con A induced proliferation in these cell populations (3X control in ATXBM mice as compared with 150X control in animals of the same age). The evidence suggests minimal T cell involvement in the proliferation.

Table 21 . Effect of Serum Deprivation on the Proliferative response to the $F(ab')_2$ of RMG.

$1X10^6$ Normal spleen cells (Expt. A) or $1X10^6$ spleen cells from ATXBM mice (expt. B) were cultured with either the $F(ab')_2$ of NRG or the $F(ab')_2$ of RMG in the presence or absence of fetal calf serum. ^3H-Thymidine incorporation was determined for the final 18 hours of a 72 hour culture.

		CPM/culture		
		A*		B*
	mcg/ml	+FCS	-FCS	-FCS
NRG $F(ab')_2$	100	1,443	487	125
	50	1,467	321	-
RMG $F(ab')_2$	100	22,614	4,629	4815
	50	14,438	5,412	-

A* cells from ATXBM $B_6D_2F_1$ spleens

B* cells from normal $B_6D_2F_1$ spleens

Table 22. The effect of T Cell Depletion on the Proliferative Response to the $F(ab')_2$ of RMG.

Expt's A and B) Normal BDF, spleen cells were treated with either NGPS or AKR anti-C3H sera + NGPS, washed and cultured in the presence of $F(ab')_2$ of either RMG or NRG. The number of cells per culture was equivalent to $1X10^6$ viable spleen cells prior to treatment.

Expt. C) Normal BDF_1 mice were thymectomized at 6 weeks of age, lethally irradiated two to four weeks later and reconstituted with bone marrow cells. Mice were used as spleen donors 4-6 weeks after reconstitution. $1X10^6$ spleen cells were cultured for 72 hours in the presence of the $F(ab')_2$ of RMG or NRG. ^3H-Thymidine incorporation was determined for the final 18 hour period of culture.

Source of Spleen cells	Treatment anti-Thy-1.2	Complement	CPM/culture $F(ab')_2$ of NRG	$F(ab')_2$ of RMG
A) Normal $B_6D_2F_1$ mice	-	+	118 ± 25	15,762 ± 779
	+	+	82 ± 14	15,650 ± 2,346
B) Normal $B_6D_2F_1$ mice	-	+	225 ± 52	12,102 ± 910
	+	+	79 ± 6	13,248 ± 659
C) ATXBM $B_6D_2F_1$ mice	-	-	1,443 ± 178	22,614 ± 2,539
	-	-	1,467 ± 146	14,348 ± 7,489

Plaque Forming Cells are not Induced by $F(ab')_2$ of RMF or RMG

The levels of thymidine incorporation induced by $F(ab')_2$ of RMG or RMF were often comparable to those observed in LPS stimulated cultures and an obvious question was whether the antibody also would induce the formation of PFC. Cultures which were challenged with a mitogenic concentration of the $F(ab')_2$ were assayed for increased levels of immunoglobulin synthesis by either immunofluorescence or by anti-TNP PFC. As a positive control some cultures containing LPS were examined in parallel.

In five separate experiments where treated cultures were examined for the presence of cytoplasmic immunoglobulin by immunofluorescence, those cultures receiving $F(ab')_2$ had fewer immunoglobulin containing cells ($\bar{x}$ = 0.5%, range 0.5 - 0.7%) than control cultures which received no additions other than FCS in the medium ($\bar{x}$ = 1.5%, range 0.8 - 2.2%). The LPS treated cultures showed variable levels of immunoglobulin synthesis ($\bar{x}$ = 25%, range 15-55%, immunoglobulin containing cells) but always much higher levels of synthesis than the control cultures.

The large blasts which were observed in the $F(ab')_2$ treated cultures had little or no detectable sIg whereas in the LPS treated cultures 70-80% of the blasts were sIg positive. Thus even though the evidence strongly supported the contention that the responding cells were B cells the above data suggests that the responding cells have had their sIg modulated off by the anti-Ig.

As an alternative approach normal spleen cells were stimulated with LPS or $F(ab')_2$ of RMF or RMG and assayed on day 3 for anti-TNP and anti-SRC PFC (Table 23). Once again the observation was that mitogenic concentrations of $F(ab')_2$ did not induce increased PFC but rather inhibited even the background levels of PFC. Previous studies had shown that continuous exposure

Table 23. Lack of PFC Induction by Mitogenic Concentrations of the $F(ab')_2$ of RMG

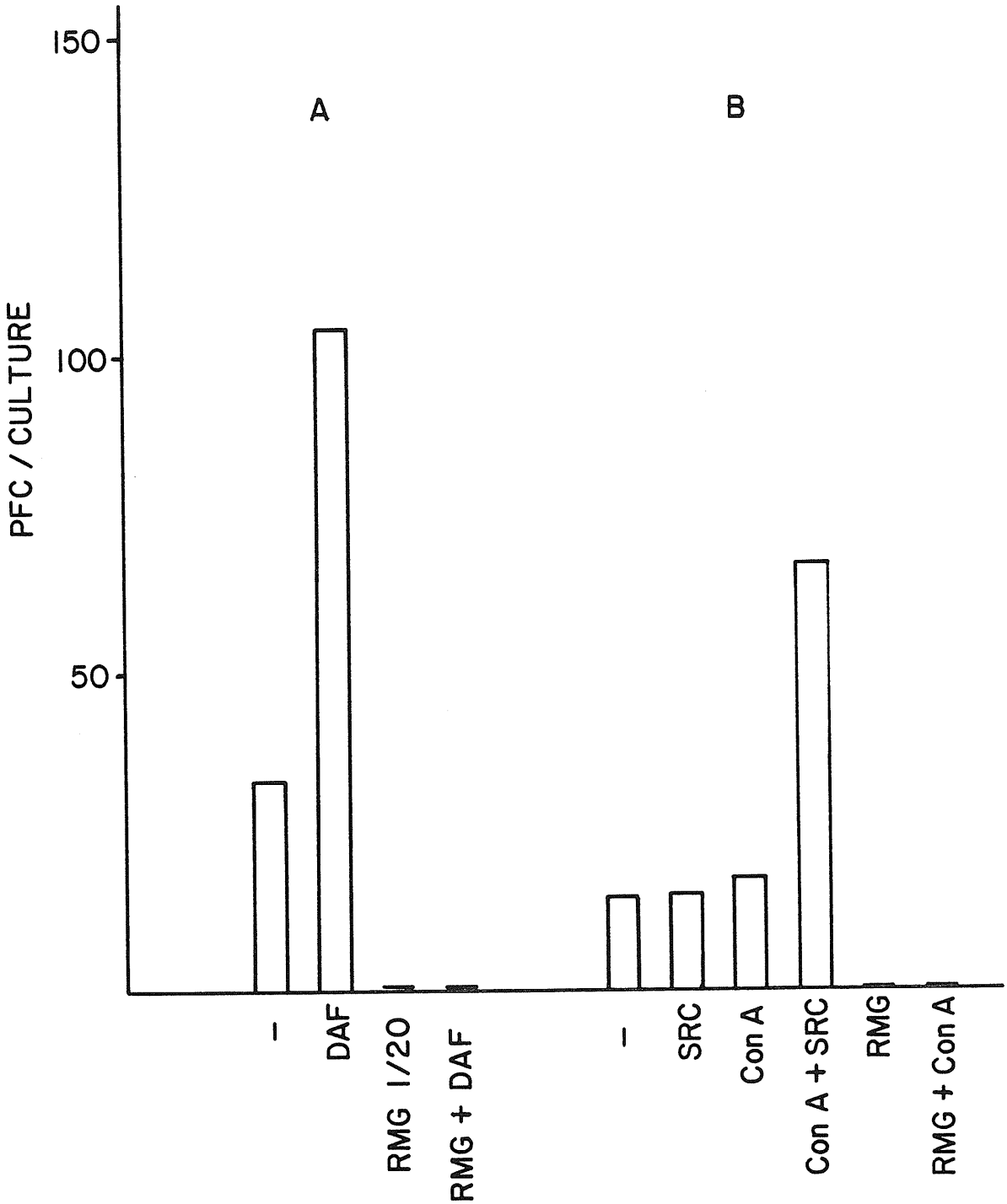
$8X10^5$ normal BDF_1 spleen cells were cultured with $F(ab')_2$ of NRG (50 mcg/culture), $F(ab')_2$ of RMG (50 mcg/culture), or LPS(10 mcg/culture). The number of direct anti-TNP PFC were assayed on day 3 of culture. The mean of sextuplicate cultures $\pm$ S.E.M.

	Anti-TNP PFC/culture	
$F(ab')_2$ of NRG	20 ± 5	6 ± 3
$F(ab')_2$ of RMG	2 ± 1	2 ± 1
LPS	456 ± 40	271 ± 13

Figure 22A. Lack of PFC induction by the $F(ab')_2$ of RMG

A) 8X105 normal spleen cells were cultured in the presence of DNP-AECM-Ficoll (10 ng/ml) $F(ab')_2$ of RMG (250 mcg/ml) or both reagents. After three days the numbers of direct anti-DNP PFC were determined.

B) $1X10^6$ normal spleen cells were challenged with SRC($1X10^6$/culture) $F(ab')_2$ of RMG 250 mcg/ml) after 24 hours 2.5 mcg of Con A was added to the cultures and 72 hours later the number of direct anti-SRC PFC were determined.


150
100
50
PFC / CULTURE
A
B
–
DAF
RMG 1/20
RMG + DAF
–
SRC
Con A
Con A + SRC
RMG
RMG + Con A

to the $F(ab')_2$ was necessary for an optimal mitogenic response, therefore if any antibody synthesis had occurred it was possible that antiI-Ig complexes would form and result in a feedback inhibition of the response. To circumvent this problem cells were cultured first with a mitogenic concentration of $F(ab')_2$ for 24-48 hours, washed and then recultured in the absence of additional $F(ab')_2$. This procedure did not result in the induction of PFC and gave consistantly lower responses than those found in control cultures.

The Effects of TRF and $F(ab')_2$ on the Responses of T Depleted Spleen Cell Cultures

As there had been no antibody response following challenge with $F(ab')_2$ alone it was questioned whether $F(ab')_2$ might require the addition of a TRF to generate a response. To test this possibility spleen cell suspensions from SRC primed animals were depleted of T cells. This resulted in a complete abrogation of the response to SRC. However if a TRF Con A preparation was added to cultures there was a partial recovery of the response. Although the response under these conditions was not always completely antigen dependent the results of treating the cultures with mitogenic concentrations of $F(ab')_2$ were always similar. The antibody did not synergize with TRF but rather it inhibited the background responses observed with TRF alone (Figures 22A,B). In some experiments the $F(ab')_2$ was removed from the cultures after 24 or 48 hours and the cells recultured in the presence of TRF (Figures 22B,23). Once again there was generally no synergism observed under these circumstances even though the SRC gave a good response under these conditions (Figure 23).

As was mentioned earlier the addition of Con A to cultures 24 hours after the initiation of the cultures has been reported as a means of

Figure 22B. The Lack of PFC Induction by Mitogenic Concentrations of the $F(ab')_2$ of RMF.

$8X10^5$ T depleted spleen cells from SRC primed mice were cultured in the presence of SRC ($2X10^5$/culture), $F(ab')_2$ of RMF (50 mcg/culture). After 24 hours of 48 hours cells were washed and recultured in the presence of $TRF_{2.5}$. The numbers of direct anti-SRC PFC were determined and day 3 (the means of sextuplicate cultures $\pm$ SEM).

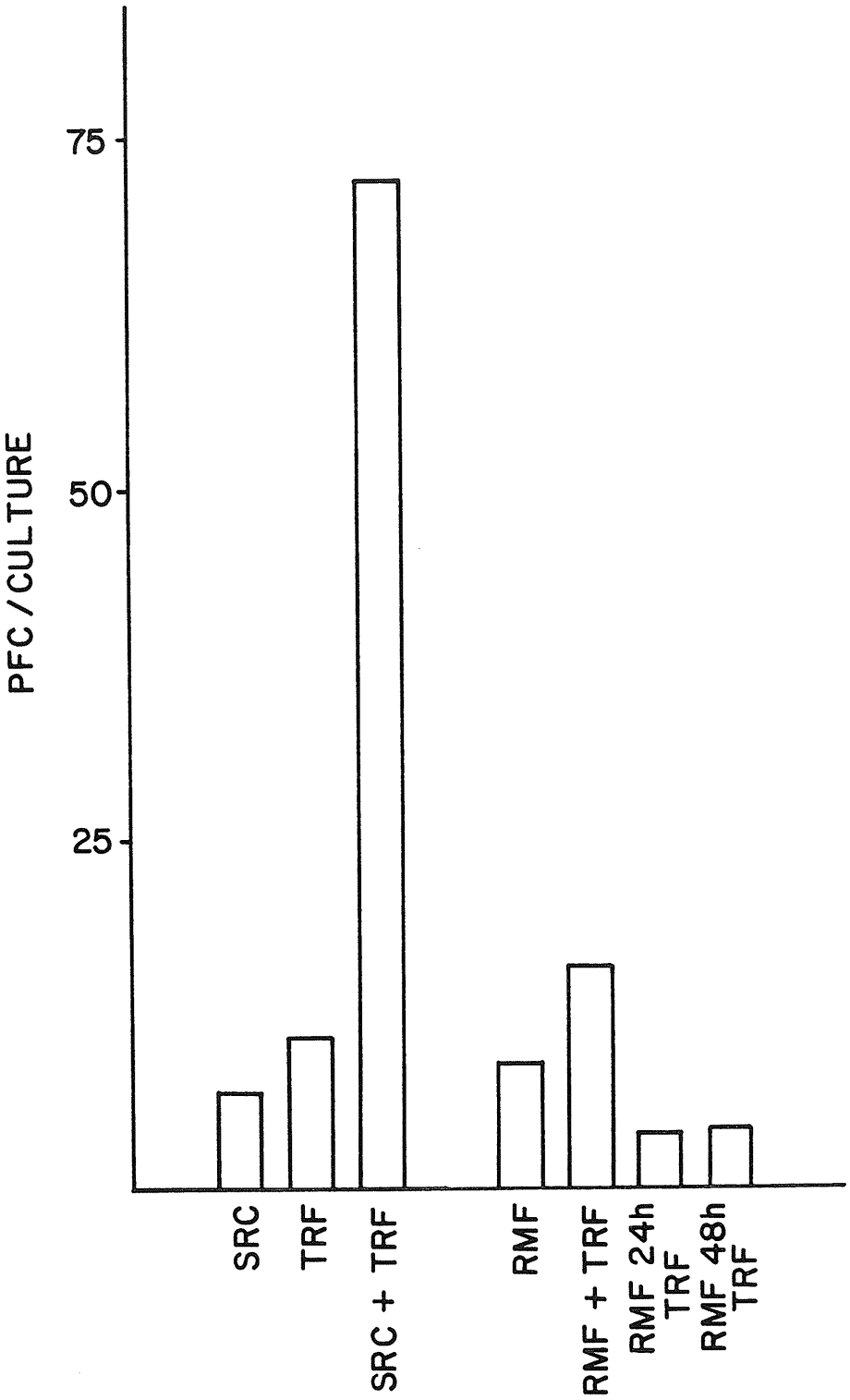
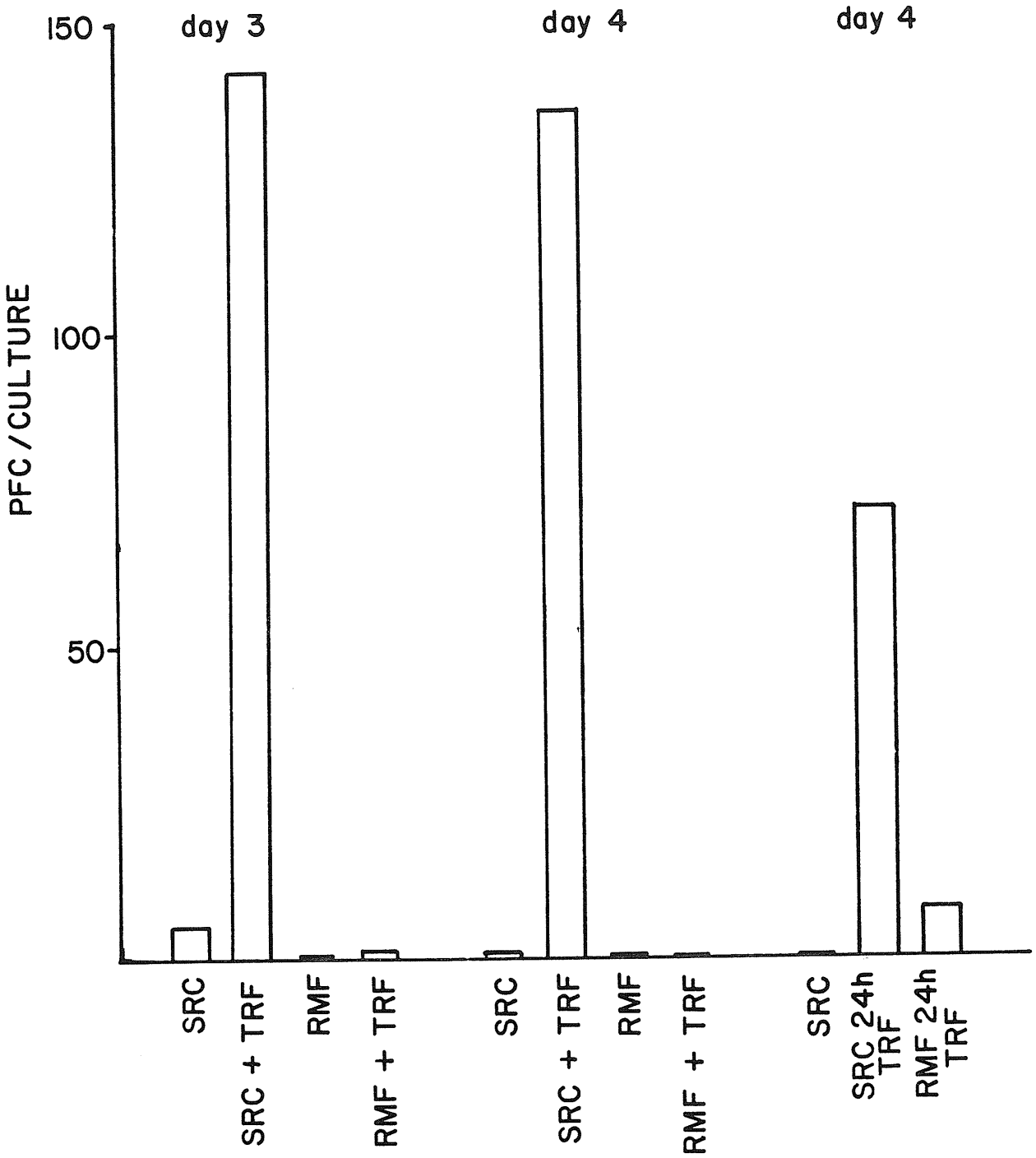

PFC / CULTURE
75
50
25
SRC
TRF
SRC + TRF
RMF
RMF + TRF
RMF 24h TRF
RMF 48h TRF

Figure 23 . Failure of Mitogenic Concentrations of the $F(ab')_2$ of RMF to Generate PFC in the Presence of TRF.

$8X10^5$ T depleted spleen cells from SRC primed mice were cultured in the presence of SRC ($2X10^5$/culture), $F(ab')_2$ of RMF (50 mcg/culture). After 24 hours in culture some cells were washed and recultured in $TRF_{2.5}$, in other cultures $TRF_{2.5}$ was added directly to the cultures without washing the cells. The numbers of direct anti-SRC PFC/culture were determined on the indicated days (the means of sextuplicate cultures $\pm$ S.E.M.).


150
100
50
PFC / CULTURE
day 3
day 4
day 4
SRC
SRC + TRF
RMF
RMF + TRF
SRC
SRC + TRF
RMF
RMF + TRF
SRC
SRC 24h TRF
RMF 24h TRF

generating nonspecific helper cells. In those few experiments where this was found to be the case there was no evidence of synergism between such helper cells and the nonspecific helper cells. The antibody reduced the levels of anti-SRC responses to less than those observed in absence of added antigen.

The Immune Capacities of $F(ab')_2$ Treated Spleen Cells

The previous series of experiments suggested that the $F(ab')_2$ of RMF and RMG were inducing a condition of reduced reactivity in the B cell pools. In order to test this possibility spleen cells were cultured with a mitogenic concentration of $F(ab')_2$ of RMF for 48 hours, washed and recultured in either fresh media alone or media plus LPS or DNP-AECM Ficoll. The ability of these cultures to respond to these antigens was then compared with those of control cultures processed in the same fashion but not having been exposed to the $F(ab')_2$ of RMF during the first culture.

The net effect of pretreating with the $F(ab')_2$ was to reduce the subsequent response to all antigens tested. Those cultures which were challenged with LPS after pretreatment with $F(ab')_2$ showed a decrease in both the levels of ^{3}H-thymidine incorporation (Figure 24) and in the numbers of anti-TNP PFC per culture relative to control cultures (Figure 25). The mitogenic responses of the treated cultures did not show as marked on inhibition as that which was observed in the plaque assays (Figures 23, 24). The anti-TNP response induced by DNP-AECM-Ficoll (Figure 24) was also reduced by the pretreatment. In a single set of experiments it was observed that the low responses of $F(ab')_2$ treated cultures were not a result of a shift in the kinetics of the response as cultures assayed the day before or after the expected peak also indicated a reduced reactivity relative to the

Figure 24 . The Effect of Pretreatment of Spleen Cells with the $F(ab')_2$ of RMF on the Proliferative Response to LPS.

Spleen cells ($5X10^6$/ml) were cultured for 48 hours with the $F(ab')_2$ of RMF (125 mcg/ml), washed and recultured (5X105/culture) with the indicated stimulus for 72 hours. ^3H-Thymidine incorporation was assayed by pulsing with ^3H-Thymidine for the final 18 hours of culture. Mean of quadruplicate cultures.

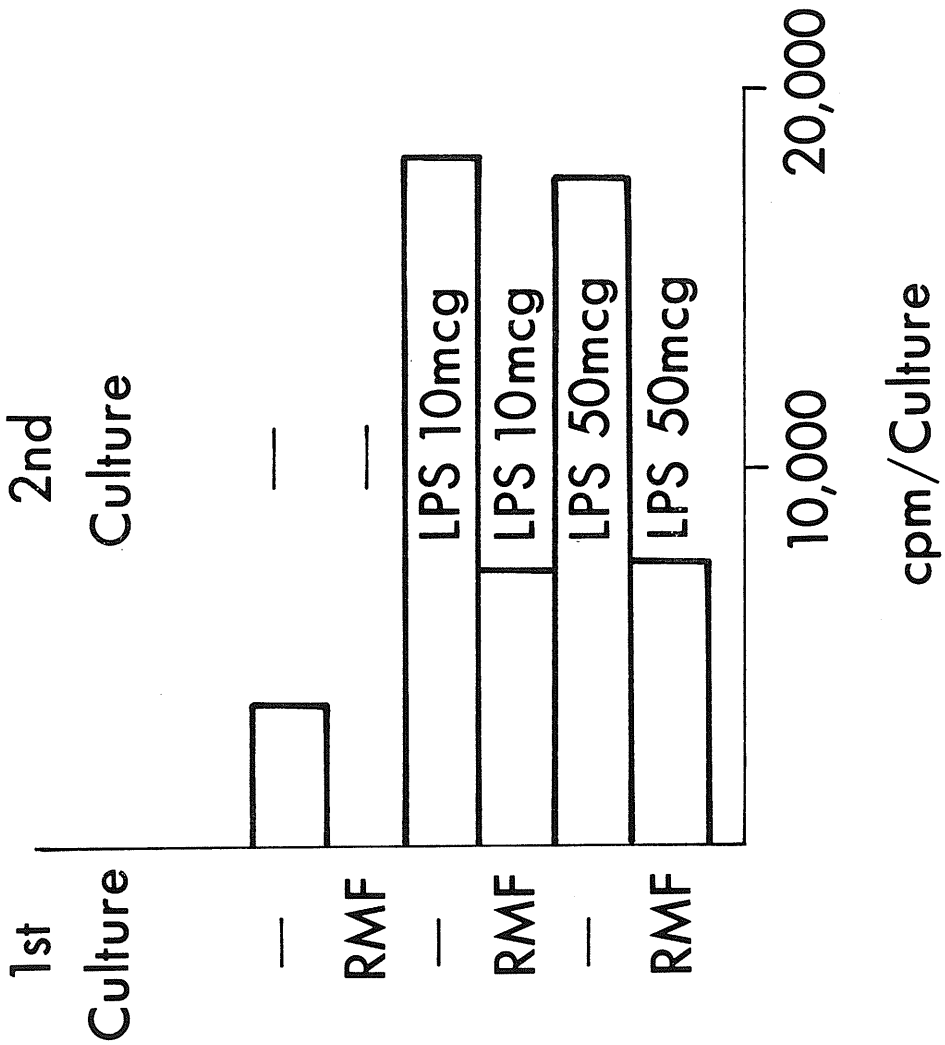
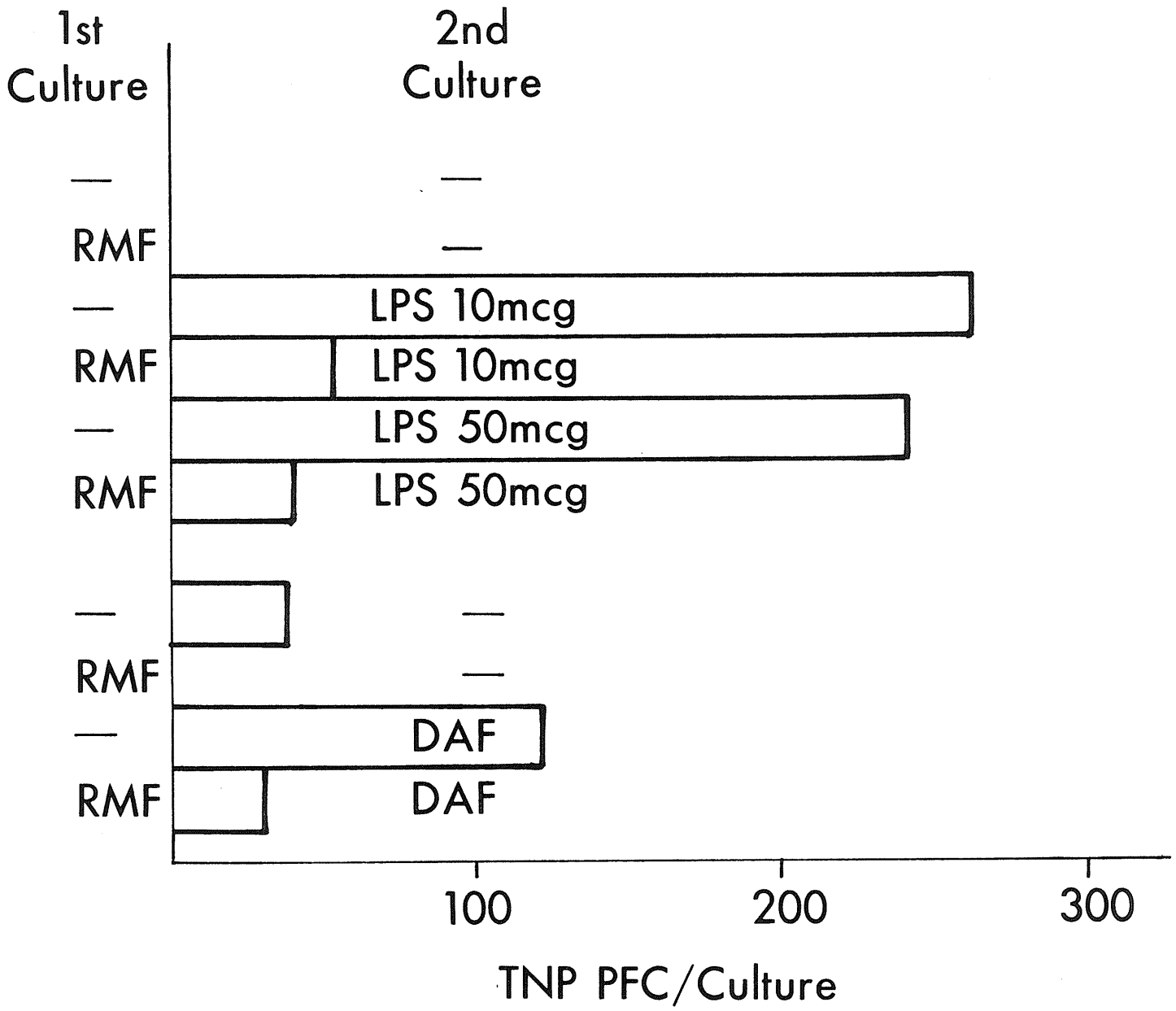

1st Culture
2nd Culture
—
RMF
—
RMF
—
RMF
—
—
LPS 10mcg
LPS 10mcg
LPS 50mcg
LPS 50mcg
10,000
20,000
cpm/Culture

Figure 35. The Effect of Pretreatment of Spleen Cells with the $F(ab')2$ of RMF on Subsequent Anti-TNP Responses Induced by LPS or DNP-AECM-Ficoll.

Normal spleen cells ($5X10^6$/ml) were cultured for 48 hours in the presence or absence of the $F(ab)_2$ of RMF. The cells were subsequently washed and recultured with DNP-AECM-Ficoll ($1X10^6$ cells/culture) of LPS ($5X10^5$ cells/culture) after 3 days the numbers of direct anti-TNP PFC were determined (each value is the mean of sextuplicate cultures).


1st Culture
2nd Culture
—
—
RMF
—
LPS 10mcg
RMF
LPS 10mcg
—
LPS 50mcg
RMF
LPS 50mcg
—
—
RMF
—
DAF
RMF
DAF
100
200
300
TNP PFC/Culture

control cultures. Similar experiments were attempted to assess the ability of cultures to respond to a SRC however it was not possible to obtain an antibody response in control cultures after such extended culture periods. The results for the LPS and DNP-AECM-Ficoll responses did however seem to confirm the previous assumption that the B cells were rendered hyporesponsive after exposure to the $F(ab')_2$ of anti-Ig's.

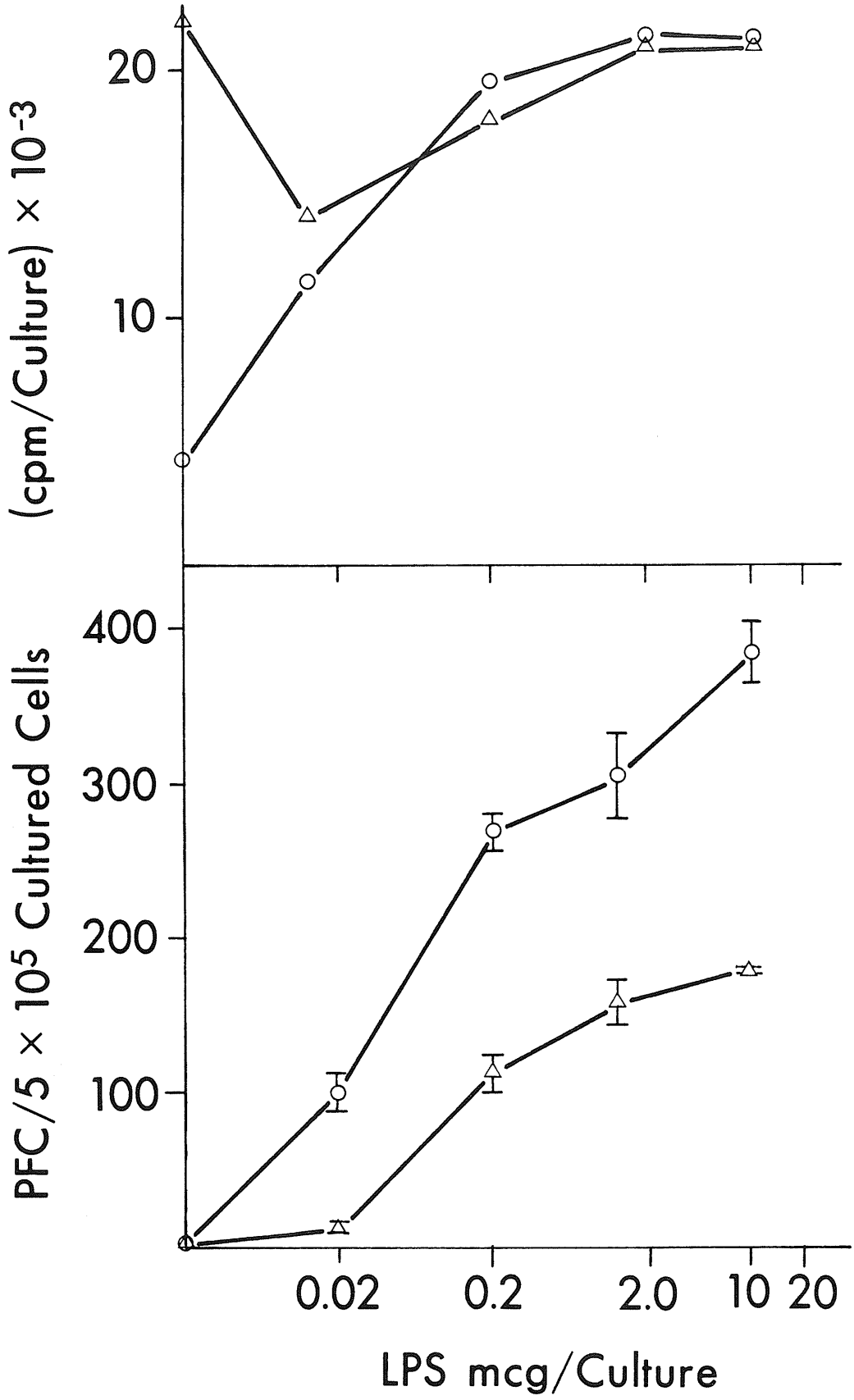
The Lack of Synergism between LPS and $F(ab')_2$ of RMF

The B cell mitogen LPS has been used in some systems (174,221) as a sourve of T cell replacing activity for the induction of PFC. It seemed possible that the previous sources of TRF which I had employed were not the appropriate ones for the induction of PFC. Therefore whole spleen cell populations were cultured with a mitogenic concentration of the $F(ab')_2$ of RMF and various concentrations of LPS and assayed for both a proliferative response and plaque forming cell generation.

The effect of exposing cultures to LPS and $F(ab')_2$ of RMF simultaneously was somewhat different from those observed by pretreating the cells with $F(ab')_2$ of RMF and the challenging with LPS. At no concentration was synergism evident. In fact, at the lowest concentrations of LPS tested (20ng/culture) there was a 30-50% reduction of thymidine incorporation relative to cultures receiving $F(ab')_2$ alone (Figure 26). However at higher concentrations of LPS there was no difference in thymidine incorporation between the cultures receiving either LPS or LPS + RMF. In contrast, the $F(ab')_2$ showed a marked inhibition of the LPS induced anti-TNP PFC at all concentrations of LPS tested (Figure 26). Thus it appears that proliferation and antibody formation had quite different requirements for their induction and maintainence.

Figure 26 . Lack of Synergism Between LPS and the $F(ab')_2$ of RMF.

$5X10^5$ Normal spleen cells were cultured with the indicated amounts of LPS in the presence (▲) or absence (●) of $F(ab')_2$ of RMF (50 mcg/culture). ^3H-Thymidine incorporation (upper figure) was assayed by adding the label for the final 18 hours of a 72 hour culture. The number of direct anti-TNP PFC/culture was determined after 72 hours of culture (the mean of sextuplicate cultures $\pm$ S.E.M.)


20
10
(cpm/Culture) × 10-3
400
300
200
100
PFC/5 × 10⁵ Cultured Cells
0.02
0.2
2.0
10
20
LPS mcg/Culture

An alternative approach to demonstrating synergism was to use a suboptimal concentration of LPS and varying amounts $F(ab')_2$ to reduce the possibility that the cells were already maximally stimulated with RMF and that the added proliferative stimulus of the LPS resulted in a shutdown of immunoglobulin synthesis. Therefore normal spleen cells were cultured with LPS (20 ng/culture) either in the presence or absence of the $F(ab')_2$ of RMF at various concentrations.

The LPS resulted in a slight depression in the $F(ab')_2$ induced mitogenesis however at no concentration of $F(ab')_2$ was there any synergism or inhibition of thymidine incorporation of the cultures relative to those receiving LPS alone (Figure 27). The $F(ab')_2$ treatment resulted in a dose dependent inhibition of the LPS induced anti-TNP PFC response at all concentrations of $F(ab')_2$ tested (Figure 28).

When control cultures receiving $F(ab')_2$ only were examined for the effects of the antibodies on the background responses a small but significant increase ($\sim$ 60 PFC/culture) was evident in the number of anti-TNP PFC measured in these cultures relative to cultures not receiving the $F(ab')_2$ (Figure 28). Clearly the response was not of a comparable magnitude to that observed with LPS (271/culture). Those concentrations of $F(ab')_2$ which caused an increase in the numbers of PFC were not mitogenic and there appears to be a dissociation of the two events i.e. antibody formation and proliferation. It should be emphasized that this was an unprimed population of spleen cells and that no additions were made to the media other than the $F(ab')_2$ and LPS.

Induction of Antibody Forming Cells by Non-Mitogenic Concentrations of RMF $F(ab)_2$

The induction of PFC were rather unexpected so the experiments were repeated using SRC as the target antigen for the assay of polyclonal antibody formation. Cultures of spleen cells from unprimed mice were set up in groups of eight with the various additions shown (Table2 24A,B) and the cultures assayed on day 4 for PFC. The variability of the primary response to SRC is again evident and as might be expected the response to SRC was better than that induced by $F(ab')_2$. The $F(ab')_2$ usually does not cause as high a level of AFC formation as the antigen SRC does but in experiment the response to $F(ab')_2$ is still 11X control responses (Table 24B).

It was quite possible that in those cultures containing $F(ab')_2$ there was insufficient help to induce full maturation to AFC therefore cultures of normal spleen cells were supplemented with $TRF_{con\ A}$ after 48 hours of culture (Table 24). The net effect was to slightly enhance the response at the highest dilutions of antibody employed but not markedly. All cultures contained $5\text{-}8x10^5$ cells which was far below the optimal cell numbers for a primary anti-SRC response. In fact, primary anti-SRC responses in microcultures of normal spleen cells were not a consistent observation. Thus even though the numbers of PFC generated/culture are very low it was not unexpected as the cultures were performed under extremely limiting conditions. As had been mentioned previously the addition of Con A to cultures of normal cells after 24 hours in the presence of antigen has been used as a means of generating non-specific helper activity. Although previous experiments had given rather variable results as to whether or not help was being generated it was decided to re-examine this approach as a possible source of non-specific T_H cells. Once again the helper activity

Figure 27 . The Effect of $F(ab')_2$ of RMF on the Mitogenic Response to a Suboptimal Concentration of LPS.

$5X10^5$ normal spleen cells were cultured in the presence of the indicated dilution of the $F(ab')_2$ of RMF in the absence (O) or presence (Δ) of LPS (200 ng/culture). ^3H-Thymidine incorporation was assayed by pulsing with the label for the final 18 hours of a 72 hour culture. Each point is the mean of quadruplicate cultures.

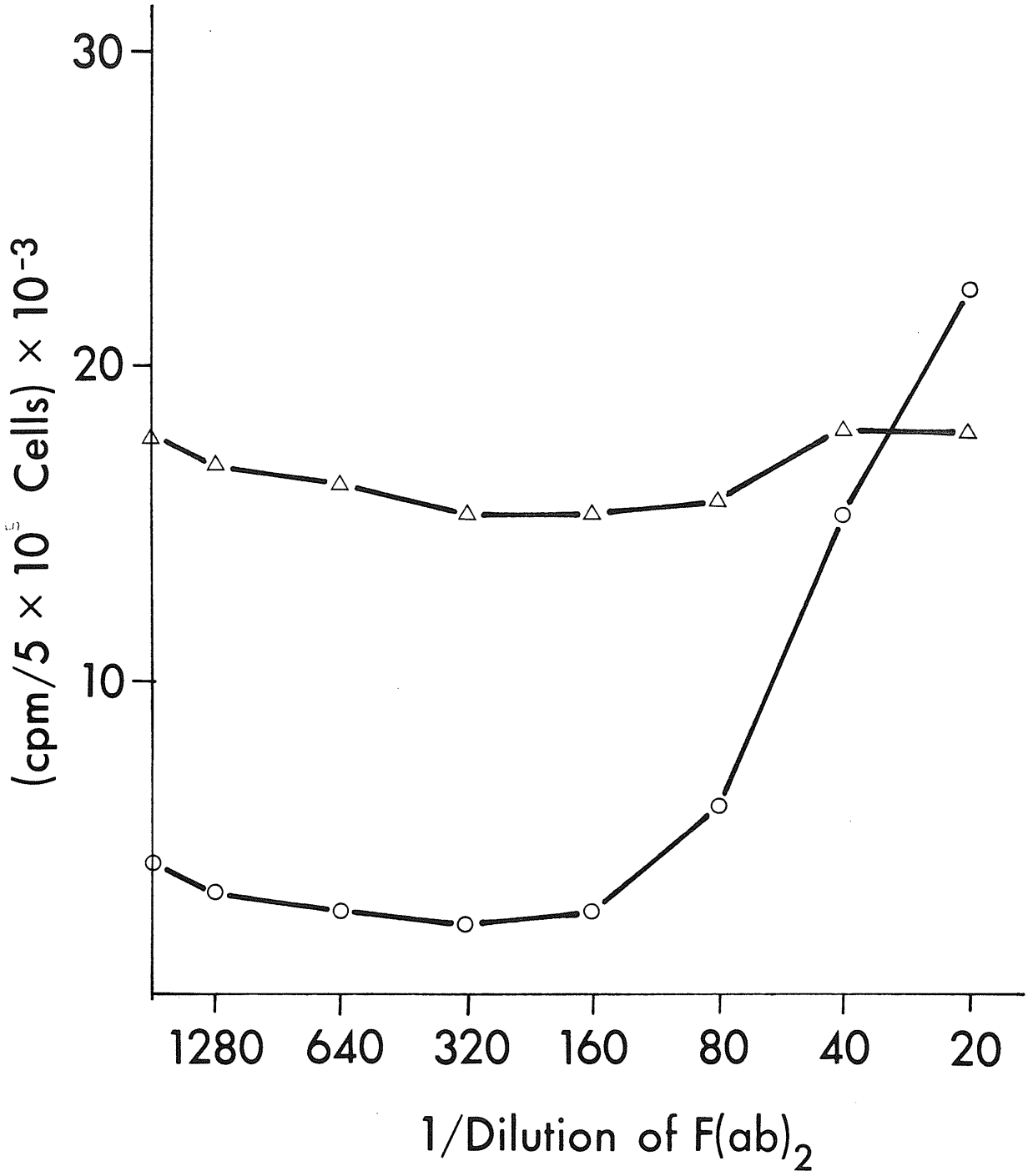

30
20
10
(cpm/5 × 10⁵ Cells) × 10⁻³
1280
640
320
160
80
40
20
1/Dilution of F(ab)2

Figure 28 . The Effect of the $F(ab')_2$ of RMF on the Anti-TNP Response to a Suboptimal Concentration of LPS.

$5X10^5$ Normal spleen cells were cultured in the presence of the indicated dilution of $F(ab')_2$ of RMF in the absence (●) or presence (▲) of LPS (200 ng/culture). Direct anti-TNP PFC were assayed after 72 hours in culture. Each point is the mean of sextuplicate cultures ± S.E.M.

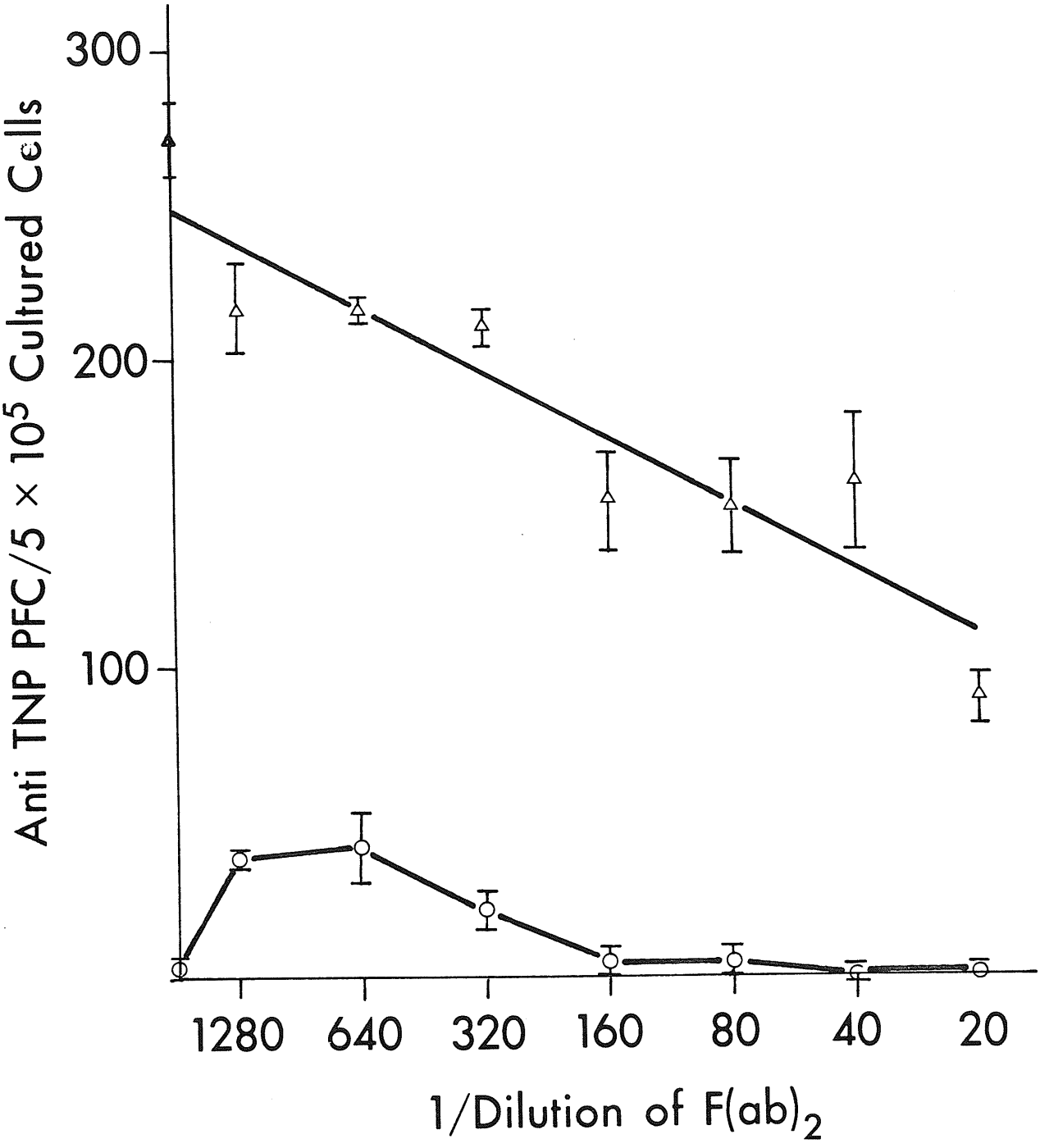

300
200
100
Anti TNP PFC/5 × 10⁵ Cultured Cells
1280
640
320
160
80
40
20
1/Dilution of F(ab)2

Figure 29 . Induction of PFC Formation in Cultures of Primed Spleen Cells.

$5X10^5$ T depleted spleen cells from SRC primed mice were cultured in the presence of $1X10^6$ SRC or the indicated concentration of the $F(ab')_2$ of RMF. $TRF_{2.5}$ was added to the cultures after 24 hours and the number of direct anti-SRC PFC/culture was determined on day 4 of culture. Each point is the mean of 8 cultures $\pm$ SEM.

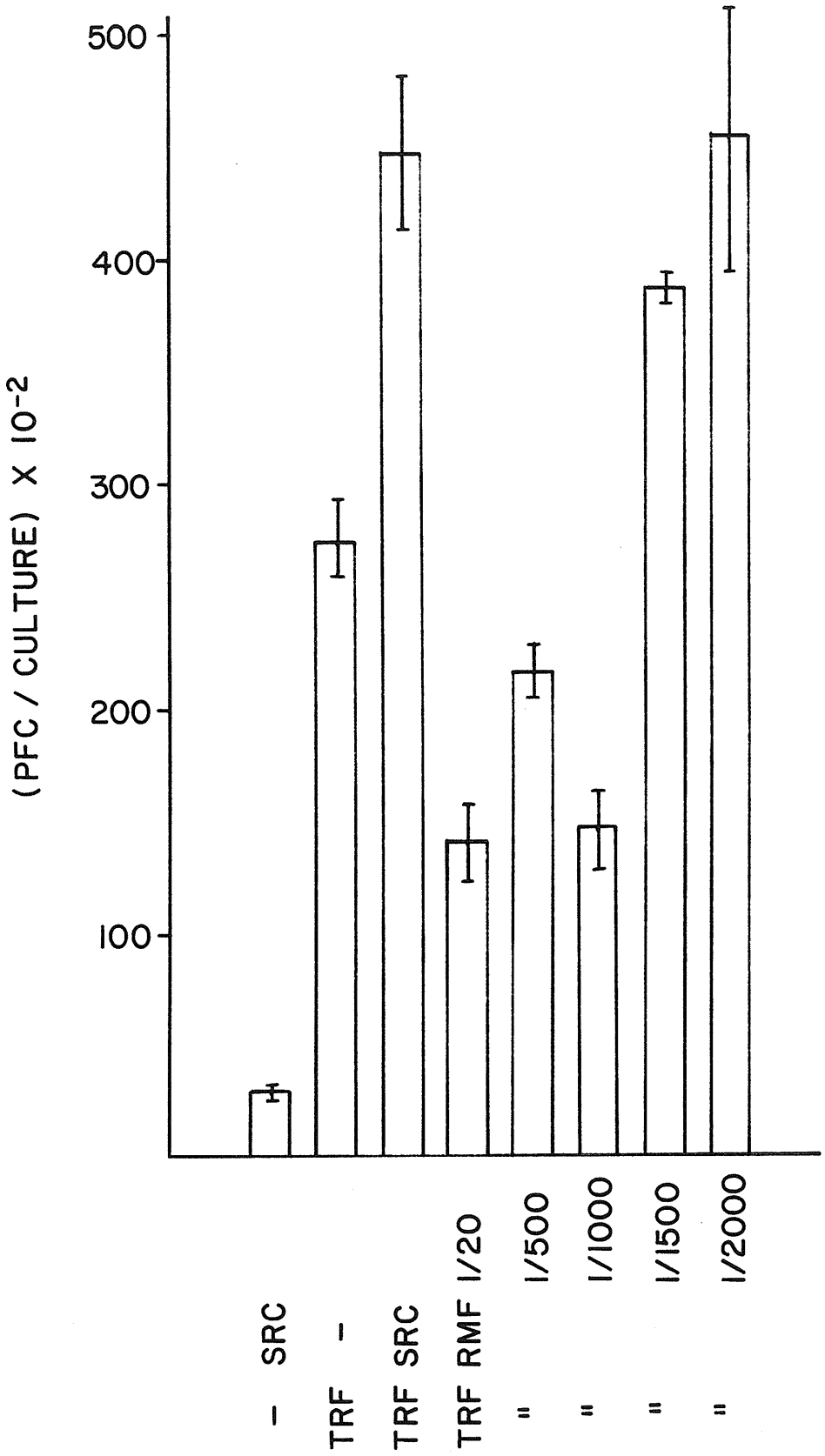

500
400
300
200
100
(PFC / CULTURE) X 10-2
- SRC
TRF -
TRF SRC
TRF RMF 1/20
" 1/500
" 1/1000
" 1/1500
" 1/2000

Figure 30 . Induction of PFC Formation in Cultures of Primed Spleen Cells.

$5X10^5$ T depleted spleen cells from SRC primed mice were cultured in the presence of $1X10^6$ SRC or the indicated concentration of the $F(ab')_2$ of RMF. $TRF_{2.5}$ was added to the cultures after 24 hours and the number of direct anti-SRC PFC/culture was determined on day 4 of culture. Each point is the mean of 8 cultures $\pm$ SEM.

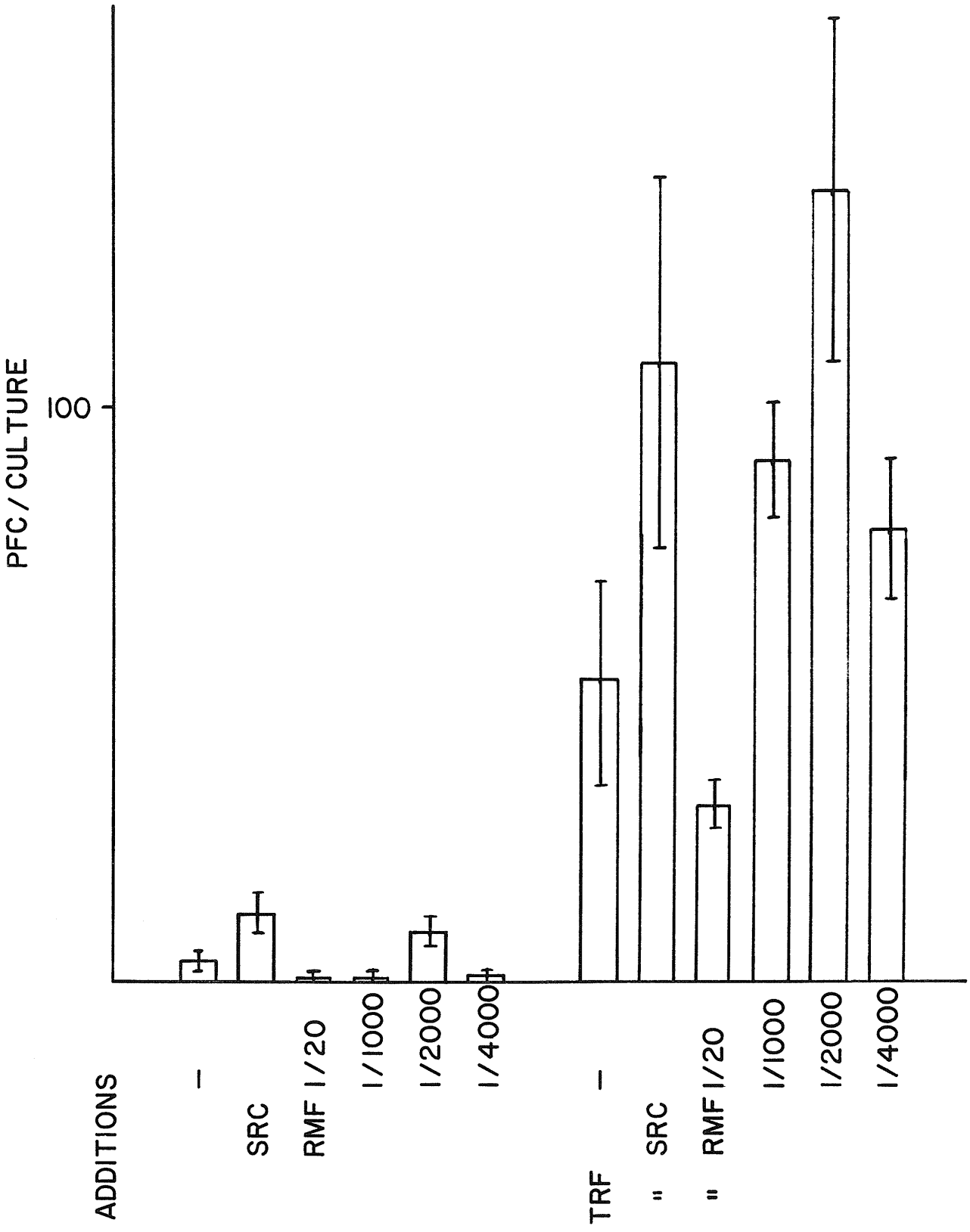

PFC / CULTURE
100
ADDITIONS
—
SRC
RMF 1/20
1/1000
1/2000
1/4000
TRF
" SRC
" RMF 1/20
1/1000
1/2000
1/4000

Table 24A, B. Induction of Anti-SRC Response by the $F(ab)_2$ of RMF.

$8X10^5$ normal spleen cells were cultured with $1X10^6$ SRC or the indicated dilution of the $F(ab)_2$ of RMF. $TRF_{2.5}$ or 1 mcg/ml of Con A was added to the cultures at 48 or 24 hours respectively. The numbers of direct anti-SRC PFC were assayed on day 4. Each value is the number of PFC for a single culture.

A

	Media	$TRF_{2.5}$	Con A mcg/ml
--	3,3,3,0 0,0,0,0	81,51,15,3 3,0,0,0	6,6,0,0 0,0,0,0
SRC	21,21,18,18 15,12,9,9	612,417,306,291 252,177,102,69	15,9,9,3 3,0,0,0
RMF $F(ab)_2$ 1/20	9,3,3,0 0,0,0,0	3,3,0,0 0,0,0,0	0,0,0,0 0,0,0,0
1/60	6,3,0,0 0,0,0,0	21,12,12,12 9,9,6,3	3,3,3,0 0,0,0,0
1/540	42,36,33,15 12,9,9,6	48,36,27,27 24,21,15,6	39,36,12,12 6,6,0,0
1/1620	51,51,45,45 27,24,6,0	78,75,75,72 60,33,30,27	84,66,24,15 15,6,3,0

B

	Media	$TRF_{2.5}$	Con A mcg/ml
--	27,27,24,12 12,12,9,6	12,6,6,3 3,3,3,0	3,3,3,3 0,0,0,0
SRC	66,54,48,24 21,21,21,12	165,150,144,111 66,45,36,30	18,9,3,3 3,0,0,-
RMF $F(ab)_2$ 1/20	0,0,0,0 0,0,0,0	3,3,3,0 0,0,0,0	6,3,0,0 0,0,0,0
1/60	6,3,3,3 0,0,0,0	6,3,0,0 0,0,0,0	6,0,0,0 0,0,0,0
1/540	108,72,6,3 0,0,0,0	51,48,18,15 12,9,6,3	9,3,0,0 0,0,0,0
1/1620	18,9,3,3 0,0,0,0	24,9,9,9 6,3,0,0	9,9,6,3 3,3,0,0

generated by this method proved to be quite variable, however, it is interesting to note that where responses were observed, the $F(ab')_2$ preparations were as effective as SRC (Table 24A).

The responses to date had been rather disappointing in the unprimed cultures and in order to increase the frequency of potential responder cells against a specific antigen mice were immunized with SRC, the spleens removed after four days, depleted of T cells and reconstituted with $TRF_{con\ A}$ in the presence of antigen or RMF. The RMF in the absence of TRF failed to induce an AFC response as did the SRC. However in the presence of TRF both SRC and RMF induced a significant response to SRC (Figures 29, 30). The effects of RMF were dose dependent with the mitogenic concentrations of $F(ab')_2$ showing an inhibitory effect on background responses (Figures 29, 30). As had previously been noted the $TRF_{con\ A}$ induced a marked increase in PFC formation in the absence of added antigen, however the response was increased significantly in the presence of both SRC and low concentrations of TRF.

Attempts to induce a response in cultures which contained low concentrations of $F(ab')_2$ for 24-48 hours, then washed and recultured in the presence of TRF consistantly failed. It is not clear as to the reason for this but it is possible that the AFC precursors were sensitive to damage at this stage or possibly that RMF must be continuously present under these conditions to supply an extended stimulus for AFC induction.

Discussion

Discussion

The aim of this project was to develop an *in vitro* model system of antigen dependent B lymphocyte activation. As was discussed in the results it was necessary to spend considerable time and effort on developing conditions which would give a reproducible antibody response *in vitro*. During the initial stages of this research it was decided that the lowest possible levels of background stimulation was a desirable property of the system, therefore fetal calf serum batches were selected which failed to support a primary anti-SRC. This was thought to be an important criterion for screening as those batches of FCS which support the above response represent the minority of FCS batches produced, and there was evidence that the supportive batches might contain bacterial products with endotoxin like activity (195, 196).

Since a means for assessing B cell function independently of T cells was required the use of a T independent antigen was essential. This class of antigens seems in general to require little if any T cell or macrophage involvement in the induction of an antibody response thus it should be possible to examine B cell function directly and to eliminate many of the collaborative events involved in T dependent responses. This is an important property as it allows for the examination of B cell responses in the absence of other cell types and also it narrows the potential sites of action of an experimental procedure to those involving B cell function; interference with T cell or macrophage function by the procedure should not effect the capacity of the antigen reactive B cells.

DNP-lysyl-Ficoll (192) was originally decided upon as the antigen of choice because of the relative ease with which it could be synthesized,

its rather simple structure and its capacity to elicit both IgM and IgG anti-DNP responses. However it became apparent that there were difficulties in controlling the degree of substitution and the amounts of crosslinking of the Ficoll molecules. This constituted a considerable problem as it would eventually be necessary to synthesize a Ficoll hapten conjugate using a hapten other than DNP and it had been expected that the Ficoll backbone would represent an invariant structure in all of these compounds. These considerations led to the decision to synthesize AECM Ficoll (90) as the matrix for future T independent antigens since the molecular composition of the AECM Ficoll could be more readily controlled.

The AECM Ficoll has indeed proven to be an excellent antigen. Since the numbers and proportions of amino and carboxyl groups on Ficoll can be controlled it is feasible to couple haptens which react with either of these functional groups. Furthermore, it has been possible to obtain highly reproducible coupling conditions such that predictable levels of haptenation are possible. It is also theoretically possible to put two different haptens on the same molecule in a controlled fashion. These last points became very important considerations in the designing of antigens to be used as specificity controls.

Once a suitable carrier had been synthesized, two noncrossreacting haptens were chosen for the production of the corresponding AECM Ficoll antigens. The dinitrophenyl group (DNP) was an obvious choice because of the ease with which it is coupled and the well established assay conditions for an anti-DNP response. The nitroiodophenylacetyl group (NIP) had been used by only a few groups and the selection of this hapten posed a somewhat difficult problem in that it was not commercially available, consequently I had to embark on the rather lengthy synthesis of the hapten.

Several factors did however favour the use of NIP; 1) several groups (84, 149) had shown that DNP and NIP were noncrossreacting, 2) the B cell precursor frequency of NIP reactive cells is relatively high and in this sense comparable to that of DNP, 3) there are several intermediates produced in the synthesis of NIP and it was therefore feasible to produce numerous variations on the hapten structure some of which were noncrossreacting, 4) the iodine in the NIP determinant allowed for the synthesis of a radioactive hapten which might prove useful for autoradiographic or antigen binding studies.

The original experimental scheme had been to simultaneously expose spleen cells to antigen and one of series of compounds which selectively modified biochemical processes in the cell (e.g. cytochalasins, colcemid, cAMP, cGMP, Ca^{++}, serine esterase inhibitors). Each of these agents affects specific areas of cell function and it was hoped that by examining the effects of these compounds on an immune response that it would be possible to identify those cellular systems(e.g.microtubules, microfilaments' cation transport) which were involved in the early events of B cell induction. This approach had been highly successful in determining the sequence of events leading to antigen mediated release of histamine from mast cells (95). Many of the activation events in mast cell degranulation had been demonstrated to be short term transient processes and it was felt that this was likely to be a property of all triggering events. If this were to be the case then B cell activation would be expected to involve similar triggering mechanisms. Most of the agents which were being planned to be used had cytotoxic effects if the cells were exposed to them for extended periods of time therefore the approach was to expose a B cell population to one of these modifying agents and antigen for a relatively short period

of time (1-5 hours), wash away the inhibitor and antigen and reculture the cells with the same antigen or a second noncrossreacting antigen. The latter antigen served as a control for the nonspecific inhibitory effects of the treatment. If inhibition of PFC formation was observed in cultures initially treated with antigen and inhibitor but not in the presence of inhibitor alone and the response to subsequent challenge with a second antigen was unaffected then one might conclude that inhibitor was acting on an antigen activated process. It was fully realized that such an approach does not prove a role for a process in the activation sequence but it does provide a means of screening for those processes which might be involved.

A very strong limitation of the above approach is the absolute requirement for a phenomenon involving a single cell type. Obviously if several cell types are required then the site of action of any of these agents might be the cell type being assayed, the B cell in this case, or any one or combination of cells involved in the collaborative events leading to the final response. This was the reason for choosing T independent antigens for the culture system. However, during the initial phases of this project it became apparent that although thymus independent antigens may not require the participation of other cell types in the generation of antibody forming cells, the B cells responding to the antigens are nevertheless sensitive to the regulatory effects of other cell types. The results of several studies (211,212) suggested that it was essential to employ purified B cell populations. Discussions with Dr. V. Paetkau who had been examining the effects of cAMP on several immune responses indicated that this indirect approach of biochemical modulation might not prove to be as useful as it had for other systems due to the cellular inter-

actions involved, the asynchrony of the responding cell population and the time scales required to generate AFC.

It had been realized from the outset of this project that direct biochemical measurements would be much more meaningful in assigning roles to biochemical processes in B cell activation; however, as was discussed in the introduction, the feasability of such measurements on whole spleen cell populations is nonexistant as the numbers of antigen reactive cells for anyone given antigen are far too low. At about the time when the difficulties in the indirect biochemical approach were becoming apparent a method appeared in the literature (82) for the purification of hapten specific B lymphocytes. The procedure consisted of allowing spleen cells to adhere to a DNP-gelatin coated culture dish, washing off the non-adherent cells at $4^{o}C$, heating the dish to $37^{o}C$ to melt the gelatin and liberating the bound cells. Using such a protocol it had been reported that a population of $>$ 85% DNP binding cells was obtained in the adherent cell population and approximately one out of every three cells could be stimulated to produce antibody by the T independent antigen DNP-POL. An examination of this method as a means of obtaining sufficient cells to permit indirect biochemical analysis or even possibly some direct analysis was undertaken. After several attempts of the procedure it was clear that unless a means of increasing the numbers of DNP reactive cells in the starting population was found the technique was proving to be too expensive and time consuming for the yields obtained. An approach which was tried was to preimmunize the donor mice with dextran sulfate a mitogen which was supposed to increase the numbers of antigen reactive cells without causing full differentiation to AFC. It became apparent that although the scheme was theoretically and operationally feasible in one group's hands

it was not practical to pursue this procedure given the resources and manpower available to me at that time. The remaining option was to isolate whole B cell populations and to continue with the indirect approach of analysis.

Currently it is only possible to obtain highly purified populations of B cells through the use of positive selection methods. Therefore, schemes for B cell isolation were examined. Parish et al (142) had reported a procedure for the isolation of large numbers of B cells on the basis of rosetting and separation of lymphocytes bearing surface immunoglobulin, and this seemed to be a reasonable approach for the isolation of B cells. However, if one considers that sIg-ligand interactions are involved in B cell activation it then becomes highly doubtful as to the merits of such an isolation scheme. Furthermore it was found that the conditions required to recover B cells from the rosettes were quite vigorous and it was questionable as to whether the basal metabolic levels of these cells were comparable to those of unrosetted B cells.

Since the utilization of an indirect approach for the examination of lymphocyte activation was only really suited for studies of homogeneous cell populations and it was not feasible to obtain such populations without some sort of positive selection method the alternate approach was to use polyclonal activating agents and perform direct biochemical measurements on the large responding populations. The effects of mitogens on B cells were being studied extensively in other laboratories and it was felt that these reagents did not offer a particularly good model for antigen dependent B cell activation due to the complexity of their interactions with numerous receptor species on the B cell surface. As was discussed earlier a polyclonal model for B cell activation involving sIg

interactions was thought to be highly desirable as it would more closely mimic the events which were thought to occur in antigen dependent B cell activation. The obvious approach was to use anti-immunoglobulin antibodies to interact polyclonally with B lymphocytes via their surface immunoglobulin and then to supply a source of T_H cells or T cell replacing factors. This was not an entirely novel scheme as it had been previously applied to rabbit lymphocytes with a limited degree of success (106).

In the Kishimoto-Ishizaka experiments lymphocytes were obtained from lymph nodes as a source of DNP primed B cells and cultured for 24 hours with a mitogenic concentration of anti rabbit immunoglobulin (105). The cells were then washed and recultured in the presence of a non-specific factor (presumably of T cell origin) for 5 days, at which time the culture supernatants were assayed for the presence of specific anti DNP antibody and total immunoglobulin. Those cultures receiving both anti Ig and NSF showed a marked increase in specific and non-specific Ig synthesis relative to cultures receiving either one of these factors. Similar results have been recently obtained using purified human lymphocytes (67). In this case human B cells isolated on anti Ig-Sepharose columns were found to be activated in the presence of supernatants derived from PWM treated cultures of human T cells. These reports were quite encouraging as they indicated that such polyclonal models of B cell activation were valid and their advantages for biochemical analysis have already been discussed.

At the time there were however only two reports of anti immunoglobulin induced lymphocyte proliferation in the murine system and both of these were dependent upon rather specialized conditions (143,225). As it has been previously shown in several different systems that proliferation

precedes the generation of AFC, it was felt necessary to establish whether antibody could induce proliferation in murine lymphocytes under more general conditions as a first step in developing a model of B cell activation.

The first six antisera used in the current study were raised against a gammaglobulin enriched fraction of normal BDF, serum (RMG) and the last two against the $F(ab')_2$ of highly purified normal mouse IgG (RMF). As an initial test of the specificities of these antisera the properties of splenic and thymic lymphocytes reactive with the antisera, as determined by immunofluorescence, were examined. All of the antisera gave comparable numbers of antibody binding cells: 50-55% of nucleated spleen cells and less than 1% of thymocytes. Similar numbers of reactive cells were obtained when these antisera were used in a rosetting assay (142) with the same cell suspensions. In an effort to establish what the cell surface target antigens of these antisera were, SDS-PAGE analysis of the immune precipitates from surface iodinated cells was performed. Three major peaks corresponding to μ, δ and light chains were observed suggesting that the binding of the antisera was a consequence of anti κ chain and/or anti Fab activity. There are however some reservations with regards to interpreting this type of data. The lactoperoxidase method selectively iodinates the tyrosine residues of proteins and the specific activity to which a membrane component is labelled will depend upon the number of accessible tyrosines present in the molecule. It is therefore possible that a critical cell surface component was not labelled very efficiently and was therefore overlooked using this technique. The patterns observed on the gels are representative of a mixture of all surface labelled components in proportions very crudely similar to those in which the various molecular

species are present on spleen cell populations. A minor component which was restricted in its cellular distribution or present in a low frequency on all of the cells would not likely be demonstrated by this technique. It is felt that these possibilities do not represent the current situation for several reasons which will be discussed below.

Initial attempts to induce mitogenesis with RMG sera or the purified IgG fractions of these sera failed even though the antibody was tested over a 10,000 fold concentration range. However it was observed that in some cultures where 2-Me was present in the media the RMG caused a slight increase in thymidine incorporation relative to control cultures receiving normal rabbit IgG. Part of the observed stimulation by the antibody was a result of a depression of background incorporation by the normal rabbit IgG. It seemed that the anti Ig molecules might be having two effects on lymphocyte proliferation, one which was possibly Fc mediated and the other through the combining site of the anti Ig antibody.

Moller (120) had reported that AgAb complexes caused increased thymidine incorporation in human lymphocytes, however, several reports have failed to confirm these results (167). It has been most frequently reported that IgG FcR interactions have an inhibitory effect (166,202). Sinclair et al (111) demonstrated that there were two types of inhibition caused by AgAb Cpx. In the case of $F(ab)_2$ antibody the effect was thought to be largely a result of increased antigen aggregation and clearance. However when antigen and intact IgG antibody was present either in vivo or in vitro there was an antigen specific inhibition of the immune response. This inhibition was mediated via the Fc of the antibody, and at least part of the inhibition was proposed as being a consequence of the binding of AgAb Cpx simultaneously to both the receptor Ig and the FcR of antigen specific B cells. Anti

immunoglobulins provide a means of mimicking such a situation on a polyclonal scale and in several cases it has been reported that anti Ig's inhibit the responses to mitogens (175, 199). However there is considerable controversy over this point as numerous other groups here obtained conflicting results (9 ,99). The recent observations of Ryan & Henkart (166) that immobilized antigen antibody complexes cause Fc dependent inhibition of mitogen induced proliferation may supply some clues as to why these discrepancies occur.

These authors employed a culture medium containing 2-Me and normal mouse serum to demonstrate the Fc mediated inhibition as cultures containing FCS rather than NMS did not show such an inhibition. A review of the literature shows that in those cases where inhibition of mitogen induced proliferation by anti Igs was observed the culture media contained FCS but notably lacked 2-Me. In contrast when inhibition by antibody was not observed 2-Me was present in the media. It would appear that there is a correlation between lack of inhibition of mitogenesis by anti Ig and the presence of 2-Me in the medium.

Recently it has been reported that 2-Me in the presence of fetal calf serum or a serum component derived from 2-Me treated serum supports the induction of anti Ig induced proliferation (74). This observation lends support to the suggestion that 2-Me alters a serum component which in some way inhibits or modifies the binding of the Fc region of the antibody to FcR. Schimpl & Wecker (171) have reported that the plasmin generated Fc of human IgG is capable of inhibiting B cell activation in the presence of specific antigen and a non-specific T cell replacing factor. These authors suggested that the TRF and the Fc were competing for a receptor on the responding B cell which is required for B cell activation. This

inhibition by the Fc was eliminated if there was 2-Me in the culture medium. The authors suggested that the Fc was modified by exposure to 2-Me, however, it is equally possible that serum components may have been altered by the 2-Me such that they would alter the binding capacity of the Fc for the FcR. Thereare, however, some recent data which would argue that the lack of inhibition by the 2-Me treated Fc fragments is not a consequence of an inability to bind to cells. Berman & Weigle (17) demonstrated that isolated, papain generated Fc fragments in the presence of 2-Me were mitogenic in both serum free cultures and those containing 0.5% NMS. The Fc of IgG from several species (mouse, human, goat) were mitogenic under these conditions whereas Fc of rabbit IgG were not, possibly due to their reduced solubility relative to the Fc fragments of other species.

The results of the studies of Fc effects are all compatible with attributing a signal transmission role for Fc receptors in B cell functioning. However it is not clear as to what role(s) should be assigned to Fc/FcR interactions. The contributions of serum components and species specific Fc variations further complicate the assessment of FcR/Fc binding and it is not presently feasible to assign the relative contributions of Fc conformational changes and Fc serum component interactions on the determination of the physiological and biological activities of the Fc. It is, however, clear that if an examination of sIg/antibody interactions is to be undertaken the Fc of the antibody molecule cannot be assigned a passive role and in light of the apparent variations in its reactivities it is best removed from the antibody to be used in such studies.

The exposure of spleen cells to $F(ab')_2$ of RMG (6 sera) or RMF (2 sera) resulted in a high level of thymidine incorporation relative to control

cultures receiving the $F(ab')_2$ of normal rabbit IgG (NRG). The normal rabbit $F(ab')_2$ were non-mitogenic and non inhibitory to basal levels of thymidine incorporation so that the two sets of controls i.e. those not receiving $F(ab')_2$ and those receiving $F(ab')_2$ of NRG showed comparable levels of thymidine incorporation. The concentration range over which the stimulation was observed was rather narrow (up to 1/80 dilution of the $F(ab')_2$), however there was no indication that a plateau for incorporation had been reached at the highest concentrations used in culture. Since the antibody had not been immunosorbent purified the specific activity of the immunoglobulin was likely quite low, and if one estimated that at most 10% of the total immunoglobulin had anti-immunoglobulin activity, the mitogenesis was observed with $\geq$ 10 mcg of $F(ab')_2$ per culture. Even without employing these calculations the concentrations of total $F(ab')_2$ employed in these studies were comparable to the quantities of immunosorbent purified antibodies used by other groups to obtain mitogenesis (199,226).

The observation that the $F(ab')_2$ of RMG induced increased thymidine incorporation in cultures of normal spleen cells from young mice was rather contradictory to the majority of previously published results using murine lymphocytes. It was therefore imperative that the mitogenic activity be shown to be associated with the anti Ig activity.

The surface iodination data as well as the immunofluorescence labelling studies have already been discussed, however, as both of these constitute indirect evidence, immunosorbent studies were undertaken. The mitogenic activity was fully removed with a NMIgG sepharose column as was the anti IgG activity as assessed by immunoelectrophoresis. Furthermore this adsorbed material failed to stain spleen cells in an indirect immunofluorescence

assay. The fact that the $F(ab')_2$ of RMF were also mitogenic lends further support to the argument that the biological activity was due to anti-immunoglobulin reactivity.

Beta-2-microglobulin is a low molecular weight protein which is present in the serum and on the plasma membranes of most nucleated cells including lymphocytes. There is considerable sequence homology between β_2M and the constant regions of immunoglobulins and antisera to human β_2M have been reported to be mitogenic for murine B cells (123). However a consideration of the cellular distribution of β_2M and the staining patterns observed with RMF and RMG in immunofluorescence would seem to rule out a cross reactivity of the anti Ig sera with β_2M as being responsible for the mitogenic activity. Beta-2-microglobulin is found in association with H-2 antigens on both B and T cells whereas neither RMG or RMF stained thymocytes or the majority of spleen cells. The SDS-PAGE analysis also failed to demonstrate any evidence of a membrane component with a M.W. comparable to that of β_2M (11,200). Thus it does not seem that cross reactivity of the RMF and RMG with β_2M is responsible for the mitogenic activity of $F(ab')_2$ of these antisera.

The possibility of contamination of the antibody preparations with bacterial products is always a real one, however, several lines of evidence argue against this being the case. The lack of synergism between $F(ab')_2$ of RMF/G and LPS at various ratios of the two reagents is not compatible with endotoxin activity being the mitogenic property in the antisera. The nature of the extensive purification procedures would be expected to remove any endotoxin activity initially present in the starting sera, and all buffers up until the final dialysis of the $F(ab')_2$ contained azide to inhibit bacterial growth. The ability to remove the mitogenic activity

with a NMIgG-sepharose column is also not consistant with an endotoxin source of mitogenic activity.

Protein A also has been reported to be mitogenic for murine lymphocytes but several lines of evidence exclude the possibility of Protein A contamination being the source of mitogenesis(123). $F(ab)_2$ which was prepared from an IgG enriched fraction of RMG and purified on a Bio-Gel P-150 column was mitogenic, thus in the complete absence of exposure to Protein A the antibody still possessed mitogenic activity. In the case of the Protein A purified $F(ab')_2$, passage of this material through a column of Protein A purified IgG coupled to Sepharose did not remove the mitogenic activity. If Protein A had been present in the $F(ab')_2$ preparations it should have been removed on the IgG-Sepharose column and the mitogenic activity of the $F(ab')_2$ been decreased or lost relative to $F(ab')_2$ passed through an OA-Sepharose column, this was not observed. Finally, the control $F(ab')_2$ used in these experiments was prepared from NRIgG which had been processed in the same way as the antisera; if Protein A contamination were occurring it should have been present in the NRG $F(ab)_2$. Yet these $F(ab')_2$ were non-mitogenic relative to control cultures receiving media alone and in fact the NRG $F(ab)_2$ were the controls used in the calculations of the stimulation indices. It would therefore appear that the mitogenic activities of the antisera are not due to endotoxin or Protein A contamination or cross reactivity with β_2 microglobulin.

Although the $F(ab')_2$ behaves in many ways as a typical polyclonal activator there are several very important differences between the two classes of activators (224). The initial kinetics of the responses are similar in both cases however the anti Ig causes a more extended stimulation than does either LPS or Con A. The optimal mitogenic response

observed at 48-72 hours with the $F(ab')_2$ requires continuous exposure of the cells to the antibody whereas periods of exposure of as little as 1 hour are sufficient to produce increases in thymidine uptake with mitogens such as LPS, PHA or Con A (4 ,151). Maximal responses for these latter agents do require exposure of 24 hours but the patterns of activation are quite significantly different from those observed with the anti Igs.

It is not clear why such an extended period of exposure to the antibody is necessary for mitogenesis. However in several cases where the requirements of the length of exposure of cells in culture to antigens have been examined similar lengths of exposure were required (133,147). The exceptions to the above have proven to be mainly the T independent antigens (POL, DNP-Ficoll) and the potent tolerogen DNP-DGL (48 ,126,145). In another case very high concentrations of FGG were found to be tolerogenic if exposed to cells for greater than 24 hours whereas as little as 1 hour exposure was sufficient to induce an eventual anti FGG response. In these cases it is,however, quite conceivable that sufficient residual antigen remained in the cultures after washing to allow for stimulation. The low rate of catabolism of TIA's as well as their extremely high biological activity ,if presented on the surface of macrophages,are also important considerations when attempting to assess the importance of extended exposure periods to antigen. If the display of antigen on the surface of macrophages is a much more efficient means of presenting antigen it might be expected that $F(ab')_2$ due to the strict orientation requirements of the antibody molecule for activation would not be capable of activating lymphocytes via this route. Activation by $F(ab')_2$ might be wholly dependent upon the binding of soluble antibody to the lymphocyte surface. This might prove to be a less efficient means of activation and thus require

extended periods of exposure while MØ bound material could be more efficient in terms of the period of stimulus and quantity of stimulus required.

The concentrations of $F(ab')_2$ required for induction of mitogenesis are very high, being sufficient to clear sIg and to maintain this cleared status of the cells throughout the culture period. If one does a crude calculation assuming 10^5 sIg receptors per B cell then a 100 mcg dose of $F(ab')_2$ constitutes a 2500 fold molar excess of antibody to sIg. The clearance of sIg by mitogenic concentrations of antibody has been reported also by another group who observed large sIg^- blasts following challenge with anti IgM. The blasts were identified as B lymphocytes by the presence of FcR and Ia antigens on the cells(185). In this study no attempt was made to identify the cells other than to demonstrate that the blasts were sIg^- and they were observed in T depleted spleen cell cultures. However the B cell nature of the responding cells was indicated by the demonstration that removal of the $F(ab')_2$ after 48 hours in culture led to the appearance of sIg blasts 24 hours later. If every sIg/anti Ig interaction is viewed as being registered as a signal by the responding B cell it seems highly unlikely that the mitogenesis as it is currently observed is a suitable model for B cell induction by antigen.

Nossal & Pike have demonstrated that when antigen enriched cell populations are treated with an immunogenic concentration of antigen (Flu-PoL) the majority of the sIg is initially cleared from the responding cells but regenerated (135). This is in contrast to the permanent disappearance of all available antigen binding sIg on those cells treated with a tolerogenic concentration of antigen. Thus the major difference between the immunogenic and tolerogenic concentrations of antigen was the ability of the cells to re-express sIg. These results are not in agreement with several

other groups where the induction of B cell tolerance seemed to correlate well with a receptor blockade type phenomenon, that is the cells seemed to be incapable of clearing the antigen/tolerogen from their surface(21,14, 47). The results of the current experiments appear to be somewhat intermediate to those discussed above, there is definitely no freezing of the membrane sIg clearance and the cells are not prevented from re-expressing their sIg.

Although proliferation is assumed to be a necessary event prior to the generation of AFC (101) it was possible that the $F(ab')_2$ induced mitogenesis might be only one part of the sequence of events leading to the induction of PFC (89). Attempts to demonstrate Ig synthesis by $F(ab')_2$ in normal spleen cell cultures on several days (2-7) consistantly failed. In fact, the only consistant observation was an inhibition of background PFC responses by the antibody. The addition of $TRF_{Con\ A}$ to cultures containing $F(ab')_2$ continuously or for 24 to 48 hours did not demonstrate any synergism between these two reagents and the $F(ab')_2$ generally proved to be inhibitory to the increased background responses caused by the TRF. The results with the $F(ab')_2$ and TRF were rather disappointing as it appeared that only half of the system had been realized and that the treatment of cultures with mitogenic concentrations of $F(ab')_2$ were immunosuppressive.

When cells were treated with a mitogenic concentration of $F(ab')_2$ for 48 hours, washed and rechallenged with LPS or DNP-AECM-Ficoll for various periods of time it was found that the capacity of treated cells to respond to a subsequent challenge when compared with control cultures was markedly reduced.

Experiments similar to those described above gave essentially identical

results even though the numbers of PFC generated per culture was 10-15X higher than that observed in the unprimed case. The reason for not detecting PFC was not a consequence of class switching of the PFC to IgG as indirect PFC were not present in these cultures whenever they were assayed. The conclusion from these experiments was that RMF/RMG $F(ab')_2$ treated spleen cells did not form PFC in the presence of the non-specific factors $TRF_{Con\ A}$. This did not however mean that the cells were necessarily tolerized but might mean instead that they had been switched into a differentiation state which was no longer responsive to the stimuli used.

Several groups have employed LPS as a stimulus which will synergize with antigen (174), anti-idiotypic antibody or hapten (221) to generate a specific PFC response. However attempts to demonstrate synergism between LPS and a mitogenic concentration of $F(ab')_2$ were unsuccessful. The $F(ab')_2$ markedly inhibited LPS induced PFC and showed no synergistic effects on the thymidine incorporation of such cultures. The reverse approach that is culturing cells with a fixed suboptimal concentration of LPS, and adding varying dilutions of the $F(ab')_2$ to these cultures resulted in a dose dependent inhibition of the LPS induced PFC with no effect on the LPS induced proliferation.

The lack of additivity between LPS and $F(ab')_2$ proliferative responses is not a consequence of limiting culture conditions as Con A induces 5-10X greater stimulation than either of these reagents. It seems that a more likely explanation for the lack of synergism between the $F(ab')_2$ and the LPS is that they both act on the same cell population. Cooper et al have reported that high concentrations of anti μ in the presence of LPS resulted in an increase in thymidine incorporation even though the anti μ alone is

non-mitogenic (99), however at lower concentrations the antiμ is slightly inhibitory to LPS mitogenesis. This synergism between anti Igs and LPS has been observed also by Melchers et al (9) but again requiring very high concentrations of antibody. It may be that in the present experiments sufficiently high antibody concentrations were not employed to demonstrate the synergy even though inhibition of AFC was observed. It has been suggested that B cells in the presence of antigen under conditions of reduced T cell function may differentiate to memory B cells (45 ,164,176). Also in a system involving anti idiotypic antibody Eichmann & Rajewsky (54a) found that the antibody could lead to the generation of memory B cells but it could not induce a primary antibody response. However in the case of Cooper et al it (99) would seem that extensive proliferation can lead to a cell type more resembling an end stage of a differentiation sequence incapable of producing antibody. The point which is relevant in all of these studies is that there are at least two distinct pathways of differentiation for B cells following stimulation and it is difficult to predict or assess the immune status of those cells which have only proliferated using present methods.

The observation that the $F(ab')_2$ of RMF at concentrations of 50 to 100 fold less than those required for mitogenesis was rather unexpected. Extensive proliferation is thought to be generally a prerequisite for induction of AFC. The responses are admittedly quite low however they are real and significant as in those experiments where antibody formation was observed the results are the means of 6-8 individual cultures each separately assayed. Furthermore if one considers the data in light of results obtained with anti idiotypic antibody or often antigens the numbers of PFC/culture quite comparable especially since the cultures employed here contained 5-8X

10^5 cells per culture. A difficulty with employing the unprimed cells for cultures is the low numbers of responding cells to any given specificity especially since there was no evidence of proliferation in the cultures. However when primed cells were used for culture there was very little increase in the frequency of cells responding to the $F(ab')_2$ in the absence of TRF. This might be expected if antibody and T help were required for activation as the majority of T helper cells in the culture were reactive with the priming antigen but not with the $F(ab')_2$. The two sources of reported non-specific help used Con A induced T_H cells and Con A induced TRF, were unfortunately not adequate sources of help. The Con A helper cell induction was not reproducible and in fact resulted in suppression of control responses more often than the generation of help. The $TRF_{Con\ A}$ did produce a response in primed cultures however a significant proportion (30-50%) of the response was apparently antigen independent.

It is not clear as to what the cause of high background with the $TRF_{Con\ A}$ is as several groups have used factors induced under similar conditions which were reported to be antigen dependent. One possibility was the source responding B cells. It may be that recently primed cells contain a large component of cells which have passed through an antigen dependent stage and are dependent upon T cells only for their subsequent differentiation to AFC. Askonas et al (132) have demonstrated that cells from primed animals which were boosted shortly before sacrifice produce a very large antigen specific IgM and IgG response. The majority of the IgM response is however antigen independent and it might be that a similar situation arises in the recently primed animals. In those cases where antigen requirements have been examined in vitro it would seem that detectable quantities of antigen are required only during the first 48 to 72 hours of culture after

which T cells are definitely required but the need for antigen is reduced (101). Therefore it may be that during the 4 day culture period some B cells may have reached this stage. Despite the high background induced by the TRF it is apparent that there is a synergism between the $F(ab')_2$ and the TRF.

A recent report has demonstrated that a large proportion of primed B cells can be activated in the absence of additional antigen if carrier specific T cells are present (30), thus lending further support to the above hypothesis.

There have recently been several reports of anti Ig induced proliferation in murine lymphocytes and considering that several groups have attempted over at least a 10 year period to achieve this result it may be worthwhile to discuss some of the possible reasons for the sudden high frequency of successes. Perhaps the results most consistent with my own data are those of Weiner et al (225). These workers observed that some but not all rabbit antisera raised against immune complexes of bacteria and antibody were mitogenic, subsequently other antisera prepared against an IgM myeloma protein were found to be consistently mitogenic thus demonstrating that the critical specificity was due to μ chain reactivity. These authors originally reported that anti-Ig responsiveness appeared with increasing age however they have recently reported that the $F(ab')_2$ of the antisera are mitogenic for spleen cells from mice of all ages (186). Unfortunately this group has not presented any data on the effects of anti-Ig treatment on immune reactivity of the responding cells.

The two other reports of anti-Ig induced proliferation have the common features of 2-Me being present in the media and intact antibody being as mitogenic as $F(ab')_2$. Sidman and Unanue (20) observed that the IgG fraction of a rabbit anti-IgM was mitogenic if the media contained 2-Me FCS

or a factor isolated from 2-Me treated serum. Several other antisera including a polyvalent anti-Ig and an anti δ antiserum failed to induce proliferation under comparable conditions. All of the antisera inhibited LPS induced PFC formation however only the anti-IgM sera showed a slight enhancement of the LPS proliferative response. Sieckmann et al (199) were able to demonstrate very high levels of thymidine incorporation induced by a goat anti μ, goat anti κ and a rabbit anti μ sera. The anti μ sera had the highest mitogenic activity and the rabbit anti μ sera had the lowest activity. A comparison of the mitogenic acticity of the IgG fraction of goat anti μ and its $F(ab')_2$ showed no differences when compared on a molar basis. Part of the reason for the high biological activity of there antibody preparations may be that immunosorbent purified material was used for most of the experiments. Interestingly Sieckman et al reported that their antisera were non-mitogenic if cultured in serum free medium in the absence 2-Me .

The data from the above reports suggests that anti IgM and possibly anti L chain reactivity is necessary for the induction of mitogenesis. However the culture conditions can markedly effect whether mitogenesis is observed. It would appear that the presence of 2-Me in the culture media, which has come into widespread use only over the last 4-5 years, may partially negate Fc effects thus allowing for the detection of positive anti Ig effects. Also the species in which the antisera are raised may have an influence on whether or not the Fc are biologically active in mouse spleen cell cultures. It seems quite probably that over the years numerous mitogenic antisera may have been produced but that Fc effects may have masked the proliferation. It is not implied that all anti Ig sera will necessarily be mitogenic as there may be certain critical deter-

minants for activation on immunoglobulins and not all antisera may have antibody activity against these determinants. Only now that a means of assessing the biological activity of antisera can this possibility be examined in detail.

Although the magnitudes of $F(ab')_2$ induced responses in unprimed populations were quite low and those observed in TRF treated primed cultures suffer from high background levels of PFC the results of the present study are very encouraging as to the feasibility of eventually attaining a polyclonal model for B cell activiation involving anti-immunoglobulin stimulation. However the development of this approach would be greatly facilitated if a reverse plaque assay were adopted for assessing B cell immune function and if an antigen specific TRF were employed. A TRF to the $F(ab')_2$ of NRIgG would supply a physiological means of utilizing T cell products and thus eliminate any reservations which might be held towards the use of non-specific TRF's. Genetics has proven to be the most powerful means of analysing immune phenomena and the reports of H-2 restricted anti Ig responsiveness offer great potential in the examination of the molecular processes involved in B cell activation. A similar system may also be applicable to the study of human lymphocyte function as it has been reported that there is an age dependent increase in the responses of human lymphocytes to anti immunoglobulins. This system clearly presents many intriguing possibilities and deserves further examination.

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