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MODULATION OF THE IN VITRO HUMORAL IMMUNE RESPONSE

BY CYCLIC AMP

by

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B.S., University of California Davis 1973

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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of the

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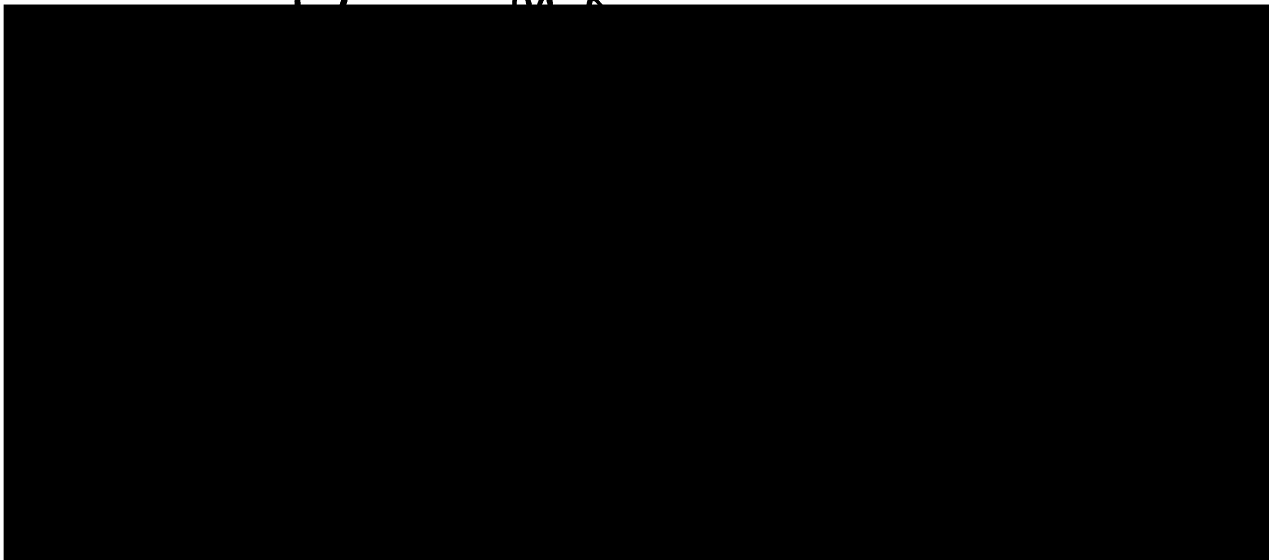

ABSTRACT

The purpose of this work was to investigate the cellular and the biochemical mechanisms by which agents that have the ability to elevate intracellular levels of cyclic AMP modulate the induction of the humoral immune response. *In vitro* studies were conducted that showed that various agents that elevate cyclic AMP could stimulate the induction of the primary humoral immune response to sheep erythrocytes (SRBC), when they were present during an early stage (hour 0-18 of a 96 hour response) of immune induction. Those agents that were most capable of maintaining a sustained elevation of cyclic AMP levels were also most capable of augmenting humoral immunity. Thus, DBcAMP and isobutyl-methyl-xanthine (MIX) were more effective than isoproterenol or PGE_1 in producing augmentation.

Because previous investigations had shown that elevation of cyclic AMP levels in cultured lymphocytes produces inhibition of proliferation, the possibility that anti-proliferative effects of cyclic AMP might account for its stimulatory effects on immune induction were considered. Several agents that have the ability to produce inhibition of proliferation of cultured spleen cells were shown to produce time-dependent stimulation and inhibition of the humoral immune response to SRBC. Colchicine and colcemid, cytosine-arabinoside (ARA-C), and hydroxy urea (HU) all exerted dose-

dependent stimulation of the SRBC response, when they were present only during the early stage of immune induction. The time-dependence for stimulation of the SRBC response by agents that elevate cyclic AMP and anti-proliferative agents were remarkably similar. These results suggested that cyclic AMP may augment humoral immunity via effects on cellular proliferation. Detailed investigation of the cellular basis for stimulation of the SRBC response by DBcAMP and ARA-C revealed that both of these agents could augment humoral immunity via effects of the T cell deficient (B cells + macrophages) population of cells. In addition, ARA-C was found to exert stimulation of the SRBC by effects on T cells.

Investigation of DBcAMP and anti-proliferative agents for their effects on the induction of humoral immune responses to T-independent antigens (DNP-Lysine-Ficoll) showed that such responses were augmented by DBcAMP, but not by colcemid, ARA-C, or HU. These results showed that simple inhibition of proliferation can not account for the ability of agents that elevate cyclic AMP to augment humoral immunity. Further experiments are needed to resolve the mechanism by which cyclic AMP and anti-proliferative agents augment the induction of humoral immune responses.

DEDICATION

To Judy, with my deepest love, and in gratitude for the many sacrifices that she has made and will continue to make on my behalf;

To our parents, whose love and guidance we will always cherish; and

To those of Trinity Baptist Church in Vacaville, California, whose love and support we will never forget.

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ABBREVIATIONS

APC	antibody producing cell
ARA-C	cytosine-arabinoside
B cell	bone marrow-derived lymphocyte
BM	B cells and macrophages
8BrcAMP	8 bromo-adenosine 3':5' cyclic monophosphate
BSS	balanced salt solution
C'	complement
CMI	cell-mediated immunity
CML	cell-mediated lympholysis
CT	cholera toxin
cyclic AMP	adenosine 3':5' cyclic monophosphate
cyclic GMP	guanosine 3':5' cyclic monophosphate
DBcAMP	N^6,O^2-dibutyryl adenosine 3':5' monophosphate
DLF	DNP-lysine-Ficoll
DMSO	dimethyl sulfoxide
HRBC	horse red blood cells
HU	hydroxy urea
IgG	immunoglobulin G
IgM	immunoglobulin M
ISO	DL-isoproterenol
LPS	lipopolysaccharide
MIX	1-isobutyl, 3-methyl xanthine
PFC	plaque forming cell
PGE_1	prostaglandin E_1
RAMB	rabbit anti-mouse brain antisera
SRBC	sheep red blood cells
SBSS	sterile balanced salt solution
T cell	thymus-derived cell
T_H	helper T cell
T_S	suppressor T cell
TNP	2,4,6 trinitrophenol

GENERAL INTRODUCTION

A. The Humoral Immune Response

The immune system is a highly organized and integrated network designed for the protection of the body against infectious disease and, quite possibly, malignant transformation. The immune response refers to those processes designed to carry out the function of the immune system and, in general, can be divided into two main components: 1) cell-mediated immunity, and 2) the humoral immune response. The basis for this division may be somewhat simplistic, as some immune responses require interactions between both components, however for the purpose of this discussion this distinction will be helpful. The main point to be made is that some immune responses operate via direct cellular interactions with the agents that elicited the response, while other processes function via the synthesis and secretion of humoral effector agents. As the purpose of this study is to investigate the modulation of the humoral immune response by pharmacological agents, this discussion will focus on the latter division.

The humoral immune response can be defined as those processes responsible for the induction, synthesis, and secretion of antibody, resulting in the specific combination of antibody with that which elicited its production

(antigen). Antibodies are protein molecules that are structured in such a manner that they contain specific sites for combination with antigenic determinants (Edelman, 1973). The consequences of antibody combining with antigenic determinants is often cell lysis via activation of the complement system when these antigens are on cell surfaces (Müller-Eberhard, 1968). However antibodies also function in cell-mediated cytotoxic reactions, phagocytosis, and neutralization of virus and toxin activities. Thus, antibodies can act via several mechanisms to eliminate or destroy those agents that elicit humoral immunity.

1. Cells of the Humoral Immune Response

It is recognized that the immune system must have a discriminatory mechanism(s) by which it responds only to certain agents. Failure of such a system to make selective discrimination might result in the destruction of self components. It now seems clear that the basis for the specific induction of humoral immunity resides in cell surface receptors that are present on certain types of lymphocytes, and that each lymphocytes expresses only one type of antigen receptor (Warner, 1974). A selective process whereby antigen induces only those specific clones of lymphocytes that bear complementary antigen receptors has now been generally accepted as being the

mechanism by which specific antibody is made (Jerne, 1955; Burnet, 1959). As the number of antigenic, or foreign determinants to which the immune system can respond is large, it follows that only a very small percentage of total lymphocytes can specifically respond to any single antigen. Because of this limitation, during the induction of humoral immunity the number of antigen responding cells increases dramatically due to clonal expansion (proliferation) of activated lymphocytes. This increase in the number of responding cells allows for increased responsiveness.

Following initial exposure of immunocompetent cells to antigen only a portion of activated cells actually proceed to develop into antibody producing cells, while the remainder of those cells capable of antibody synthesis go on to form a memory population of cells which will respond to subsequent antigenic exposure. The initial exposure of cells to antigen results in what is termed a primary response. This response is characterized by the production of immunoglobulin of the M type (IgM). Secondary responses are all subsequent responses that result from antigen stimulation of memory cells. This response is more vigorous in both onset and magnitude and results from the induction of another class of antibodies known as immunoglobulin G (IgG). Thus, there is both a qualitative and a quantitative distinction between primary and secondary responses.

During recent years it has become clear that the induction of humoral immunity is not as simplistic as the combination of antigen with an appropriate cell surface lymphocyte receptor. It is now known that only certain types of lymphocytes bear antigen receptors and can make antibodies, while other lymphocytes and hematopoietic cells function in an accessory manner (Katz and Benacerraf, 1972). Those cells that make antibody have been named B cells because, in birds, immature lymphocytes that later differentiate in the Bursa of Fabricius represent the precursors of antibody forming cells (Miller, et al, 1969). The specificity for antibody production, therefore, resides in cell surface receptors present on clones of B cells. The antigen receptor on B cells is known to be an immunoglobulin that has a sequence homology with the antibody that it is capable of synthesizing (Warner, 1974).

The need for accessory cells in production of antibody has been shown in that the removal of certain types of lymphocytes and hematopoietic cells, that are not B cells, results in diminution of humoral immunity to certain antigens (Katz and Benacerraf, 1972). Several investigators have shown that induction of humoral immunity to many antigens requires the presence of T lymphocytes, so-called because these lymphocytes require the thymus for differentiation (Claman and Chaperon, 1969; Miller and Mitchell, 1969). As T cells are required for many humoral

immune responses, they are termed helper T cells. During recent years other roles for T lymphocytes besides aiding in the induction of humoral immunity have been recognized:

1) T cells mediate cell-mediated immunity. These are the cells that are responsible for such reactions as delayed-type hypersensitivity, allograft rejection, and graft versus host reactions. These T lymphocytes have been termed cytotoxic T cells.

2) T cells exert helper effects on the induction of humoral immunity and perhaps on certain cell-mediated responses. These are the helper T cells previously discussed.

3) T cells have been shown to modulate immune expression by exerting inhibitory effects on humoral immunity and perhaps on cell-mediated responses. These T cells have been termed suppressor T lymphocytes (Gershon, 1974).

That these various T cell activities described are actually mediated by succinct populations of T cells has been shown by the ability to identify different alloantigens on their cell surfaces (Cantor and Boyse, 1975) and the ability to physically separate these cells in density sedimentation gradients (Tse and Dutton, 1976).

The mechanism(s) by which T cells exert their effects are unclear, but the specificity with which they produce their effects suggests that they bear cell surface receptors for antigen. The nature of the T lymphocyte antigen

receptor is till unresolved (Warner, 1974; Paul and Benacerraf, 1977). Many investigators have shown that T cells may exert helper effects on humoral immunity via the secretion of soluble helper factors. Some of these factors have been shown to be antigen non-specific (Dutton, et al, 1971; Schimpl and Wecker, 1972; Gorcynski, et al, 1973; Watson, 1973; Armerding and Katz, 1974; Rubin and Coons, 1972; Harwell, et al, 1976) while other invetsigators have shown helper factors to exert effects with antigen specificity (Feldman and Basten, 1972; Munro, et al, 1974; Tada, et al, 1973). Other T cell products have been shown to exert suppressive effects on the induction of humoral immunity (Tada, et al, 1975; Tadakuma, et al, 1976; Feldman, 1974; Thomas, et al, 1975; Kapp, et al, 1976). Suppressive factors are believed to be the humoral effector agents of the suppressor T cell population of lymphocytes.

The need for another accessory cell in the induction of humoral immunity has also been determined (Unanue, 1972). Thus, it has been shown by many investigators that macrophages are also necessary for the induction of humoral immune responses, but their role in immune induction is not clear. Macrophages apparently do not function in an antigen specific manner, but may rather serve a function of antigen processing, clearance, or presentation (reviewed in Unanue, 1972).

In summary, the induction of humoral immunity is known

to involve several distinct populations of lymphocytes and hematopoietic cells. The interactions of these cells with antigen can proceed with great specificity via combination with cell surface receptors. Recent evidence suggests that collaboration between the various cell types requires cellular interactions via membrane components that may display genetic restriction (Paul and Benacerraf, 1977). Thus, immunocompetent cells must not only be able to recognize foreign antigenic determinants, but they must also be able to recognize each other. The requirements for several different populations of cells for the induction of humoral immunity and the low frequency with which antigen specific cells are present has made it difficult to examine the biochemical basis for induction of humoral immune responses.

2. Concepts of B Lymphocyte Activation

A number of investigators have proposed models for what they believe represent the minimal requirements for B cell activation (Möller, 1975). It is unclear which, if any of these models is correct, but in general these models can be divided into two types of theories:

1) A one-signal model for B cell activation (Coutinho, 1975; Möller, 1975; Diener and Feldmann, 1972)

2) A two-signal model (Bretscher, 1975; Bretscher and Cohn, 1970; Schrader, 1973 and 1974; Watson, et al, 1973).

The main source of controversy in distinguishing between these models is whether or not there truly exist antigens that can induce humoral immunity in the absence of T cells.

Evidence has been presented by Feldmann, et al that several long polymers of protein or polysaccharide can induce humoral immunity in the absence of T cell help by virtue of the ability of these antigens to interact with a high epitope density on the cell surface of B cells (Feldmann, et al, 1975). These investigators claim that if antigen can bind to the B cell surface to a sufficient extent that cross-linking of antigen receptors occurs, then activation of the B cell will occur. In this system the role of accessory cells (T cells and/or macrophages) would be that of presenting antigen in such a manner that cross-linking of receptors would occur.

Other investigators claim that the binding of antigen to the antigen receptor is not involved directly in the activation of the B cell, but rather serves to focus T cells that can deliver one non-specific signal of activation to the B cell (Coutinho, 1975; Moller, 1975). These investigators suggest that T cells are obligatory in the induction of humoral immunity, therefore there are no such thing as T-independent antigens.

Proponents of a two-signal model suggest that antigen binding to the B cell receptor constitutes the first signal of immune induction and that the second signal is given by

the T cell. It is postulated that when signal one is given in the absence of signal two, immune paralysis (tolerance) is produced. Watson, et al suggest that the second signal, that which is normally given by a T cell, is a mitogenic signal and he has shown that a B cell polyclonal activator (LPS) can substitute for T cell help (Watson et al, 1973). Watson, et al suggest that T-independent antigens to do not need T cell help because they are, in themselves, mitogenic; however, the mitogenicity of all T-independent antigens has been shown to be questionable (Feldmann, et al, 1975).

On the basis of all available information it is not possible to conclusively prove that either the one or the two signal theory for induction of humoral immunity is correct. Much of the evidence can support either contention, and it is never possible to be absolutely certain that all T cells have been removed when studying T cell depleted cultures for the effects of T-independent antigens. Many investigators have relied upon a T cell deficiency disease in mice (athymic "nude" mice) to obtain what they thought were immunocompetent cells that had an absolute absence of T cells. However, it is now apparent that such mice do possess T cells or T cell precursors (Zinkernagel, 1974). It is fair to say that if so-called T-independent antigens do require T cell help for the induction of humoral immunity, that this require-

ment is much less than that for the induction of T-dependent responses.

Although there is a strict requirement for the presence of macrophages for the induction of humoral immunity, their role in this process is unclear. It now seems apparent that macrophages are required for the induction of both T-dependent and T-independent responses, and for polyclonal activation by mitogens (Unanue, 1972). The role of the macropahge and its interactions with other immunocompetent cells in the induction of humoral immunity is an area of intensive investigation in contemporary cellular immunology.

Thus far, activation of B cells has been discussed in terms of the induction by antigens; however, it is clear that B cells can be activated by agents other than antigens. For instance, polyclonal B cell mitogens are known that can activate B cells in the absence of antigen specificity (Möller, 1975). It is not within the scope of this brief review to specifically discuss those experiments that have studied B cell activation with mitogenic agents. In general, such experiments have been conducted in an attempt to understand the biochemical basis for antigen activation of B cells and T cells; however, the exact relationship between antigen and mitogen activation is unclear. A recent review focuses on the activation of lymphocytes by mitogenic agents (Möller, 1973).

In summary, the results of previous investigations suggest that B cell activation requires the cooperative interaction of different cell types. Evidence suggests that direct cell-cell interactions may not be needed for cell cooperation, as various soluble factors have been described that can substitute for cellular requirements. However, whether the induction of soluble mediators requires cell-cell interactions is unclear. In consideration of all of the various components for immune induction, it is possible that pharmacological modulation of the humoral immune response could be achieved via interaction with any one of several cell types and/or by effects on the synthesis, secretion, or activity of various soluble immune mediators.

3. Modeling of the Humoral Immune Response *In Vitro*

In vitro systems have long been realized to offer great advantage in the analysis of complicated *in vivo* responses, in that one is quite often able to define and manipulate those conditions under which responses are generated. For a variety of reasons, establishment of an *in vitro* method by which to study the induction of the humoral immune response was delayed; however, various attempts were ultimately successful (Dutton, 1967). Some of the difficulties in generating primary immune responses

in vitro resulted from inadequate culture conditions and damage to cells during isolation procedures, but it became clear that the precise architecture of lymphoid organs need not be maintained, as dissociated spleen cell suspensions were found capable of mounting *in vitro* humoral immune responses (Mishell and Dutton, 1966).

The *in vitro* humoral immune response has proven to be a good model system by which to study the effects of chemical agents on the induction of humoral immunity. By its very nature, *in vitro* responses are removed from the bodily influences that normally provide the environment in which humoral immune responses are generated. It is unclear what role the endocrine and/or nervous system play in the humoral immune response. It has been recently demonstrated that this response exhibits a circadian rhythm of activity, suggesting that these two systems may possess a modulatory capacity (Fernandes, et al, 1976). If such a capacity does exist, then chemical agents may exert effects via these mechanisms *in vivo*, while such an activity would be absent in the *in vitro* situation.

It is also to be realized that chemical agents may produce different effects *in vivo* or *in vitro* due to differences in bioavailability of the agent. For instance, those processes of absorption, distribution, metabolism, and excretion may limit drug activity *in vivo*; therefore, drug effects might be seen *in vitro*, but not *in vivo*. The

converse of this situation is that some drugs (such as cyclophosphamide) might require *in vivo* processes, such as bioactivation by liver microsomal or other enzymes, to become pharmacologically active. Thus, activity would be seen *in vivo*, but not *in vitro*. These considerations point out the need to confirm results from *in vitro* studies in *in vivo* systems, and that conditions for drug activity (dose and time-dependence) may vary markedly.

In spite of the preceding considerations, it is clear that the *in vitro* humoral immune response has proven to be a good model for prediction of *in vivo* responsiveness and that it allows for precise manipulation of culture conditions to study the effects of pharmacological agents on the induction of humoral immunity. These studies will involve the use of murine spleen cells in the induction of humoral immunity because such cells are readily available and are reasonably well defined for their responses *in vitro*.

B. The Pleiotypic Response

To account for the apparent coordination of cellular responses resulting from humoral and environmental influences and causing programmed changes in cellular biochemical activity, Tomkins and his colleagues have proposed that cell function is regulated via a pleiotypic program (Hershko, et al, 1971). The pleiotypic program is postulated to be modulated via an intracellular mediator, and evidence has been presented that cyclic AMP may fulfill such a role (Kram, Mamont, and Tomkins, 1973). The role of a pleiotypic mediator might be to coordinate biochemical processes necessary for cell growth, proliferation, and differentiation (ie. a positive pleiotypic mediator); or it might function in a conservatory manner to protect the cell from environmental changes (ie. a negative pleiotypic mediator). The pleiotypic response may be relevant to the induction of the humoral immune response because this process requires the coordination of the processes of cellular synthesis, proliferation, and differentiation resulting from inductive stimuli. Conversely, immune induction must be regulated to limit the extent of responsiveness. Thus, if such a mechanism exists, it should be possible to exert effects via these agents to coordinately stimulate or inhibit immune induction.

1. The 2nd Messenger Concept

It is now well recognized that many hormones that interact with cell membrane receptors require the generation of an intracellular signal to transmit information of membrane-associated events to the cell nucleus and that, in many instances, cyclic AMP fulfills a role as a 2nd messenger (Robison, et al, 1971). Those agents that can increase intracellular concentrations of cyclic AMP can often mimic hormone effects to bring about cellular responses. Intracellular levels of cyclic AMP are maintained by a steady state resulting from synthesis and degradation; therefore, several biochemical mechanisms can produce elevation of cyclic AMP concentrations:

1) Administration of an agent that activates the cyclic AMP-generating enzyme (adenylate cyclase) via combination with a coupled receptor. Such agents include: isoproterenol (stimulates the beta-adrenergic receptor), histamine (stimulates a histamine receptor), prostaglandin E_1 (stimulates a prostaglandin receptor), and cholera toxin (receptor type uncertain).

2) Inhibition of metabolism of cyclic AMP by the enzyme phosphodiesterase. Agents capable of this activity are methyl xanthines, such as theophylline and isobutyl methyl xanthine.

3) Administration of exogenous cyclic AMP derivatives that can be directly absorbed into intracellular cyclic AMP

pools. Such agents include dibutyryl cyclic AMP (DBcAMP) and 8-bromo-cyclic AMP (8BrcAMP).

With specific regard to leukocytes, many of these types of agents have been shown to increase intracellular levels of cyclic AMP (Bourne, et al, 1971; Franks, et al, 1971; Makman, 1971). In addition, it has been found that not all leukocytes bear receptors for certain cyclic AMP-elevating agents (Weinstein, et al, 1973; Melmon, et al, 1974). The non-random distribution of these receptors suggests that such receptors play a physiological role in modulation of leukocytic function. It might also be predicted that some agents that act indiscriminately in elevating cyclic AMP levels (such as DBcAMP and 8BrcAMP) may have a different spectrum of pharmacological activity than those agents that act selectively on lymphocytes with an appropriate receptor (such as histamine, isoproterenol, and PGE_1). In all investigations of the role of cyclic AMP in modulation of physiological responses, it is necessary to correlate the effects of these agents with their ability to affect intracellular concentrations of cyclic AMP.

2. Cyclic AMP and the Immune Response

It is now well established that cyclic AMP can exert modulatory effects on various aspects of the immune response (Bourne, et al, 1974; Parker, et al, 1974; Braun,

1974; Watson, 1975), but the mechanism(s) by which this is accomplished is unclear. The purpose of this section will be to briefly review those experiments that suggest that cyclic AMP may modulate the immune response.

With the increasing realization of the ubiquitous nature of cyclic AMP and implications regarding its biological role in mammalian cells, it was logical that cyclic AMP would be suspected as a mediator of immune induction. Two main reasons why this was suspected were: 1) the induction of the immune response requires a complex series of events that must be coordinately regulated, ie. it requires a pleiotypic program; and 2) triggering of the immune response can be achieved by a cell membrane-associated inductive event; this might require an intracellular relay signal from cell membrane to the cell nucleus. Evidence for the latter is derived from experiments that show that polyclonal activators that are conjugated to Sepharose beads can activate T and B lymphocytes in the absence of penetration of the cell surface (Greaves and Bauminger, 1972).

In general, a role for cyclic AMP in modulation of immune induction has been suggested on the basis of two types of observations: 1) by measuring changes in intracellular concentrations during various stages of immune induction, or 2) by showing that pharmacological agents that increase intracellular levels of cyclic AMP can exert

effects on the immune response.

a. Changes in Intracellular Levels of Cyclic AMP During Immune Induction.

In recent years, this approach has become controversial due to inconsistencies in experimental results between groups. Several investigators have claimed that cyclic AMP levels are elevated by polyclonal activators in lymphocytes (Smith, et al, 1970, and 1971; Webb, et al, 1974; Winchurch and Actor, 1972); however, this has been disputed by other workers (Novogrodsky and Katchalski, 1970; McGrey and Rigby, 1971; Hadden, et al, 1976; Watson, 1974). If mitogens activate lymphocytes via a cyclic AMP-mediated mechanism, then cyclic AMP should be able to potentiate submitogenic doses of polyclonal activators; however, results from these types of experiments have shown that cyclic AMP actually inhibits polyclonal activation (Smith, et al, 1971; Hirschorn, et al, 1970 and 1974; Webb, et al, 1973; Watson, 1974 and 1976; De Rubertis, et al, 1974). These results are paradoxical if cyclic AMP truly mediates mitogenic activation. More recent investigations have shown that cyclic GMP may mediate a mitogen activation signal; however, these results have also been the subject of much controversy and will not be considered in greater detail here (Hadden, et al, 1976; Parker, 1974; Watson, 1975;

Goldberg, et al, 1974).

One other series of investigations have attempted to correlate direct measurement of cyclic AMP with lymphocyte activation. In these experiments, antigen was injected into mice, and then spleen cells were removed at various intervals and were examined for their cyclic AMP levels. The results of these studies showed antigen-dependent increases in splenic cyclic AMP concentrations (Pleiscia, et al, 1975; Yamamato and Webb, 1975). Interpretation of these results is difficult, for if this change reflects an antigen-specific inductive signal, it is hard to imagine that such a change could be detected due to the low frequency of antigen-specific cells.

In summary, the results of studies attempting to correlate induction of the immune response with their ability to elevate cyclic AMP levels are inconclusive. It is unclear whether induction of the immune response by antigen (specific induction) or activation by polyclonal activators (non-specific induction) trigger lymphocytes via the same mechanism. Generalization of the results obtained from polyclonal studies to a mechanistic interpretation of how antigen triggers immune induction must be viewed with caution until the validity of such an association is established. Therefore, until methods for obtaining large numbers of enriched antigen-specific cells are available, it will be difficult to use a direct measurement of

cyclic AMP as evidence for antigen-specific activation.

b. Effects of Agents that Elevate Cyclic AMP on Immune Induction

The ability of various agents to produce elevation of intracellular levels of cyclic AMP in lymphocytes provides another avenue by which to pursue the role of cyclic AMP in immune induction. As a great many investigations have employed this method for studying effects on the induction of various immune responses, these experiments will be briefly discussed according to the particular response studied. These experiments will show that cyclic AMP exerts modulatory effects on the induction of the immune response via several different biochemical mechanisms and will suggest that modulation of the humoral immune response may result from effects on several components.

i. IgE-Mediated Histamine Release

Immediate hypersensitivity reactions result from combination of antigen with cytophilic antibody (immunoglobulin type E) attached to mast cells or basophils, resulting in the vesicular release of stored histamine (Austen and Humphrey, 1963). The release of histamine into the surrounding tissues elicits those responses known as acute anaphylaxis

or allergy, depending upon the severity of the response. Previous to the discovery of cyclic AMP, it had been known that various adrenergic agents could inhibit or ameliorate these allergic responses, and later studies supported these findings (Schild, 1936; Mannaioni, et al, 1964; Tabachnick, et al, 1965). However, it was not until 1968 that Lichtenstein and Margolis reported that inhibition of this reaction in sensitized leukocytes by beta-adrenergic agonists, methyl xanthines, and DBcAMP, that the role of cyclic AMP was suspected (Lichtenstein and Margolis, 1968). Following this initial finding, many investigations were conducted and verified that elevation of cyclic AMP levels in sensitized tissues results in inhibition of the immunological release of histamine and other mediators (Lichtenstein, 1971; Bourne, et al, 1972; Ishizaka, et al, 1970; Orange, et al, 1971). With these studies began an era, which now continues, for investigation of the role of cyclic AMP in the modulation of the immune response.

ii. Modulation of Cell-Mediated Immunity by Cyclic AMP

Cell-mediated immunity (CMI) represents those immunological reactions that are directly mediated by sensitized T lymphocytes. The role of the CMI is believed to be that of protection from tumors and viral, fungal, and mycobacterial infections. This system is also responsible for

allograft rejection and graft versus host reactions. The recent establishment of an *in vitro* system by which to study the induction of cytotoxic lymphocytes and their lytic effect on target cells has allowed examination of the role of cyclic AMP in this process (Brunner, 1970; Cerrottini, et al, 1970). In general, the results of such studies show that agents that elevate cyclic AMP inhibit cell-mediated lympholysis (CML) (Henney and Lichtenstein, 1971; Henney, et al, 1972; Henney, 1974; Strom, et al, 1972 and 1974). The mechanism by which elevation of cyclic AMP levels inhibits the effector phase of the CML is unclear, but some evidence suggests that cyclic AMP may inhibit the secretion of a cytolytic lymphokine (Henney, 1974). This concept is supported by the observations that colchicine and cytochalasin B also inhibit this stage of the CML; however, such experiments are far from conclusive (cerottini and Brunner, 1972; Plaut, et al, 1973). In consideration of the mechanism by which cyclic AMP modulates the immune response, effects on secretory activity must be considered whenever humoral factors are important in immune induction or immune expression.

iii. Modulation of the Humoral Immune Response by Cyclic AMP

At the same time that cyclic AMP was being implicated

in modulation of cell-mediated immunity and allergic responses, several investigators began testing various agents that elevate cyclic AMP for their ability to affect the humoral immune response. Braun and his colleagues found that theophylline could potentiate the adjuvant effects of poly A:U, when given at the time of immunization with sheep erythrocytes in vivo or in vitro (Ishizuka, et al, 1970; Braun and Ishizuka, 1971; Ishizuka, et al, 1971). Their conclusion from these experiments was that poly A:U produces elevation of cyclic AMP levels which is potentiated by theophylline. However, evidence to support this concept is lacking, as poly A:U appears unable to elevate cyclic AMP levels (Mozes, et al, 1973). Data showing that various nucleic acids can exert an adjuvant effect on the induction of humoral immunity suggests that poly A:U may act independent of a cyclic AMP mechanism (Merritt and Johnson, 1965), but the finding that theophylline augments humoral immunity was the first evidence for cyclic AMP modulation of the humoral response. Following these initial findings, several investigators showed that agents that elevate cyclic AMP, such as DBcAMP (Uzunova and Hanna, 1973) and cholera toxin (Chisari, et al, 1974), could also augment the in vivo humoral immune response to SRBC, when given at the time of immunization.

In an attempt to understand the cellular and biochemical basis for effects of cyclic AMP on the humoral immune

response, various investigators also studied the effects of cyclic AMP-elevating agents in _in vitro_ systems. In general, the results of these studies showed that various cyclic AMP-elevating agents could exert biphasic effects on the induction of humoral immunity to T-dependent antigens; that is, cyclic AMP elevation during an early stage produced stimulation, whereas cyclic AMP elevation during later stages produced inhibition (Watson et al, 1973; Teh and Paetkau, 1974 and 1976a; Kishimoto and Ishizaka, 1976; Winchurch, 1973). From these experiments, the mechanism by which cyclic AMP exerts stimulatory effects on immune induction was unclear, but suggestive evidence was given for the ability of cyclic AMP to directly activate B cells (Watson, et al, 1973; Kishimoto and Ishizaka, 1976) or to exert effects via regulatory cells (Teh and Paetkau, 1976b). The latter evidence for cyclic AMP augmentation of humoral immunity via a regulatory cell mechanism is tenuous, but this conclusion is supported by the observations that cells that are responsvie to certain cyclic AMP-elevating agents can be removed by fractionation over insolubilized histamine columns and that the humoral immune potential of the non-adherent cells is increased. Thus, regulatory cells may have cell surface receptors for cyclic AMP-elevating agents (Shearer, et al, 1972 and 1974; Weinstein and Melmon, 1976).

Several investigators have shown that cyclic AMP ele-

vation during late stages of immune induction result in inhibition of humoral immunity (Watson, et al, 1973; Teh and Paetkau, 1974; Kishimoto and Ishizaka, 1976). As these investigators did not consider effects of cyclic AMP-elevating agents on cell viability, and these agents are known to decrease cell recoveries, inhibition of the humoral response may be due to cytotoxic effects of these agents (Berenbaum, et al, 1976).

All of the preceding studies that have demonstrated stimulation of humoral immunity with cyclic AMP-elevating agents have shown that these agents must be removed following an exposure at the initiation of cell cultures. These investigations show the importance of the time during which cyclic AMP levels must be elevated to produce augmentation. Other investigators have shown that appropriate doses of DBcAMP can produce augmentation in the absence of drug removal (Marchalonis, et al, 1976). As the dose that these investigators find produces stimulation is less than that which produces augmentation when the drug is removed, it might be suspected that the half life of DBcAMP in culture is such that an effective level is maintained only during the early time period. In general, high doses (10^{-4} to 10^{-3}M) have been shown to produce maximal augmentation of humoral immunity. These doses are supra-physiological; however, intracellular levels of DBcAMP are probably much lower due to limitations in absorption and the stability of

the agent in culture.

Taken collectively, the data from many laboratories suggest that cyclic AMP may exert modulatory effects on immune induction, but it is not possible to satisfy all of the criteria proposed by Robison, et al for implicating cyclic AMP as a 2nd messenger (Robison, et al, 1971). This inability stems from the need to work with heterogeneous populations of cells to study humoral immune induction. This has made interpretation of the biochemical basis for immune induction difficult. An assumption that is made in studying the biochemistry of cell populations is that individual cells respond in a manner consistent with the general population. This assumption is not always valid, especially when dealing with heterogeneous populations of cells.

3. Cyclic AMP and Cell Differentiation

During recent years, evidence has accumulated to support the concept that cyclic AMP may play a role in the modulation of cellular differentiation. Prasad and Kumar have reviewed the evidence that shows that cyclic AMP may induce differentiation in cultured neuroblastoma cells (Prasad and Kumar, 1974). Such cells have altered morphology following treatment with agents that elevate intracellular levels of cyclic AMP, but the stability of this differentiated state

is less clear.

Evidence from several investigators has shown that cyclic AMP may mimic the effects of lymphocyte differentiation hormones (Scheid, et al, 1973; Kook and Trainin, 1975). Various methods are now available to study the expression of cell surface phenotypes, and investigations have shown that cyclic AMP can induce the expression of T and B cell specific alloantigens (Scheid, et al, 1975; Hammerling, et al, 1975). As it is not possible to monitor the expression of these phenotypes in long-term cultures, it is not clear whether drug-induced expression of alloantigens results in heritable changes in cell surface phenotypes.

It is likely that various cell surface phenotypes are expressed only during certain stages of cellular differentiation (Warner, personal communication). Very recent evidence suggests that such stage-dependent expression may exist for hormonal receptors, such as the beta-adrenergic and the histamine receptors. Roszkowski, et al have shown that lymphocytes are differentially sensitive to isoproterenol and histamine, as measured by cyclic AMP accumulation, during different stages of lymphocyte activation (Roszkowski, et al, 1977). It has been observed by Melmon and his colleagues that B cells adhere to insolubilized histamine columns apparently only after they have become activated. This was demonstrated by the ability of histamine columns to remove plaque forming cells from a primed spleen cell population (Melmon, et al, 1974).

The results of these previous studies on the role of cyclic AMP in lymphocyte differentiation suggest that these cells may be sensitive to pharmacological agents only during certain stages of maturation. Cyclic AMP modulation of the induction of lymphocyte differentiation continues to be an active area of research investigation, and is likely to provide basic information concerning the regulation of cell growth and development.

4. Cyclic AMP and the Control of Cell Proliferation

Several lines of evidence suggest that cyclic AMP may be an important modulator of cellular proliferation:

1) Cyclic AMP-elevating agents are found to inhibit the proliferation of many cultured cells (Burk, 1968; Ryan and Heindrich, 1968; Sheppard and Bannai, 1974; Coffino, et al, 1975; Kurz and Friedman, 1976).

2) Rapidly growing cells, such as transformed cells, have low levels of cyclic AMP when compared to normal cells that have slower rates of proliferation (Otten, et al, 1971; Pastan, et al, 1974).

3) Cyclic AMP inhibits mitogen-induced proliferative responses (Smith, et al, 1971; Hirschorn, et al, 1970 and 1974; Webb, et al, 1973; Watson, 1974 and 1976; DeRubertis, et al, 1974).

4) Inhibition of proliferation normally occurs when cells reach high densities in tissue culture; cyclic AMP can decrease the density at which cells become contact inhibited, though its ability to restors contact inhibition in transformed cells is questionable (Johnson and Pastan, 1972; Pastan, et al, 1974).

5) Cyclic AMP levels vary during different stages of the cell cycle, and some investigators believe that these levels regulate the passage of the cell through the cell cycle. Therefore, when cyclic AMP is elevated in cells, its effects could depend upon the particular stage of the cell cycle that the cells were in at the time of drug administration (Pastan, 1975).

Taken collectively, the evidence that cyclic AMP can exert effects on cell proliferation is quite impressive, but the mechanism(s) by which this is accomplished is unresolved. Evidence has been presented that both cyclic AMP and calcium ions work together to modulate cell proliferation (Whitfield, 1976). The relationship between these two agents has not been determined. The results of studies showing effects of agents that elevate cyclic AMP on cell proliferation of cultured lymphocytes makes it evident that such effects must be considered when studying the effects of cyclic AMP elevation on the induction of humoral immunity *in vitro*.

C. Statement of Intent

The preceding sections have provided a brief review of the major areas in which cyclic AMP has been shown to exert modulatory effects on various responses of lymphoid cells. In general, cyclic AMP has been shown to exert effects on the release of endogenous mediators and humoral agents, lymphocyte activation, differentiation, and proliferation. Using this information as a foundation, the present studies were conducted to investigate the mechanism(s) by which agents that elevate cyclic AMP can augment the induction of the humoral immune response.

Previous investigations have shown that cyclic AMP-elevating agents can augment the *in vitro* and *in vivo* humoral immune response when these agents were present at appropriate times. The mechanism(s) by which this was accomplished was unclear. This present study has been designed to establish an *in vitro* system in which to study the effects of agents that elevate cyclic AMP during specific time intervals, in an attempt to determine the relationship between the time of elevation of cyclic AMP levels and the concomitant effects on the induction of humoral immunity.

Evidence has been discussed, in a preceding section, that cyclic AMP may exert effects on the proliferation of cultured lymphocytes. Because the induction of the humoral immune response requires the clonal expansion of activated

lymphocytes, agents that exert effects on cellular proliferation may also affect humoral immune responsiveness. A purpose of this presnt study was to determine if agents that elevate intracellular levels of cyclic AMP can exert modulatory effects on humoral immunity via effects on proliferation. To test this hypothesis, several anti-proliferative agents were tested for their ability to augment humoral immunity during time frames when cyclic AMP was found to produce stimulation. In addition, the relative ability of these agents to inhibit the humoral response was investigated. The results of these studies will show that while anti-proliferative agents and cyclic AMP-elevating agents both have the ability to augment humoral immunity, when they are present during an early stage of immune induction to T-dependent responses, anti-proliferative effects are insufficient to account for augmentation produced by cyclic AMP.

In an initial attempt to resolve the cellular basis for stimulation of humoral immunity by agents that elevate cyclic AMP and anti-proliferative agents, the effects of DBcAMP and cytosine-arabinoside were tested on B versus T cells. The results of these experiments showed that anti-proliferative agents can augment humoral immunity via an effect on both B cells and T cells, whereas cyclic AMP effects were limited to the B cell population. The results of

these experiments will be discussed in terms of current concepts of B cell activation and modulation of the humoral immune response.

A. Time-Dependent Stimulation and Inhibition of the Humoral Immune Response by Agents that Elevate Cyclic AMP

1. Introduction-Purpose

Specific examples of previous studies implicating a role for cyclic AMP in the modulation of the humoral immune response have been discussed in a preceding section. In general, many investigators have relied upon the use of certain prototype agents that elevate intracellular concentrations of cyclic AMP. However, no attempt has been made to correlate the differential ability of these agents to modulate humoral immunity. Such a correlation is necessary, if cyclic AMP levels are deemed responsible for modulation of humoral responsiveness.

Investigators have found that some agents that elevate cyclic AMP are without effects on induction of humoral immunity, while other agents can exert time-dependent stimulation or inhibition of this response (Kishimoto and Ishizaka, 1976). Thus, while DBcAMP, theophylline, and prostaglandin E_1 (PGE_1) exerted significant augmentation of IgG production, isoproterenol was found to be without effect. Theories that attempt to explain the mechanism by which agents that have the ability to elevate intracellular levels of cyclic AMP augment humoral immunity must account for the differential effects of these agents.

Not only have differential effects of agents that elevate cyclic AMP been observed on the induction of humoral immunity, but it is apparent that the effects produced by these agents is a function of the time during which cyclic AMP levels are elevated. The effects of these agents have been termed biphasic, because these agents can exert stimulatory effects when they are present during an early stage of immune induction; whereas they produce inhibition when they are present during later times (Watson, et al, 1973; Teh and Paetkau, 1974; Kishimoto and Ishizaka, 1976). Although time-dependence has been recognized as important is previous studies, no attempt has been made to determine whether critical time periods exist during which cyclic AMP produces augmentation of humoral immunity. Such studies would aid in the understanding of the role of cyclic AMP in immune induction and immune modulation.

With the preceding considerations in mind, the purpose of the experiments described in this section was to both confirm and extend the results of previous investigators. Following the establishment of an *in vitro* system in which to study the effects of pharmacological agents on the induction of humoral immunity, the goal of these studies was to determine those time frames during which stimulation versus inhibition of the humoral immune response was produced. The effects of various agents that elevate cyclic AMP were then correlated for their ability to effect both intracellular

concentrations of cyclic AMP and their ability to modulate humoral immunity. Because cyclic AMP-elevating agents have been shown to produce significant cytotoxicity, these effects were considered when the inhibitory effects of these agents were investigated (Berenbaum, et al, 1976).

2. Materials and Methods

Mice. Female BDF_1 ($C_{57}BL_6$ x DBA/2) mice of 6-8 weeks age were obtained from Simonsen Laboratories (Gilroy, CA) and were acclimatized in our animal facilities for at least one week before use. All animals were maintained by the University of California at San Francisco Vivarium in environmentally controlled rooms. Animals were maintained under a 12 hour light- 12 hour dark lighting schedule and were allowed free access to water and food.

Antigens. High responder (see Mishell and Dutton, 1967) sheep red blood cells (SRBC) were obtained from Colorado Sera, Inc. (Denver, Colo.) twice a month and were stored at 4°C. Only SRBC that were less than two weeks old were used as antigen. Before each use, SRBC were washed three times in a sterile balanced salt solution (SBSS) to remove Alsever's solution (see Mishell and Dutton, 1967). One drop of a 0.5% vol/vol SRBC suspension was used to inoculate both the 1 ml and the 0.5 ml cultures (approximately 2×10^6 cells/culture).

Cultures. Spleen cell cultures were prepared according to the method of Mishell and Dutton (Mishell and Dutton, 1967). Briefly, spleens were extirpated from cervically dislocated mice and were placed in 5 ml of SBSS in a sterile 60 mm tissue culture dish (Falcon, #3002). No more than 3 spleens were placed in a single dish. Spleens were then teased with sterile forceps to cause the release of spleen cells from the surrounding capsule and membranes. Cells were then gently mixed with a sterile pasteur pipet to form a dissociated spleen cell suspension. Connective tissue and aggregated cellular debris was removed by placing this suspension into a sterile 15 ml centrifuge tube (Corning, #25310) and allowing the large particulate matter to sediment for approximately 5 minutes at room temperature. The spleen cell suspension was then removed from the debris, was mixed with 3 volumes of SBSS in a sterile 50 ml centrifuge tube (Corning, #25330), and was then centrifuged at 600 x g for 10 minutes at 4°C. The supernatant was then removed and the cells were suspended in complete culture media. Complete culture media consisted of RPMI-1640 (GIBCO, #187G) supplemented with 0.1% glutamine (GIBCO,#503L), 1% penicillin-streptomycin (GIBCO, #507), 5×10^{-5}M 2-mercaptoethanol (Sigma, #M-6250) in SBSS, and 5% fetal calf sera (GIBCO, #614; Lot #A952020). The fetal calf sera was previously screened to determine that this lot could support *in vitro* responses. Spleen cells were suspended at 1.25×10^{7}

viable cells/ml for culture, and were routinely 90-95% viable at the initiation of culture. One ml spleen cell suspensions were cultured in 35 mm tissue culture dishes (Falcon, #3001) for approximately 96 hours. Cultures were kept in plexiglass gas boxes (C.B.S. Scientific, Del Mar, CA) in a 10% CO_2, 7% O_2, and 83% N_2 atmosphere (Ohio Medical Supply, Berkeley, CA) at 37°C, and were continually rocked on a platform rocker (Bellco Glass, Inc., #7740). All cultures were run in triplicate and were fed daily with a nutritional cocktail mixture (Mishell and Dutton, 1967).

Harvesting Cells and Assay of Plaque Forming Cells. After approximately 96 hours, cultures were gently scraped with a polyethylene policeperson and the spleen cell suspensions from triplicate cultures were pooled into 10 ml of BSS (ie., non-sterile SBSS) in a 15 ml centrifuge tube (Corning, #25300). Cells were then centrifuged at 600 x g for 10 minutes at 4°C. After the supernatant was discarded, the cell pellet was resuspended in a volume of BSS equal to twice that of the original pooled culture volume (ie., the pellet from 3 pooled cultures was resuspended in 6 ml). Cells were then assayed for direct plaque forming cells (PFC) by the slide modification of the Jerne hemolytic plaque assay (Mishell and Dutton, 1967; Jerne and Nordin, 1963). Briefly, an aliquot of the cell suspension (usually 0.05 ml or 0.1 ml) was added to 0.5 ml of an agarose solution at 43°C

containing 0.05 ml of 1-15 diluted washed SRBC. Agarose solution was prepared by dissolving 0.5 g agarose (L' Industrie Biologique Francaise, Indubiose #A 37) in 80 mls of BSS, by heating in a boiling water bath and then maintaining at 43°C until needed for the PFC assay. Immediately following addition of the spleen cell suspension to the agarose-SRBC suspension, the contents were gently mixed and then spread evenly over the surface of a pre-treated microscope slide (agar-coated). Agar was then allowed to gel at room temperature, and then the slides were incubated in an agar-upright position on slide racks (see below) in a humified box at 37°C for 90 minutes. Following this incubation, slides were then inverted and incubated with 1-20 diluted complement solution for 90 minutes on special racks designed to bathe agar-suspended cells in the complement solution (C.B.S. Scientific, Del Mar, CA). Fresh frozen guinea pig complement (GIBCO, #919F) was absorbed with SRBC and HRBC before use and was aliquoted and stored at -70°C (Revco, #ULT 985-B). Complement was thawed and diluted with BSS (1-20) immediately before each use. Direct PFC were counted by visualization with a dissecting microscope (Bausch and Lomb, Stereozoom series 31). Triplicate slides were counted from the pooled culture suspension.

Pharmacological Agents. All agents were prepared as sterile or near-sterile (depending upon the particular agent) stock reagents that were diluted 1-50 to achieve final concen-

trations in culture. Filtration sterilization was performed as indicated with pre-washed 0.22 micron Millex (Millipore Corp., #19882) syrringe filters. Each filter was washed with 25 mls of boiling distilled water, followed by a 25 mls wash with SBSS to remove detergents from the filter that might affect lymphocyte cultures. Filters were voided of residual wash solution before drug solutions were collected. Because of the large variation in the solubility and stability of the various agents employed, different solvents were necessarily used to prepare stock solutions of drugs, although 50X stock solutions were always made in SBSS. The controls for potential solvent effects are included where appropriate. DL-isoproterenol (ISO) (Sigma, #I-5627) was prepared immediately before use due to chemical instability. ISO was dissolved in SBSS and was sterilized by filtration as indicated. N^6,O^2-dibutyryl adenosine 3':5" cyclic monophosphoric acid (DBcAMP) (Sigma, #D-0620) and 8-bromo adenosine 3':5' cyclic monophosphoric acid (8BrcAMP) (Sigma, # B-7880) were dissolved in SBSS and were filter sterilized. DBcAMP and 8BrcAMP stock solutions were stored at -20°C for weeks without loss in activity, although fresh stock solutions were periodically made. 3-Isobutyl-1-methyl xanthine (MIX) (Sigma, #I- 5879) was prepared as a 0.5 M stock solution in dimethylsulfoxide (DMSO) (Aldrich, #M8,180-2) and was stored at room temperature. MIX was diluted before each use to a 50X stock solution in SBSS. Prostaglandin E_1

(PGE_1) was generously provided by Dr. John Pike (Upjohn Co., Kalamazoo, Mich.) and was dissolved in 95% ethanol at 10 mg/ml. PGE_1 was stored at -20°C and was diluted to a 50X stock immediately before use in SBSS. Cholera toxin (CT) (Schwartz-Mann, #90-0593) was dissolved in sterile water at 10 mg/ml, stored at 4°C, and was diluted to a 50X stock in SBSS before each use.

Drug Incubations with Cultured Cells. In order to study the effects of agents that elevate cyclic AMP on the generation of PFC during various time frames of immune induction, a method for washing drugs from cultured cells, followed by resuspension of cells for the remainder of the culture period was determined. At appropriate time intervals for removal of drugs from culture, 1 ml cultures were gently scraped with a sterile polyethylene policeperson, and the cell suspensions from triplicate cultures were placed into 10 mls of SBSS in a 15 ml centrifuge tube (Corning, #25310). Special care was taken to maintain the PH of the culture media in which cells were suspended, by harvesting only 2 sets of triplicate cultures at a time and each time re-gassing the gas box that contained the cultures. Cells were then centrifuged at 600 x g for 10 minutes at 4°C, supernatants were then removed, cells were resuspended for a second wash in 10 mls of SBSS, and were then re-centrifuged. This washing procedure was found to be effective in reducing the concentration of all agents tested to a "no-effect" level. Following the second

wash and removal of supernatant, the cells were resuspended in fresh complete media to 70% of original culture volume. This suspension was then re-cultured as 0.5 ml cultures in 24-well culture plates (Falcon, #3008) for the remainder of the 96 hour culture period. The 70% resuspension was found to be optimal for reculture when cells were present at an inital density of 1.25 x 10^7 cells/ml (Data not shown). No detectable differences were observed between the magnitude of the responses of 0.5 ml and 1 ml cultures when they were run in parallel. When SRBC were used as antigen, no re-addition was necessay at the time of re-culture; as SRBC were found to pellet with the spleen cells. However, when DNP-Lysine-Ficoll (DLF) was used as an antigen, DLF was re-added at the time of reculture in the same concentration as used at culture initiation. Because washing-resuspension of cultures during various time frames could, in itself, exert significant effects on the induction of humoral immunity, appropriate wash control, in the absence of any added drug, are included.

Cell Viability. Cell viabilities were assessed by the trypan blue exclusion method (Boyse, et al, 1964). Aliquoted cell suspensions were mixed with an equal volume of 0.4% trypan blue (GIBCO, #525) in a test tube. Two samples of each suspension were then counted under phase contrast in an Improved Neubauer hemacytometer (Scientific Products, #B3189-1). All viability data is reported as the average of

duplicate samples not varying by more than 15%. When several samples were assayed for viability, trypan blue was added immediately before making cell counts and, in all instances, was not in contact with the cells for longer than 5 minutes before cell counts were made. Following 96 hours of culture, cells were routinely 25-35% viable, compared to the number of initially cultured cells. However, various manipulations and drug additions could effect cell viabilities, therefore this data is included where it is appropriate. It is important to note that trypan blue assessment of cell viability is here used only as a relative index of cytotoxicity. It has recently been shown that trypan blue exclusion may be a high estimate of cell viability, and that fluorescein diacetate (FDA) may be a better indicator of cytotoxicity (Berenbaum, et al, 1976). Therefore, on a few occasions the trypan blue and the FDA method were compared (Takasugi, 1971). It was found that both methods gave analogous results, therefore the trypan blue method was routinely used with the understanding of its possible limitations.

Expression of Data and Statistical Analysis. Although many of the agents used in this study exert significant cytotoxic effects, no correction for these effects has been made when expressing PFC data. The reason for this is that cytotoxic effects in antigen specific cannot be determined. In most experiments, the effects of agents on cell viability are

shown separately. Instances where cytotoxic agents augment humoral immunity may, therefore, be low estimates of actual drug stimulation. However, cytotoxic effects of such agents may contribute significantly to apparent inhibition of humoral immunity. All data is expressed in terms of PFC per total number of initially cultured cells (PFC/CULTURE), assuming 10^7 cells/culture. All confidence intervals represent standard deviations calculated from the variation seen between replicate slides in the PFC assay on the basis of pooled cultures. In these experiments, comparison between treated versus untreated cultures is made on the basis of dose and time-dependence for effects. Some experiments make single point comparisons, that are justified on the basis that these effects have been consistently observed. In this latter instance, a prototype experiment is shown that yields results that are consistent with those obtained from several experiments.

Effects of Pharmacological Agents on Intracellular Levels of Cyclic AMP in Cultured Spleen Cells. The effects of various agents on intracellular levels of cyclic AMP were determined by incubation of these agents with cultured spleen cells, at varying concentrations for various lengths of time. Conditions were identical to those used for the induction of *in vitro* humoral immune responses, except that no antigen was added and tissue culture flasks were used for cell suspensions (Falcon, #3024). Two ml aliquots were removed and cen-

trifuged at 600 x g for 10 minutes at appropriate time intervals. The supernatant was then discarded, the cell pellet was resuspended in 0.5 ml of sodium acetate PH 4 containing 10^{-4}M isobutyl methyl xanthine (MIX), and then these samples were placed immediately into a boiling water bath for 10 minutes. Samples were then freeze-thawed three times by alternately freezing in an acetone-dry ice bath and thawing in a boiling water bath, to ensure complete cell lysis. Thawed samples were then centrifuged for 15 minutes at 2,000 x g and supernatants were assayed for cyclic AMP concentrations. Cyclic AMP concentrations were assayed by the competitive binding protein method of Gilman (Gilman, 1970). Samples were assayed in duplicate that gave values within the linear range on a cyclic AMP standard curve. A standard curve was determined for each experiment using known concentrations of cyclic AMP (Sigma, #A-9501). A best-fit standard curve was calculated using a Tektronix 31 lab computer. All cyclic AMP data is expressed as the average value obtained from duplicate samples not varying by more than 10%.

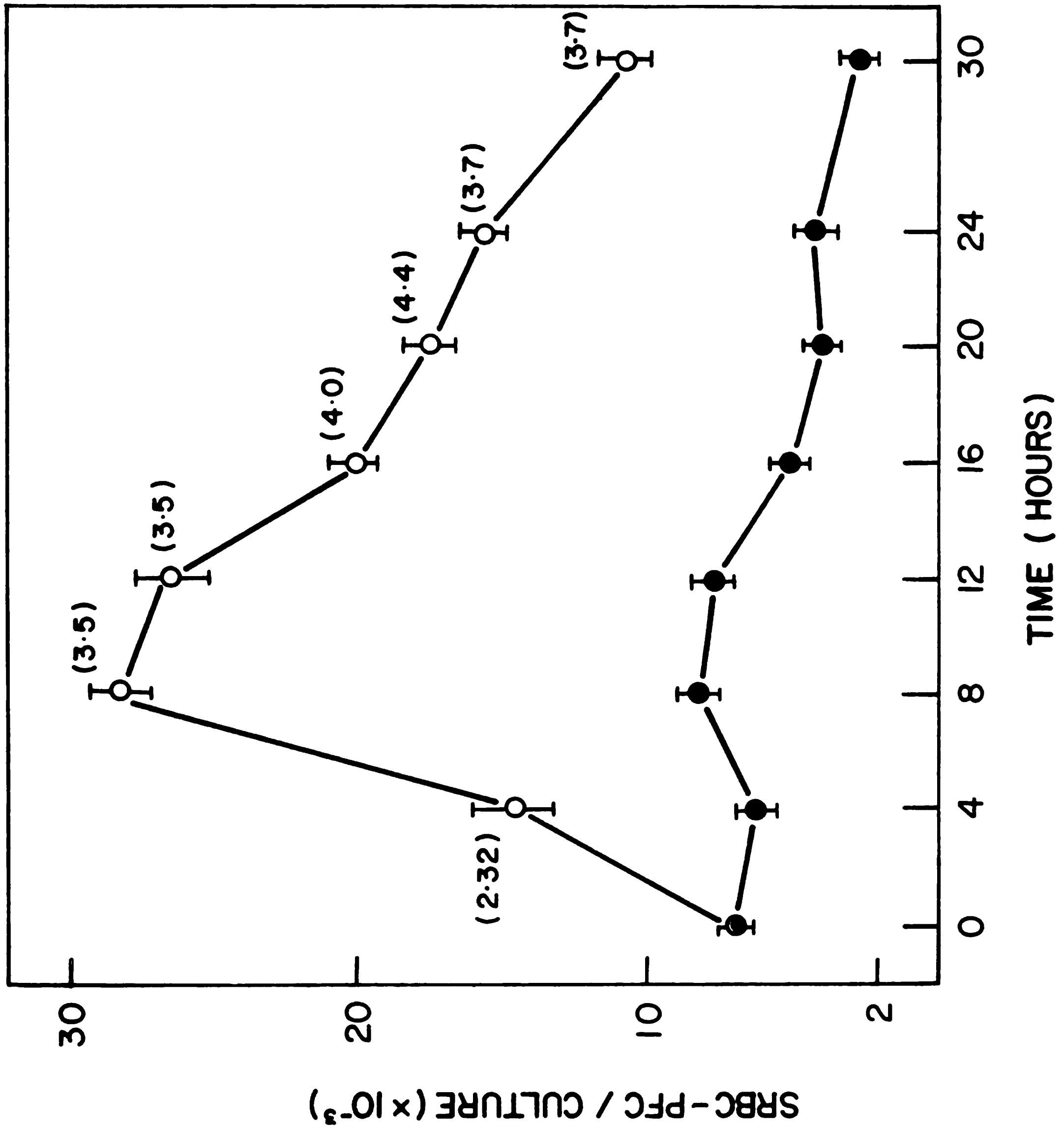
3. Results

Time-Dependent Stimulation of Humoral Immunity by DBcAMP. When present at 5×10^{-4}M, DBcAMP produces a time-dependent stimulation of the PFC response to SRBC (Figure 1). In this

experiment, DBcAMP was added at the initiation of the culture period (time 0) and was washed out at the times indicated. Although DBcAMP produces an apparent maximal effect when it is incubated form hour 0 to hour 8 (ie., hour 0-8), when compared to control washed and resuspended cultures, it is clear that effects equal or greater in magnitude are produced with longer incubations. Comparison of the effects of pharmacological agents on the induction of humoral immunity are facilitated by the expression of the data in terms of a stimulation index (SI). The SI represents the ratio of drug-treated responses to the appropriate control response.

Effects of Washing-Resuspension on PFC Responses. As noted previously, washing-resuspension of cultures can, in itself, exert significant effects on the induction of humoral immunity. The time-dependent inhibition of the SRBC response observed in Figure 1 caused by washing-resuspension is reproducible, and has been observed when other antigens are studied. These inhibitory effects have not been rigorously examined, but, in general, are thought to result from the mechanical disruption of cell-cell interactions necessary for the induction of humoral immunity. That this effect is not due to a cytotoxic effect resulting from cell manipulation is suggested, because recovery of viable cells is not markedly different from that of non-washed cultures. In addition, this inhibition is seen only during intermediate time frames of immune induction (Figure 5). This inhibitory effect is also

Figure 1. Stimulation of the SRBC-PFC response by 5×10^{-4}M DBcAMP, when it is added at the initiation of cultures (time 0) and is washed out at the times indicated (○—○). The effects of washing-resuspension at various times during immune induction is shown (●—●). Parenthesis indicate the stimulation indexes for comparison of drug effects to the appropriate wash controls. Confidence intervals are $\pm$ standard deviations for the PFC assay.

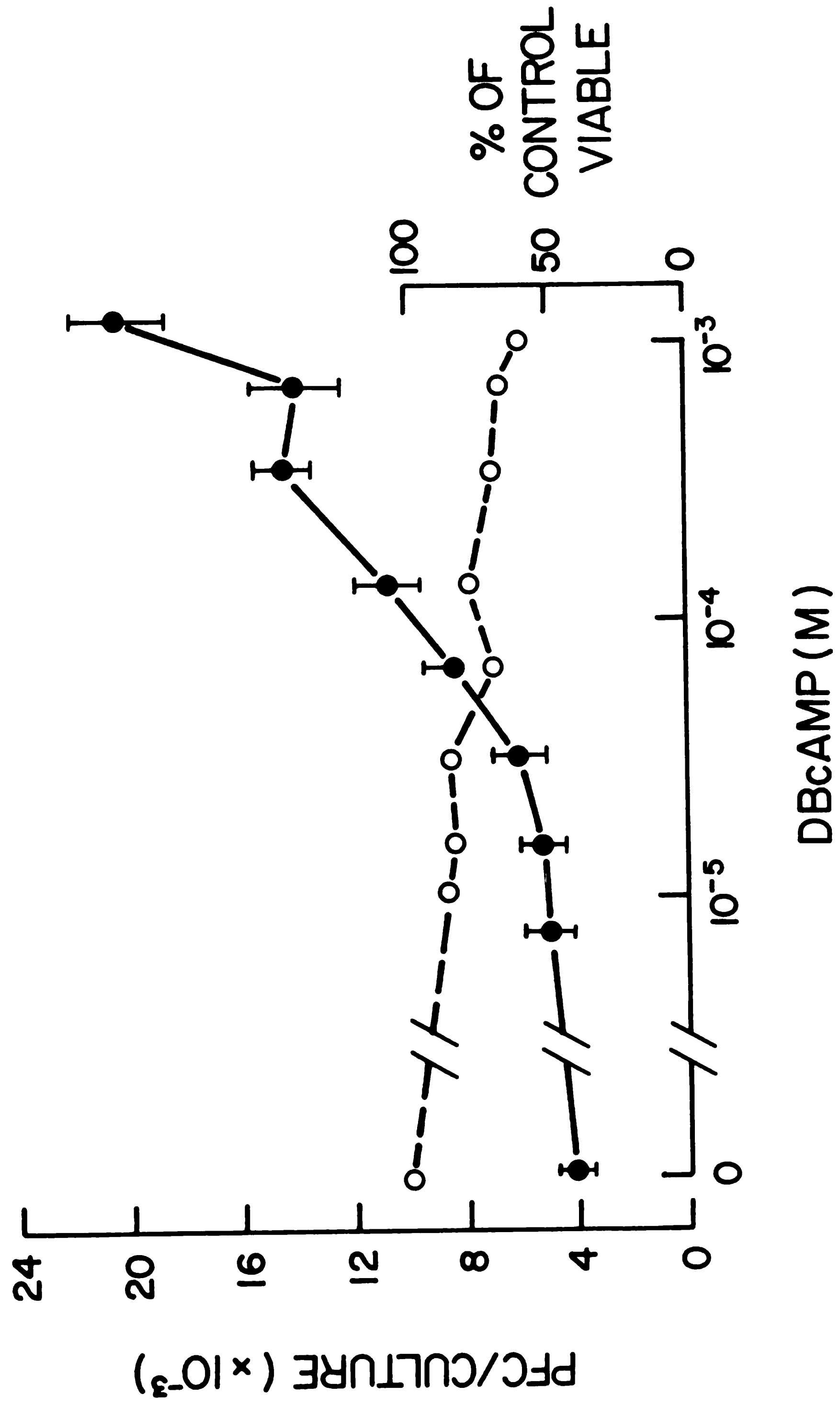

SRBC-PFC / CULTURE (x 10⁻³)
30
20
10
2
TIME (HOURS)
0
4
8
12
16
20
24
30
(2·32)
(3·5)
(3·5)
(4·0)
(4·4)
(3·7)
(3·7)

apparently not antigen dependent, as inhibition is seen regardless of whether antigen is re-added at the time of re-culture, as is the case when DNP-Lysine-Ficoll is used as an antigen (Figure 16).

DBcAMP Produces Dose-Dependent Stimulation of the PFC Response to SRBC. When present at varying concentrations in a 0-18 hour incubation, DBcAMP produces a dose-related increase in the number of PFC to SRBC and also decreases the percentage of recovered viable cells. In this experiment DBcAMP produced a 5-fold stimulation of the SRBC response when present at 10^{-3}M. At this same concentration, DBcAMP causes nearly a 50% decrease in the number of recovered viable cells at hour 96. Because PFC data is expressed in terms of the number of originally cultured cells (PFC/CULTURE), the cytotoxic effects of DBcAMP may reduce the apparent magnitude of stimulation. Expressing PFC data in this manner should be viewed as a conservative estimate of drug effects. Possible explanations for the need of high concentrations (10^{-3}M) of DBcAMP and other exogenous sources of cyclic AMP have been discussed in a previous section.

Stimulation of Humoral Immunity by Agents the Elevate Cyclic AMP. Although DBcAMP has been shown to produce dose-dependent stimulation of humoral immunity during a 0-18 hour incubation, it is necessary to show that other cyclic AMP-elevating agents can produce stimulation during a similar time frame, in order to implicate a possible involvement of

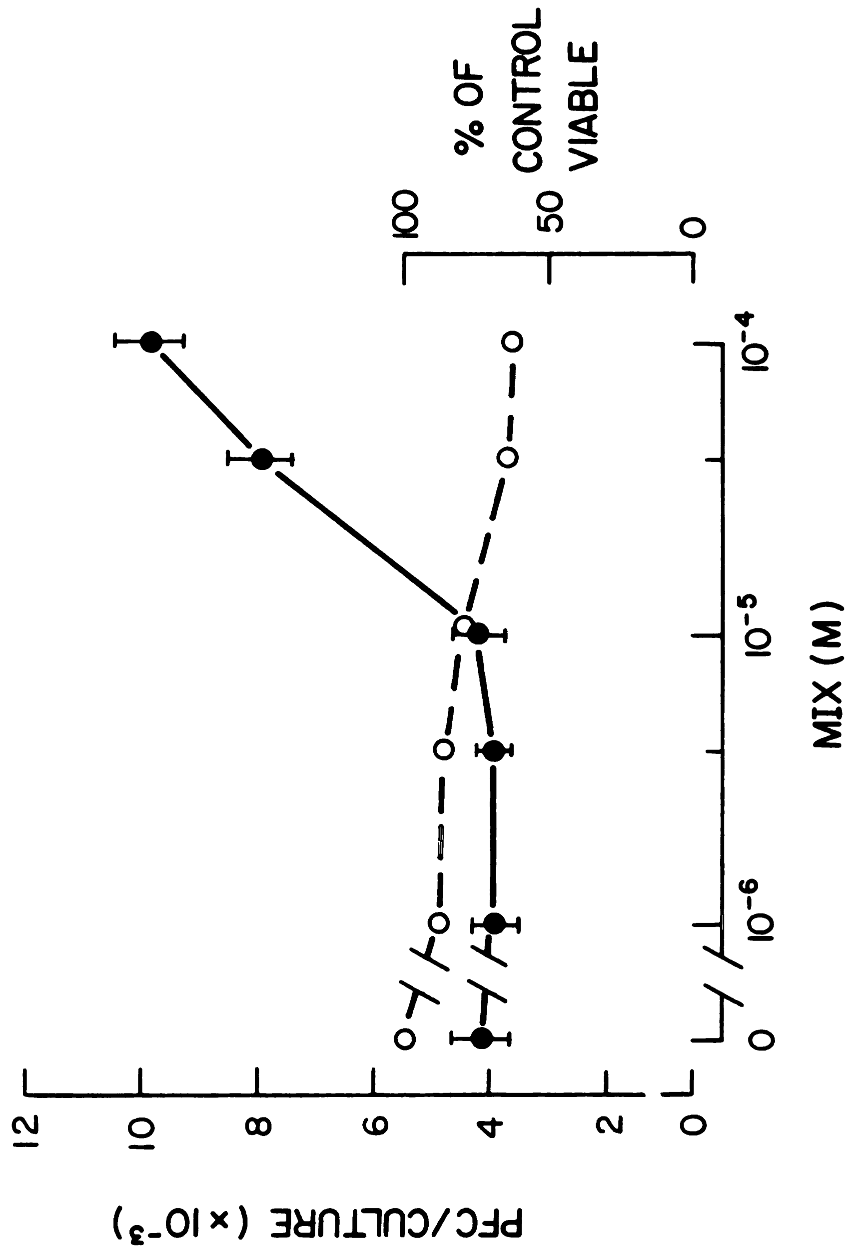
Figure 2. Dose-dependent stimulation of the SRBC-PFC response by DBcAMP, when it is present in a 0-18 hour incubation (●—●). Cytotoxic effects of DBcAMP are expressed as the percentage of control, using average values obtained from duplicate cell counts not varying by more than 15% (O--O). Confidence intervals are ± standard deviations for the PFC assay.


PFC/CULTURE (× 10⁻³)
24
20
16
12
8
4
0
0
10⁻⁵
10⁻⁴
10⁻³
DBcAMP (M)
100
50
0
% OF CONTROL VIABLE

endogenous cyclic AMP in the process. Both isobutyl-methyl xanthine (MIX) and cholera toxin (CT) are shown to produce dose-dependent increases in the generation of PFC to SRBC (Figures 3 and 4). When present during a 0-18 hour incubation, maximal effects of MIX are produced at 10^{-4}M; this concentration being the solubility limit for MIX in the culture medium. At 10^{-4}M, MIX produces a 2.5-fold stimulation of humoral immunity and a 50% decrease in the viable cell recovery (Figure 3). Likewise, cholera toxin causes a 2.5-fold enhancement of the PFC response when present at 10 ng/ml, but its cytotoxic effects are more pronounced than MIX, with only a 25% recovery of viable cells (Figure 4). Although cultures containing CT were washed at hour 18, previous reports indicate that cholera toxin is not readily removed from cultured cells (Bourne, et al, 1973). This may contribute to the increased cytotoxicity seen and may make it difficult to study the time-dependence of CT effects.

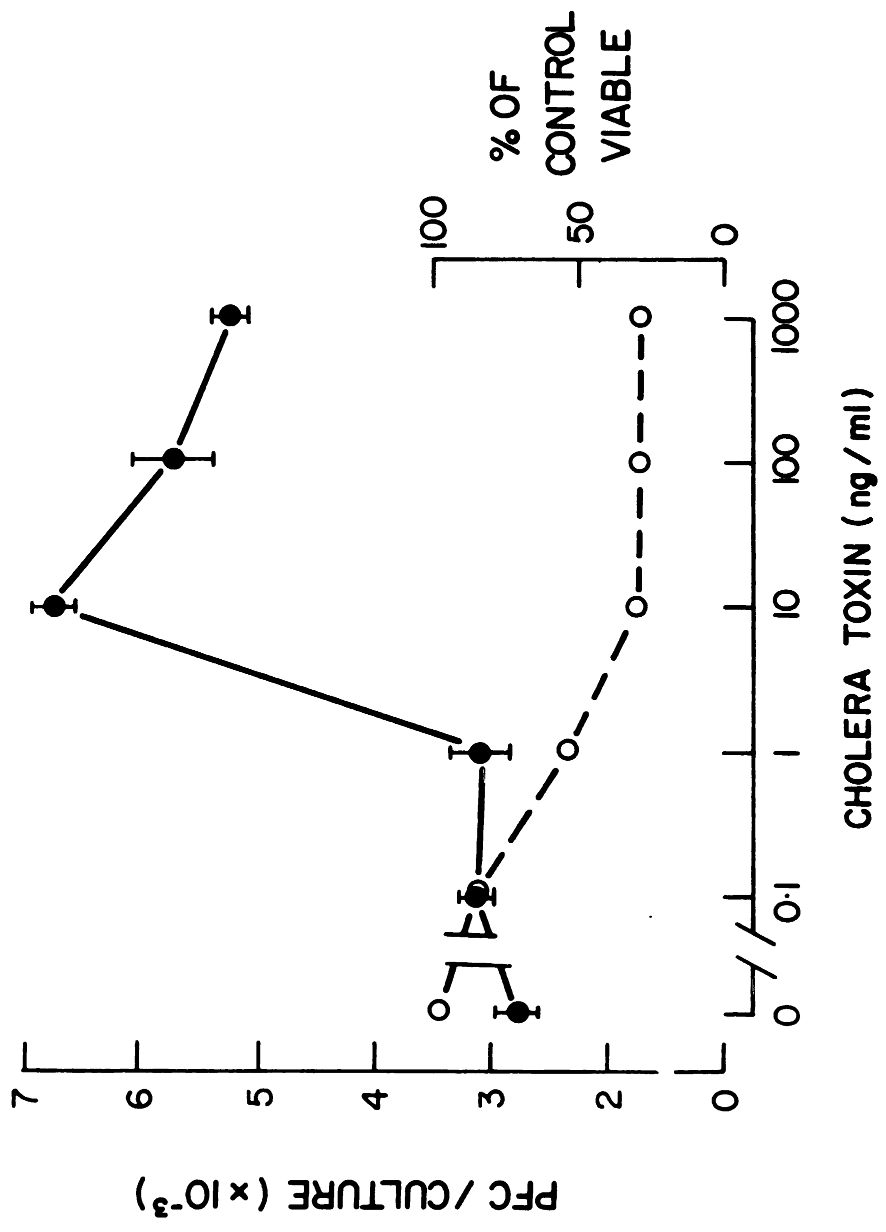
Time-Dependent Stimulation and Inhibition of Humoral Immunity by DBcAMP. DBcAMP has been tested for its ability to affect the induction of a SRBC response by adding it at time 0 and washing it out at various times up to hour 30 (Figure 1). It was of interest to determine the effects of cyclic AMP elevation during various time frames of immune induction throughout the entire culture period. Therefore, for this experiment, DBcAMP was added and removed from culture during specified time periods. The results of this

Figure 3. Dose-dependent stimulation of the SRBC-PFC response by isobutyl-methyl-xanthine (MIX), when it is present in a 0-18 hour incubation (●—●). Cytotoxic effects of MIX are expressed as the percentage of control, using data values obtained from cell counts not varying by more than 15% (○--○). Confidence intervals for the PFC data are ± standard deviations for the PFC assay.



PFC/CULTURE (× 10^-3)
12
10
8
6
4
2
0
0
10^-6
10^-5
10^-4
MIX (M)
100
50
0
% OF CONTROL VIABLE

Figure 4. Stimulation of the SRBC-PFC response by cholera toxin, when it is present during a 0-18 hour incubation (●—●). The dose-dependent cytotoxic effects are shown as the percentage of recovered viable cells, determined by the average of duplicate cell counts not varying by more than 15% (○--○). Confidence intervals are ± standard deviations for the PFC assay.



PFC / CULTURE (x 10^-3)
0
2
3
4
5
6
7
CHOLERA TOXIN (ng/ml)
0
0.1
1
10
100
1000
% OF CONTROL VIABLE
0
50
100

study showed that DBcAMP produced both stimulation and inhibition of humoral immunity. Its effects were dependent upon both the time and total length of exposure. In general, DBcAMP was found to stimulate the generation of SRBC-PFC when it was present during early time periods, such as in hour 0-18 or hour 0-30 incubations. DBcAMP produced inhibition or little effect when it was present during later time frames. From this experiment it can also be seen that cultures are particularly sensitive to washing-resuspension effects during intermediate time periods (see controls for hour 30, 42, and 50).

Because DBcAMP exerts marked cytotoxic effects when it was present during various time frames, it is possible that drug-induced cell death may be responsible for or contribute to the inhibitory effects seen with this agent. A comparison of these two effects can be made by calculating a correlation coefficient for effects on cytotoxicity versus PFC responses. Since early exposure resulted in stimulation of humoral immunity, these time points were not used in determining the relationship between cytotoxicity and inhibition of the SRBC response. Using the data from the 50, 72, and 96 hour time points from Table I, a correlation coefficient of $R=0.7$ was calculated on a Tektronix 31 lab computer. Statitistical analysis of this data shows that this correlation is significant at the $p<0.01$ level for $N=15$. The relationship between cytotoxicity and inhibition of humoral immunity is

TABLE I

Time-Dependent Stimulation and Inhibition of the SRBC-PFC Response by DBcAMP[a]

Time (hours)	Viability (%)	PFC/CULTURE	S.I.[b]
18 Control	100 (33)[c]	1960 ± 360[d]	-
0-18	57	4440 ± 720	2.3
30 (control)	100 (21)	640 ± 80	-
0-30	28	840 ± 160	1.3
18-30	45	280 ± 60	0.4
42 (control)	100 (28)	360 ± 80	-
0-42	18	280 ± 40	0.8
18-42	35	200 ± 40	0.6
30-42	34	560 ± 80	1.6
50 (control)	100 (32)	1000 ± 80	-
0-50	25	400 ± 80	0.4
18-50	28	240 ± 40	0.2
30-50	36	720 ± 20	0.7
42-50	70	840 ± 320	0.8
72 (control)	100 (24)	2440 ± 160	-
0-72	46	680 ± 40	0.3
18-72	17	360 ± 120	0.1
30-72	36	800 ± 80	0.3
42-72	41	1360 ± 400	0.6
50-72	37	2560 ± 80	1.0
96 (control)	100 (31)	2000 ± 280	-
0-96	21	680 ± 180	0.3
18-96	17	280 ± 40	0.1
30-96	23	400 ± 80	0.2
42-96	28	400 ± 120	0.2
50-96	32	760 ± 120	0.4
72-96	58	1440 ± 120	0.7

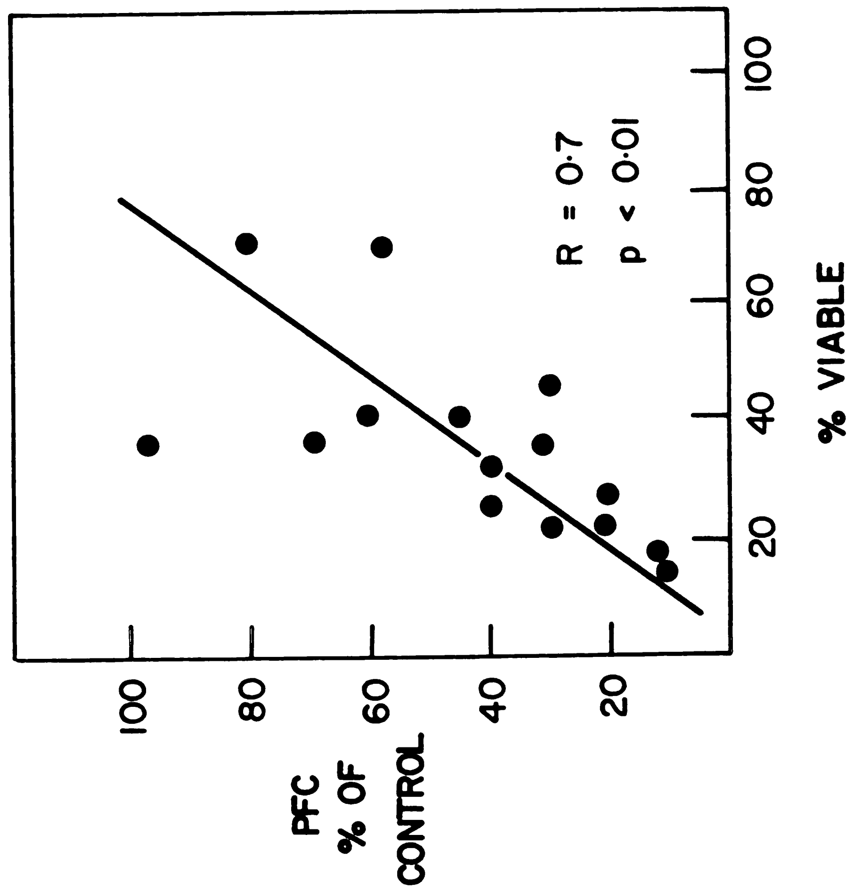
[a] Final concentration of DBcAMP is 5×10^{-4}M

[b] Stimulation index

[c] Parenthesis indicate percent viable of total cultures cells

[d] Standard deviation for the PFC assay

Figure 5. Correlation between the ability of DB-cAMP (5 x 10^{-4}M) to inhibit the SRBC-PFC response and its ability to produce cytotoxicity. Data is taken from Table I, using the 50, 72, and the 96 hour time points. A best-fit curve and a correlation coefficient (R) were calculated on a Tektronix 31 lab computer.


PFC
% OF
CONTROL
100
80
60
40
20
% VIABLE
R = 0·7
p < 0·01

shown in Figure 5, using the computer to determine the best fit curve. On the basis of this comparison it can be seen that a relationship exists between cytotoxicity and inhibition of humoral immunity with DBcAMP. This correlation does not prove that DBcAMP inhibits the SRBC response via a mechanism of cytotoxicity, but it shows that the magnitude of cytotoxicity might be sufficient to account for inhibitory effects. On the basis of these findings, it is difficult to ascribe an immunoregulatory role for cyclic AMP in inhibition of humoral immunity. This conclusion is supported by evidence that shows that while DBcAMP is effective in augmenting humoral immunity *in vivo*, it is not effective in inhibiting this response (Krasny, personal communication).

Differential Stimulation and Inhibition of Humoral Immunity by Cyclic AMP-Elevating Agents. To facilitate the comparison of several cyclic AMP-elevating agents for time-dependent effects on the generation of PFC, three time periods were defined for exposure of cultures to pharmacological agents: 1) an early stage (hour 0-18), 2) a late stage (hour 48-72), and 3) a combined stage (hour 0-96). These time periods were defined on the basis of effects observed with DBcAMP (Table I) that show time-dependent stimulation and inhibition of humoral immunity. In addition, these time periods were chosen because the effects of washing-resuspension were less marked.

All cyclic AMP-elevating agents tested can augment the humoral immune response to SRBC, when they are present during the early stage of immune induction (Table II). The results of this experiment are consistent with the results of several other identical experiments that showed a differential ability of cyclic AMP-elevating agents to augment the SRBC response. From these experiments, it has been found that DBcAMP and 8BrcAMP are most effective in augmenting the PFC response. Isobutyl-methyl-xanthine (MIX) is routinely slightly less effective than DBcAMP and 8BrcAMP, while isoproterenol (ISO) and prostaglandin E_1 (PGE_1) are much less effective in augmenting humoral immunity. The effects of cholera toxin (CT) are variable, perhaps due to the inability to completely remove CT from culture or because of its marked cytotoxicity. On some occasions CT is found to have effects similar in magnitude to MIX (Figure 4), but in other experiments its effects on augmentation of the humoral response are less marked (Table II).

It is notable that dimethyl sulfoxide (DMSO) and 95% ethanol (EtOH), at final concentrations of 0.02%, can each exert stimulatory effects on the induction of humoral immunity to SRBC. These effects have been consistently seen, are dose-related, and must be considered when interpreting the effects of agents which are dissolved in these solvents. For this reason, the effects of MIX and PGE_1 are compared to the appropriate solvent when calculating a stimulation

TABLE II

Time-Dependent Stimulation and Inhibition of Humoral Immunity by Agents that Elevate Cyclic AMP

Treatment	Viability (%)	PFC/CULTURE	S.I.[a]
0-18 (control)	100	920 ± 160[b]	-
DBcAMP (10^{-3}M)	60	4680 ± 600	5.1
8BrcAMP (10^{-3}M)	59	4000 ± 240	4.3
DMSO (.02%)	90	1680 ± 120	-
MIX (10^{-4}M)	72	4360 ± 240	2.6[c]
95% Ethanol(.02%)	100	1560 ± 280	-
PGE_1 (5 x 10^{-6}M)	88	2840 ± 80	1.8[d]
ISO (5 x 10^{-6}M)	100	1360 ± 280	1.5
CT (10 ng/ml)	31	1360 ± 120	1.5
48-72 (control)	100	2360 ± 280	-
DBcAMP	20	1240 ± 240	0.5
8BrcAMP	37	2000 ± 240	0.8
DMSO	62	3040 ± 240	-
MIX	28	920 ± 200	0.3[c]
Ethanol	72	2440 ± 320	-
PGE_1	61	1400 ± 120	0.6[d]
ISO	52	1560 ± 120	0.7
CT	22	1960 ± 80	0.8
0-96 (control)	100	2360 ± 160	-
DBcAMP	33	680 ± 80	0.3
8BrcAMP	40	2680 ± 280	1.1
DMSO	75	1640 ± 160	-
MIX	33	720 ± 80	0.4[c]
Ethanol	63	2520 ± 200	-
PGE_1	51	1720 ± 400	0.7[d]
ISO	53	2320 ± 400	1.0
CT	27	2600 ± 160	1.1

[a] Stimulation Index

[b] Standard deviation for the PFC assay

[c] S.I. compared to the DMSO control

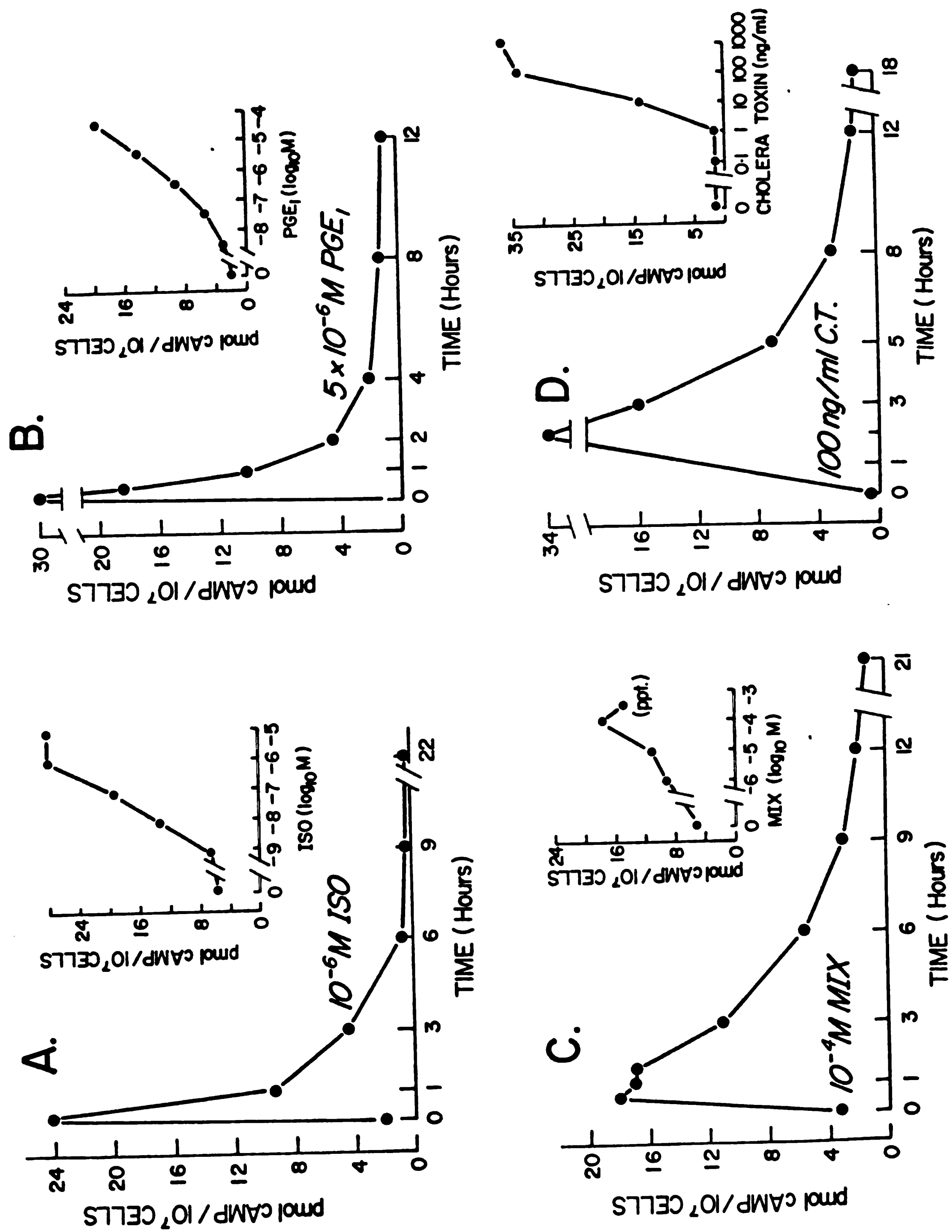
[d] S.I. compared to the Ethanol control

index. The stimulatory effects of DMSO on immune induction in other systems has been previously reported (Burtfield and Goldstein, 1975; Dennis and Wilson, 1975).

When present during the late or combined stages of immune induction, cyclic AMP-elevating agents either inhibit or exert no effect on the SRBC response. Based on the previous suggestion that inhibition of the humoral response with DBcAMP may be related to a cytotoxic mechanism, those agents that produced inhibition were examined for their effects on cell viability. Examination of this relationship reveals that all agents that inhibit the humoral response to SRBC also produce sufficient cytotoxicity that might account for the decreased responsiveness. It is notable that some agents (such as 8BrcAMP) produce significant cytotoxicity, but they are not effective in inhibiting the PFC response.

Differential Effects of Cyclic AMP-Elevating Agents On Cyclic AMP Levels in Cultured Spleen Cells. Several agents that each have the ability to increase cyclic AMP levels by different biochemical mechanisms have been shown to exert time-dependent stimulation of the humoral immune response (Table II). If cyclic AMP is the common denominator for effects produced by these agents, then it should be possible to correlate the differential ability of these agents to affect intracellular levels of cyclic AMP with their differential ability to affect humoral immune induction.

Figure 6. The differential ability of agents that elevate cyclic AMP to affect intracellular levels of cyclic AMP in spleen cells. Dose and time-dependence for affects on cyclic AMP levels by: isoproterenol (ISO), A; prostaglandin E_1 (PGE_1), B; isobutyl-methyl-xanthine, C; and cholera toxin (CT), D. Each point represents the average of two samples, each run in duplicate, and that varied by less than 10%. For experimental conditions, see text.


A.
10^{-6}M ISO
pmol cAMP / 10^7 CELLS
TIME (Hours)
ISO (log_{10} M)
B.
5 x 10^{-6}M PGE_1
pmol cAMP / 10^7 CELLS
TIME (Hours)
PGE_1 (log_{10}M)
C.
10^{-4}M MIX
pmol cAMP / 10^7 CELLS
TIME (Hours)
MIX (log_{10} M)
(ppt.)
D.
100 ng/ml C.T.
pmol cAMP / 10^7 CELLS
TIME (Hours)
CHOLERA TOXIN (ng/ml)

It is again emphasized that any biochemistry done using heterogeneous populations of cells provides only suggestive evidence and interpretations must, therefore, be qualified.

When isoproterenol (ISO), prostaglandin E_1 (PGE_1), isobutyl-methyl-xanthine (MIX), and cholera toxin (CT) are examined for dose and time effects on intracellular levels of cyclic AMP, it is clear that these agents can all produce dose-related increases in cyclic AMP, but that a dichotomy in the time responses exists (Figure 5). While ISO and PGE_1 are found to produce a very transient increase in intracellular concentrations of cyclic AMP, MIX and CT maintain elevation of cyclic AMP levels with a half-life for maximal elevation of approximately 3 hours. ISO and PGE_1 have half-lives for elevation of cyclic AMP of about 1 hour. These results suggest that if the duration of elevation of cyclic AMP levels is important in stimulation of humoral immunity, it is predicted that MIX and CT would be more effective than ISO or PGE_1 in producing effects (See Discussion).

4. Discussion

The purpose of these experiments was to establish an *in vitro* system in which the effects of agents that elevate cyclic AMP could be studied for their ability to modulate

the induction of the humoral immune response. In comparison with previous investigations, the results from these experiments can be characterized as both confirmation and extensions of previous findings.

It is generally agreed that high concentrations of DBcAMP are necessary to affect the induction of humoral immunity (Watson, et al, 1973; Teh and Paetkau, 1974; Kishimoto and Ishizaka, 1976). This present study has confirmed the observations that DBcAMP needs to be present in the dose range of 10^{-4}M to 10^{-3}M to exert effects. Possible explanations for the need for such high concentrations of DBcAMP have been considered in a preceding section.

Previous studies have not systematically compared the effects of various types of agents that elevate cyclic for their effects on the induction of humoral immunity. In addition, no attempt has been made to correlate the effects of agents on cyclic levels with their effects on the humoral response. In this study, agents that elevate cyclic AMP via several different biochemical mechanisms have been tested for their ability to affect both responses. Dose levels that produced maximal, or near-maximal, effects on cyclic AMP accumulation were used to study the effects of cyclic AMP-elevating agents on immune induction.

All agents that have the ability to elevate cyclic AMP that were tested could produce stimulation of the PFC response to SRBC, when they were present during the early

stage of immune induction. However, these agents possessed different abilities to augment humoral immunity. A comparison between those agents that were most capable of producing augmentation with those that were less effective reveals that, in general, those agents which can maintain a more sustained elevation of cyclic AMP levels were most effective in augmenting the PFC response. DBcAMP and 8BrcAMP were most effective in producing augmentation, but their effects on intracellular levels of cyclic AMP can not be directly determined. It is believed that these two agents would be effective in maintaining sustained elevation of cyclic AMP levels. Because MIX and CT have half-lives for elevation of cyclic AMP that are longer than those for ISO and PGE_1, they are more effective in augmenting humoral immunity. These effects are consistent with a temporal comparison for drug effects on the humoral immune response. The final piece of evidence that supports the concept that the duration of elevation is important for stimulation of humoral immunity is shown by the observation that DBcAMP produces sub-obtimal stimulation of the SRBC response when it is present during shortened time periods. Thus, when DBcAMP is present in a 0—4 hour incubation (Figure 1), it produces stimulatory effects that are similar to MIX and CT in magnitude. As MIX and CT can only maintain cyclic AMP elevation for approximately 4 hours following exposure to spleen cells, this observation suggests that these agents do not have differential

effects on the induction of humoral immunity because they affect different populations of cells, but rather that this differential ability results from their ability to maintain sustained elevation of cyclic AMP levels.

The inability of agents such as ISO and PGE_1 to maintain sustained elevation of cyclic AMP levels may result from several mechanisms (See Shear, et al, 1976):

1) A general loss in responsiveness resulting from lymphocyte culture conditions.

2) An agonist-specific loss in responsiveness (tachyphyllaxis).

3) The induction of the cyclic AMP-metabolizing enzyme, phosphodiesterase.

Initial evidence suggests that each of these mechanisms contribute to the inability of ISO and PGE_1 to maintain sustained elevation of cyclic AMP, and, theoretically, their decreased ability to augment humoral immunity (Data not shown).

A limiting piece of information in making such temporal comparisons concerns the affects of CT on the induction of humoral immunity. The results from Table II show that under certain conditions CT produces stimulation of the SRBC response of the same magnitude as ISO and PGE_1. Theoretically, CT should produce stimulatory effects similar in magnitude to MIX. This observation may not necessarily invalidate a temporal comparison for several reasons: 1) the effects of CT are variable, as on some occasions it is found to have

effects that are similar to MIX (See Figure 4), 2) it may be difficult to removed cholera toxin from cultured cells by washing (Bourne, et al, 1973), thus residual amounts of CT may reduce stimulatory effects; and 3) the cytotoxic effects of CT are more pronounced than MIX, perhaps resulting from consideration (2).

The results of the time-dependent studies are, in general, consistent with the results from previous investigations, with a few exceptions. Watson, et al observed that, in a system similar to that used in the present study, the effects of DBcAMP were inhibitory following a 0-10 hour incubation (Watson, et al, 1973). These results are inconsistent with those of this study, but it is believed that residual amounts of DBcAMP, due to inefficient washing, may have resulted in inhibitory effects being seen at such early times. The results from these investigators were consistent with those of this study in showing that stimulation could be produced with shorter incubations of DBcAMP, and that washing of cultures could produce time-dependent inhibition of the PFC response.

Teh and Paetkau studied the time-dependence of aminophylline effects on the primary response to SRBC using Marbrook cultures (Teh and Paetkau, 1974). Their findings were entirely consistent with those of this study, showing a switch from stimulation (hour 0-24) to inhibition (hour 24-48) and a decrease in cell viability from 35-65%, depending upon the

particular time of incubation.

Kishimoto and Ishizaka reached a similar conclusion as that in this present study in defining the effects of agents that elevate cyclic AMP and modulate immune induction on the basis of an early versus a late stage of exposure. Their studies were performed using *in vitro* rabbit lymph node cultures of primed lymphocytes, and they measured secondary anti-hapten IgG responses. Despite these differences, the results of their study were similar to those obtained in this investigation. They found that DBcAMP, theophylline, and PGE_1 all exerted stimulatory effects on the induction of humoral immunity, when these agents were present only during the early stage. No attempt was made to correlate the differential effects of these agents. In addition, they found that ISO had little, if any, effect in their system. Their conclusions were that agents that elevate cyclic AMP probably augment humoral immunity via an effect on B cells, and that inhibition produced during the late stage probably results from inhibition of proliferation of these cells. These latter results will be considered in following sections.

In view of the findings of this and previous studies that show a great similarity in the time-dependence for cyclic AMP effects on the induction of humoral immunity, it seems reasonable to conclude that cyclic AMP may be an important modulatory agent in the induction of humoral im-

mune responses. The evidence that cyclic AMP-elevating agents produce cytotoxic effects on cultured lymphocytes, and that the magnitude of these effects may be sufficient to account for inhibitory effects produced during late time periods, makes it difficult to ascribe a role for cyclic AMP in modulating the late stage of immune induction. However, all available evidence supports the concept that cyclic AMP may modulate the early induction of humoral immunity.

In conclusion, an *in vitro* system has been established that allows for the study of the effects of pharmacological agents during various time frames of immune induction. The results of this study are consistent with those of previous investigations in showing that agents that elevate cyclic AMP exert time-dependent effects on the induction of humoral immunity. Strong evidence for a modulatory role for cyclic AMP in immune induction is provided by the observations that the differential ability for agents to elevate cyclic AMP levels can be correlated with their differential ability to affect the humoral immune response. Studies to follow will investigate the mechanism(s) by which elevation of cyclic AMP levels during the early stage of immune induction results in augmentation of humoral immunity.

B. Inhibition of Proliferation: A Possible Mechanism of Modulation of Humoral Immunity by Agents that Elevate Cyclic AMP.

1. Introduction-Purpose

In the preceding section it was shown that elevation of cyclic AMP concentrations during an early time period of humoral immune induction resulted in augmentation of the PFC response to SRBC. It was found that those agents that were most capable of maintaining elevated cyclic AMP levels were, in general, most effective in augmenting humoral immunity. In addition, agents such as DBcAMP that can, presumably, maintain sustained elevation of cyclic AMP, were most most effective in producing augmentation when they were present for approximately 18 hours at the initiation of the culture period.

This section deals with the possible mechanism(s) by which cyclic AMP elevation during the early stage of immune induction produces augmentation of the SRBC-PFC response. As previously discussed, cyclic AMP is believed to play an important role in the modulation of cell differentiation and proliferation in lymphocytes and other cells. The maintenance of high intracellular levels of cyclic AMP in cultured spleen cells is likely to exert effects on both differentiation and proliferation of these

cells. The purpose of these experiments was to determine if inhibition of proliferation was a mechanism by which agents that elevate cyclic AMP might augment humoral immunity. The strategy for testing this hypothesis was to test various anti-proliferative agents in comparison with DBcAMP for their effects on the induction of the SRBC primary response _in vitro_. Those anti-proliferative agents tested were believed to fulfill the following criteria:

1) They could exert anti-proliferative efffects _in vitro_;
2) Anti-proliferative effects were exerted via different biochemical mechanisms;
3) Effects were produced at different stages of the cell cycle; and
4) Anti-proliferative effects were produced independent of the cyclic AMP system.

A fifth possible criteria might have been that these agents exerted reversible inhibition of proliferation, but this would have been difficult to assess, as anti-proliferative agents exert significant cytotoxic effects.

Agents believed to fulfill the preceding criteria were: 1) colchicine and colcemid (inhibit the mitotic stage of the cell cycle, ie., anti-M), 2) cytosine-arabinoside (inhibits the stage of DNA synthesis, ie., anti-S), and 3) hydroxy urea (anti-S). A fourth agent tested in the following experiments was high specific activity ^{3}H-thymidine (anti-S). Because ^{3}H-thymidine inhibits DNA synthesis via destruction

of DNA, its effects would clearly be irreversible. Such an agent might be useful in providing information concerning the need for reversible inhibition of proliferation.

It should be realized at the outset that while all of these agents may exert anti-proliferative effects, they may also have other effects on the induction of humoral immunity. Although differences in the magnitude of effects are to be expected with these various agents, should all of these agents exert consistent effects on the induction of humoral immunity, such data would provide strong evidence for an anti-proliferative mechanism of augmentation of the humoral immune response.

2. Materials and Methods

The materials and methods used in this section were identical to those used in the preceding section, with the following additions:

Pharmacological Agents. All agents were prepared as sterile or near-sterile 50X stock solutions immediately before each use. Filter sterilization was performed as previously described, as indicated. Crystalline colchicine (Sigma, #C-9754) and colcemid (GIBCO, 30144) were dissolved in SBSS before each use to obtain a 50X stock

solution. Cytosine-arabinoside (Cal-Biochem, #251010) was maintained at -20 C in a dessicator and was dissolved in SBSS to achieve a 50X stock solution. The cytosine-arabinoside was then filter-sterilized with pre-washed Millipore filters. Hydroxy urea (Sigma, #H-8627), thymidine (Sigma, #T-9250), and 2-deoxycytidine (Sigma, #D-0883) were handled in a manner identical to that for cytosine-arabinoside. High specific activity ^{3}H-thymidine was obatined from New England Nuclear (NET-027X, 20 Ci/mmol) and was stored at -20 C. ^{3}H-thymidine was diluted to a 50X stock immediately before each use in SBSS. All safety procedures for working with radioactive materials were observed. Following incubation and washing of ^{3}H-thymidine from cultured cells, "cold" thymidine (100 mcg/ml) was added to resuspended cultures to help displace ^{3}H-thymidine from cellular thymidine pools (Dutton and Mishell, 1967).

3. Results

Effects of Colchicine, when Present During the Early Stage of Immune Induction, on the SRBC-PFC Response. In a preceding section (Section A), it has been shown that agents that have the ability to elevate intracellular levels of cyclic AMP can augment the induction of humoral immunity, when they are present during an early stage of immune induction. To test the hypothesis that these agents may pro-

duce their stimulatory effects via an anti-proliferative mechanism, several anti-proliferative agents were tested for their ability to augment the SRBC response during an early stage of immune induction. Colchicine is shown to produce a dose-dependent stimulation and inhibition of the SRBC-PFC response, when it was present in a 0-8 hour incubation (Figure 7). A dose of 5×10^{-7}M colchicine was found to produce maximal stimulation, whereas higher doses resulted in inhibition. As colchicine produced dose-dependent cytotoxic effects, such effects probably account for the inhibition observed at the higher doses. It is notable that cytotoxicity is observed at the doses that produce augmentation of the SRBC response. Cytotoxic effects may reduce the stimulatory effects of colchicine at the intermediate doses.

Dose-Dependent Stimulation of the SRBC-PFC Response by Colcemid. In an attempt to study the effects of colchicine-like agents for their effects on immune induction, but in the absence of marked cytotoxic effects, colcemid was examined for its effects on the SRBC response. In this experiment, colcemid was found to produce dose-dependent stimulation of the SRBC-PFC response, when it was present in a 0-18 hour incubation (Figure 8). Maximal (3-fold) augmentation was seen at a dose of 5×10^{-6}M. At this dose level a 50% decrease in the number of recovered viable cells was observed. It is notable that although colcemid

Figure 7. Dose-dependent effects of colchicine on the induction of the SRBC response, when present in a 0-8 hour incubation (●——●). Cytotoxic effects of colchicine are compared to the 8 hour wash control, using an average value obtained from duplicate cell counts that didn't vary by more than 15% (○--○). Confidence intervals are ± standard deviations for the PFC assay.

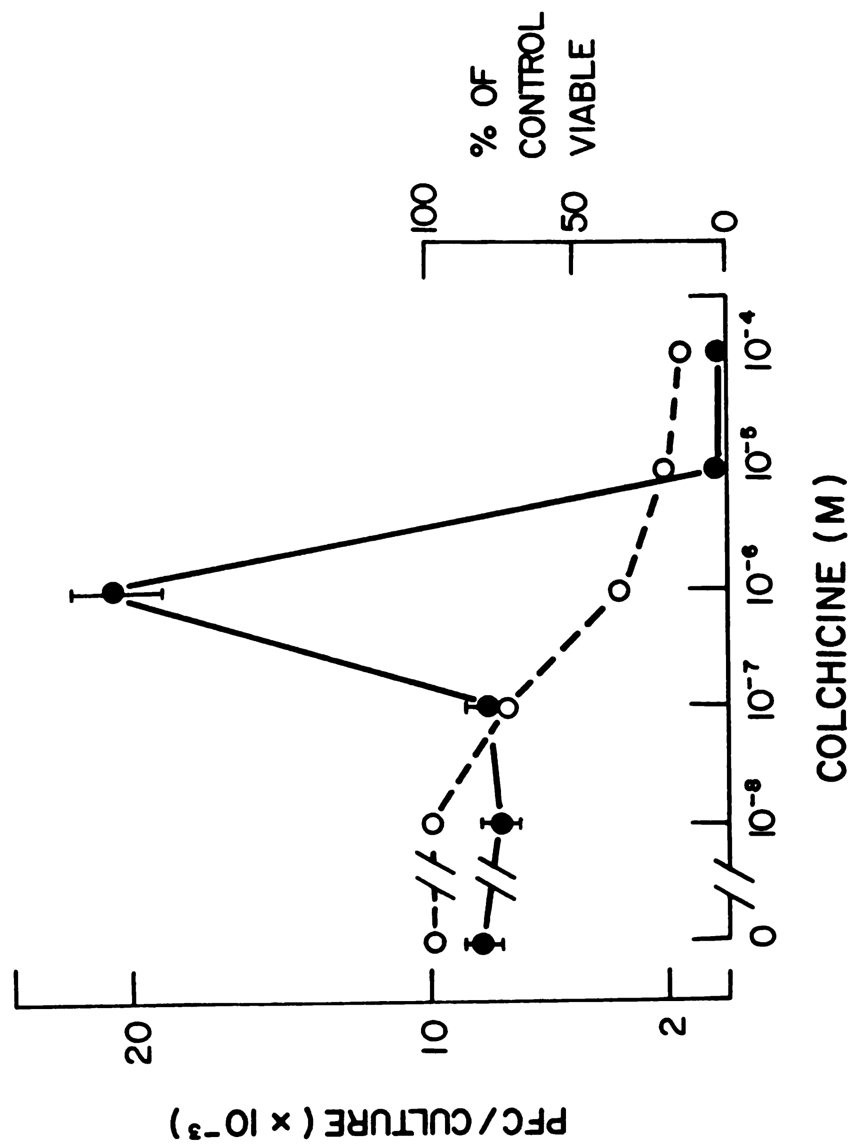
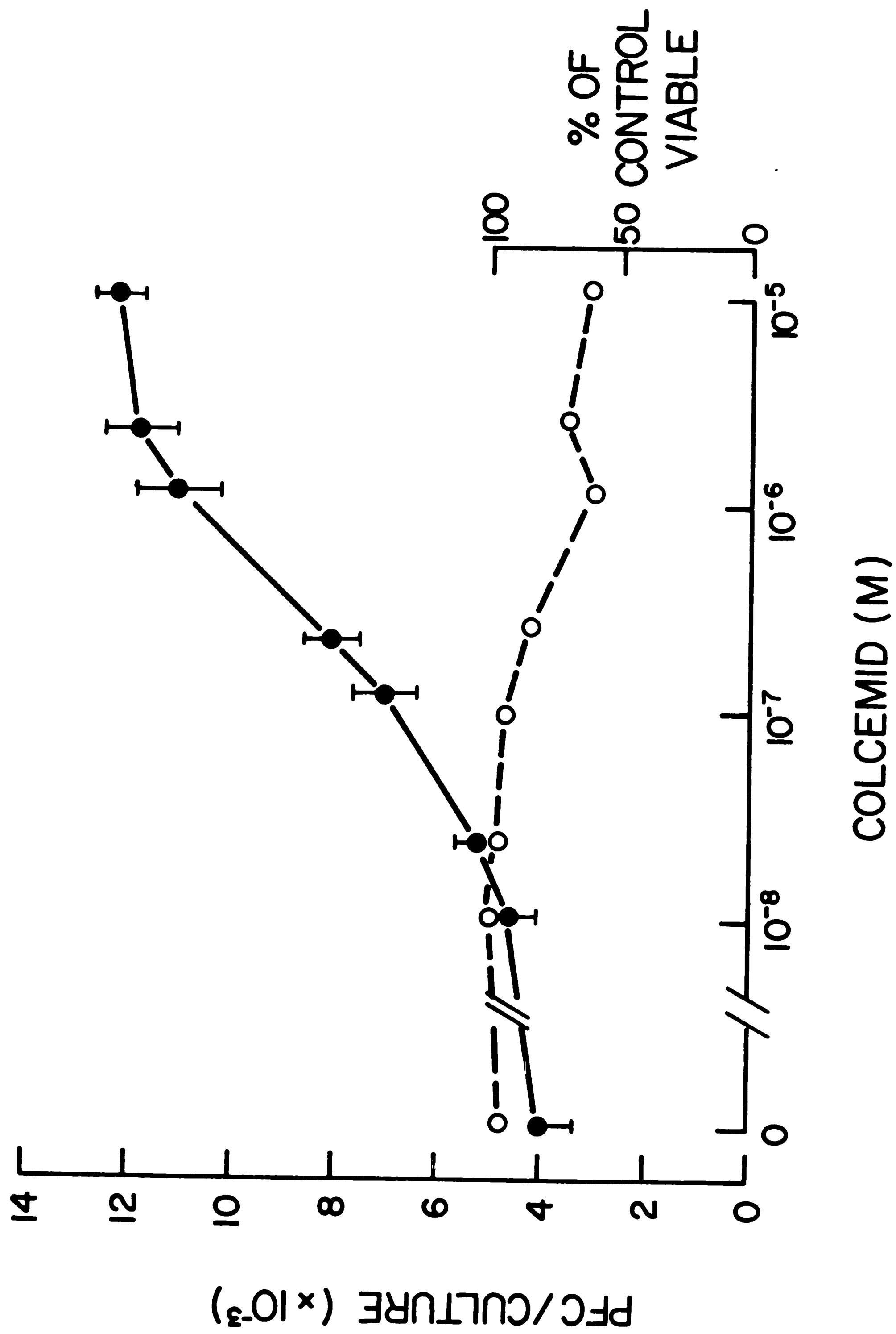

PFC / CULTURE (x 10^-3)
20
10
2
0
10^-8
10^-7
10^-6
10^-5
10^-4
COLCHICINE (M)
100
50
0
% OF
CONTROL
VIABLE

Figure 8. Dose-dependent stimulation of the SRBC-PFC response by colcemid, when present during a 0-18 hour incubation (●——●). Cytotoxicity is expressed as the percentage of control of recovered viable cells, obtained from duplicate cell counts not varying by more than 15% (○--○). Confidence intervals are ± standard deviations for the PFC assay.

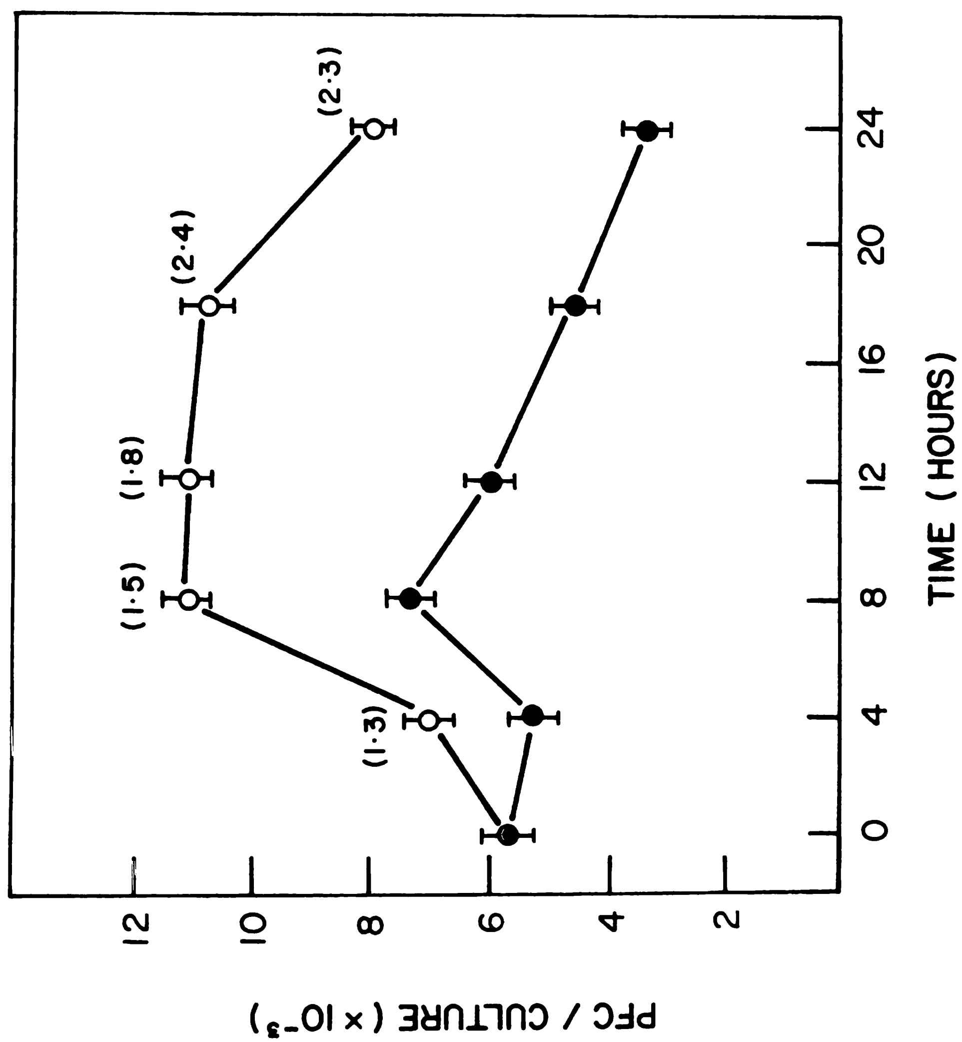


PFC/CULTURE (x 10^{-3})
0
2
4
6
8
10
12
14
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
COLCEMID (M)
0
50
100
% OF CONTROL VIABLE

produces cytotoxicity, at the higher concentrations maximal stimulation of the PFC response is maintained; whereas with colchicine, inhibition was observed at the higher dose levels.

Time-Dependent Stimulation of the SRBC-PFC Response by Colcemid. Because the time frame (hour 0-18) during which the effects of colcemid were studied in Figure 8 were arbitrarily defined on the basis of experiments performed with DBcAMP (Table I), the effect of 5×10^{-6}M colcemid, when added and removed at various times, was examined (Figure 9). In agreement with the results of previous experiments, washing-resuspension was found to produce time-dependent inhibition of humoral immunity (Compare Figure 9 with Figure 1 and Table II). Therefore, drug effects must be compared to appropriate wash controls (ie., compare stimulation indexes). The results of this experiment show that maximal stimulation with colcemid was produced during a 0-18 hour incubation. It is notable that the time-dependence for colcemid effects are very similar to that observed for DBcAMP (Figure 1). In comparison, little stimulation is seen with a short incubation, but longer incubations (such as hour 0-18 or 0-24) are found to exert maximal effects. Colcemid was found to produce a similar degree of enhancement as colchicine, when it was present in a 0-8 hour incubation (Compare Figure 9 with Figure 7).

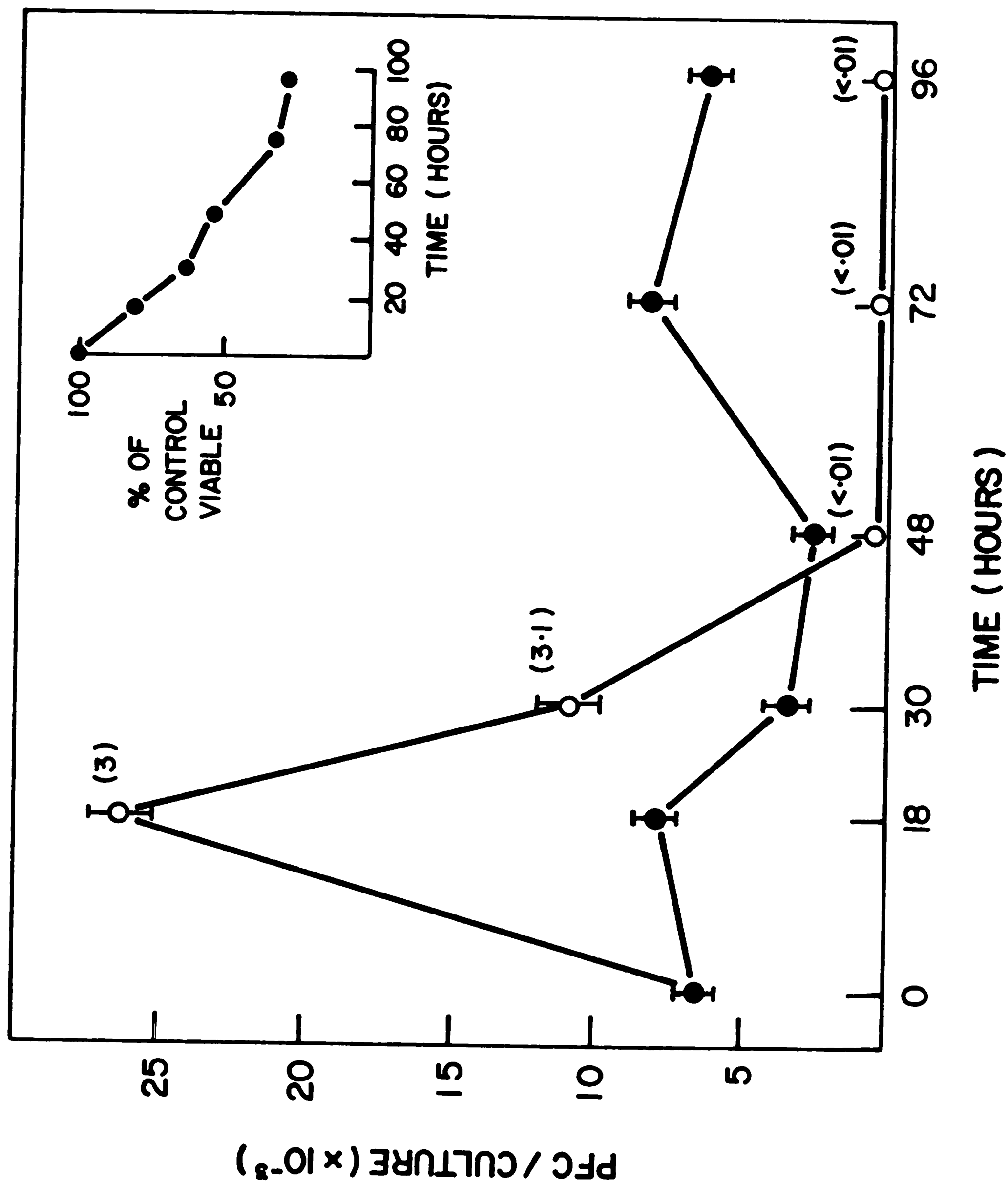
Figure 9. Time-dependence for stimulation of the SRBC response, when colcemid is added at the initiation of cultures (time 0) and is washed out at the times indicated (○—○). The effects of washing-resuspension of cultures at various times is shown (●—●). Parenthesis indicate stimulation indexes for drug effects compared to the appropriate wash control. All confidence intervals reflect standard deviations for the PFC assay.


PFC / CULTURE (× 10⁻³)
12
10
8
6
4
2
0
4
8
12
16
20
24
TIME (HOURS)
(1·3)
(1·5)
(1·8)
(2·4)
(2·3)

Time-Dependent Stimulation and Inhibition of the SRBC-PFC Response by Colcemid. Colcemid has been shown to stimulate the generation of SRBC-PFC, when it was present during an early stage of immune induction (Figure 9). The time-dependence for colcemid effects, when present at various times throughout the entire culture period were next examined (Figure 10). When added at the initiation of cultures at a concentration of 5×10^{-6}M, colcemid produced time-dependent stimulation and inhibition of the humoral immune response to SRBC. Exposure during early time periods (hour 0-18 or 0-30) resulted in stimulation, whereas exposure during later times (hour 0-48, 0-72, or 0-96) resulted in near-complete inhibition. Cytotoxic effects of colcemid were also shown to be time-dependent (See Figure 10 insert), but these effects were not of sufficient magnitude to account for the inhibitory effects of this agent.

The time-dependence for colcemid effects are very similar to those observed for DBcAMP (Table I). In subsequent experiments, the addition of colcemid at various times during the induction of the PFC response to SRBC, and its removal at various times, yielded results analogous to those obtained with DBcAMP (Data not shown). The main difference between the effects of colcemid and DBcAMP were that DBcAMP is more effective in producing stimulation, whereas colcemid was more effective in producing in-

Figure 10. Time-dependent stimulation and inhibition of the humoral immune response to SRBC by colcemid (O—O). Colcemid was added at the initiation of cultures (time 0) and was washed out at the times indicated. The effect of washing-resuspension is shown (●—●). Parenthesis indicate stimulation indexes for the comparison of drug effects to appropriate wash controls. Confidence intervals are ± standard deviations for the PFC assay. Time-dependent cytotoxic effects are shown in the insert. Each point represents the average of duplicate cell counts not varying by more than 15%.



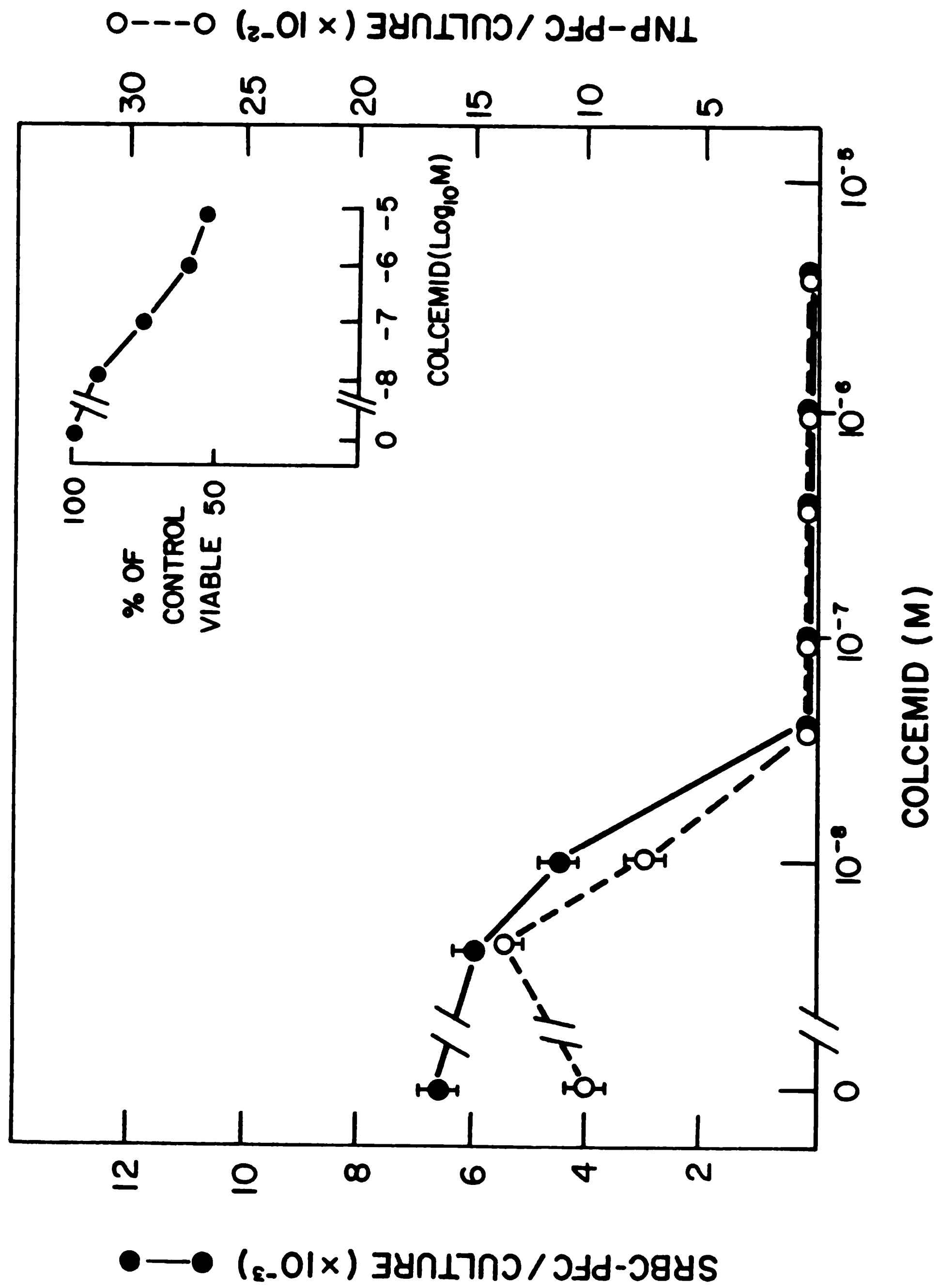
PFC / CULTURE (× 10⁻³)
25
20
15
10
5
0
18
30
48
72
96
TIME (HOURS)
(3)
(3·1)
(<·01)
(<·01)
(<·01)
% OF CONTROL VIABLE
100
50
20 40 60 80 100
TIME (HOURS)

hibition. The relative ability of anti-proliferative agents to produce stimulation versus inhibition are compared in a following experiment (Table III).

Dose-Dependent Inhibition of Humoral Immunity by Colcemid. A comparison of the dose-dependence for inhibition of the humoral immune response to SRBC and to DNP-Lysine-Ficoll (To be discussed later) PFC responses is shown in Figure 11. The results of this experiment show that when colcemid is present throughout the entire culture period it produces complete inhibition of the PFC response at a dose of 5×10^{-7}M. Cytotoxic effects (See Figure 11 insert) are insufficient in magnitude to account for inhibition of humoral immunity. From this experiment, a "no-effect" level of colcemid in the Mishell-Dutton culture system can be observed. As maximal inhibition of the PFC response is seen at 5×10^{-7}M, and no effect is seen at a dose of 5×10^{-8}M, washing of drug from cultures must be effective in reducing the concentration of colcemid to a "no-effect" level, when studying stimulatory effects produced by colcemid at higher doses during the early stage of immune induction.

Dose-Dependent Stimulation of the SRBC-PFC Response by Cytosine-Arabinoside. The finding that both DBcAMP and colcemid can both augment the SRBC response, when they are present during a 0-18 hour incubation, prompts the investigation of other putative anti-proliferative agents for their

Figure 11. Dose-dependent inhibition of the humoral immune response to SRBC (●—●) and DNP-Lysine-Ficoll (○—○), when colcemid is present throughout the entire culture period (hour 0-96). Confidence intervals are ± standard deviations for the PFC assay. The insert shows the cytotoxicity dose-response for colcemid in SRBC cultures, expressed as the percentage of control. Each value represents the average of duplicate cell counts not varying by more than 15%.



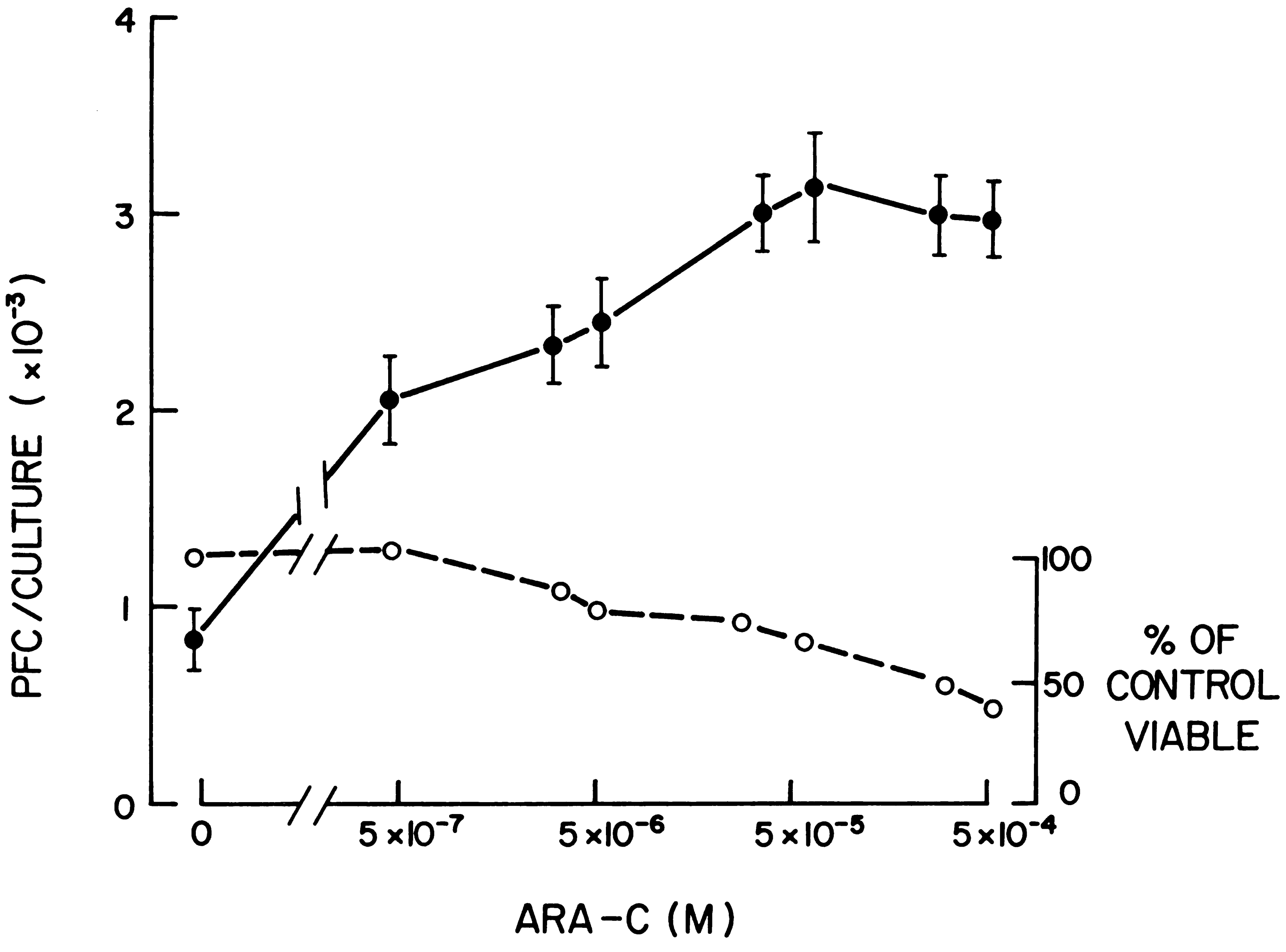
SRBC-PFC / CULTURE (×10^{-3}) ●—●
TNP-PFC / CULTURE (× 10^{-2}) o---o
COLCEMID (M)
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
2
4
6
8
10
12
5
10
15
20
25
30
% OF CONTROL VIABLE
100
50
0
-8
-7
-6
-5
COLCEMID (Log$_{10}$M)

ability to exert similar effects. Cytosine-arabinoside (ARA-C) is shown to produce dose-dependent stimulation of the SRBC-PFC response, when it is present from hour 0-18, and to cause a 50% decrease in the number of recovered viable cells (Figure 12). Thus, the effects of ARA-C are consistent with the effects of DBcAMP and colcemid in stimulating the humoral immune response to SRBC during an early time period.

Time-Dependent Stimulation of the SRBC-PFC Response by Cytosine-Arabinoside. When the effect of 5 x 10^{-5}M ARA-C added at time 0 and washed out at various times was investigated, it was found that maximal enhancement was produced from a 0-18 hour incubation (Figure 13). The time-dependence for ARA-C effects are very similar to those observed for colcemid (Figure 9) and DBcAMP (Figure 1), in augmenting the SRBC response. In the ARA-C experiments, the addition of 2-deoxycytidine to the culture resuspension media was not found to have advantage in reversing the effects of ARA-C; therefore, it was not included (Data not shown).

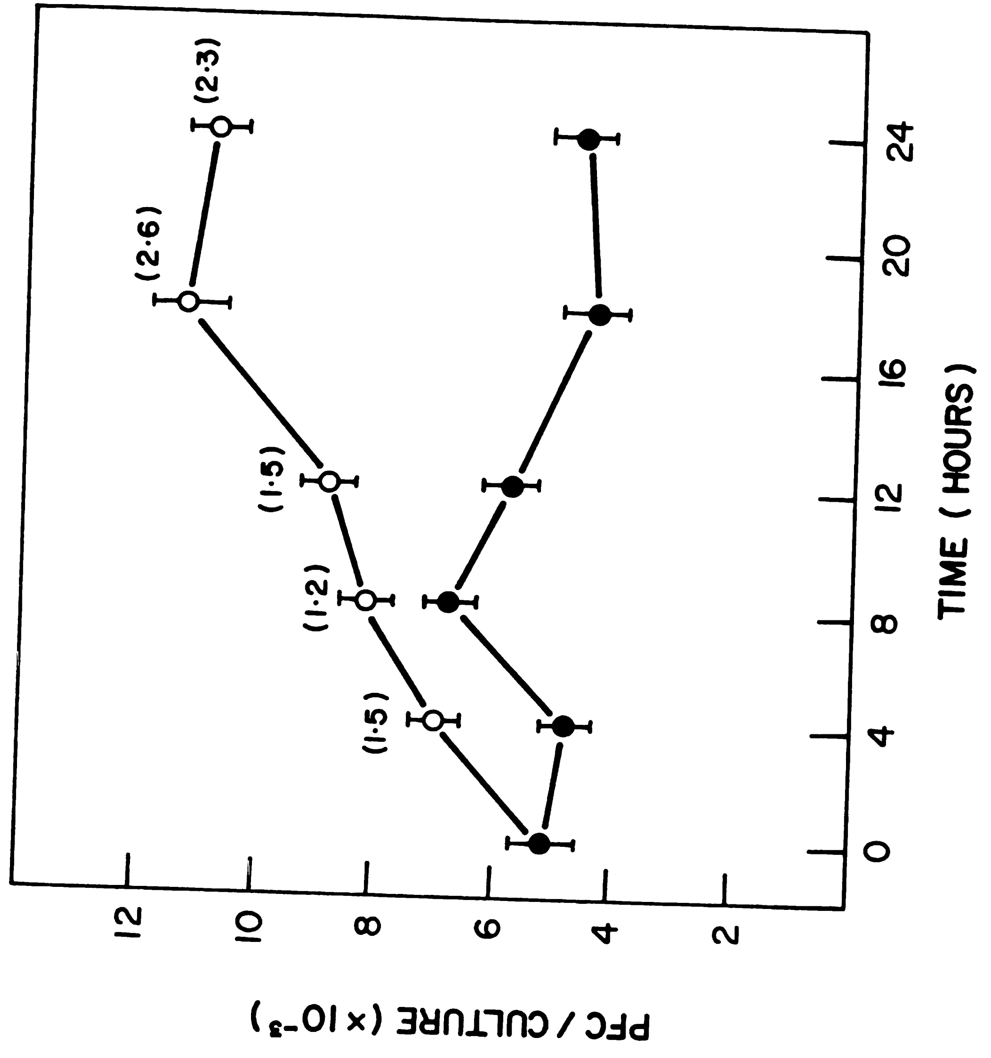
Hydroxy urea Produces a Small Dose-Dependent Increase in the Number of SRBC-PFC. Under conditions that were identical to those shown to produce stimulation of the SRBC-PFC response with DBcAMP, colcemid, and ARA-C, hydroxy urea (HU) was found to exert a small dose-dependent increase in the number of SRBC-PFC (Figure 14). At a dose of 10^{-3}M, HU

Figure 12. Stimulation of the SRBC-PFC response by various doses of cytosine-arabinoside (ARA-C), when present in a 0-18 hour incubation (●——●). Cytotoxicity produced by ARA-C at each dose is expressed as the percentage of control, based upon duplicate cell counts not varying by more than 15% (○--○). Confidence intervals are ± standard deviations for the PFC assay.



PFC/CULTURE (×10⁻³)
4
3
2
1
0
0
5×10⁻⁷
5×10⁻⁶
5×10⁻⁵
5×10⁻⁴
ARA-C (M)
100
50
0
% OF CONTROL VIABLE

Figure 13. Time-dependent stimulation of the humoral immune response to SRBC by cytosine-arabinoside (ARA-C) when added at the initiation of cultures (time 0) and washed out at the times indicated (○——○). The effect of washing of cultures at various times (●——●). Parenthesis indicate stimulation indexes for comparing ARA-C effects to appropriate wash controls. Confidence intervals are ± standard deviations for the PFC assay.

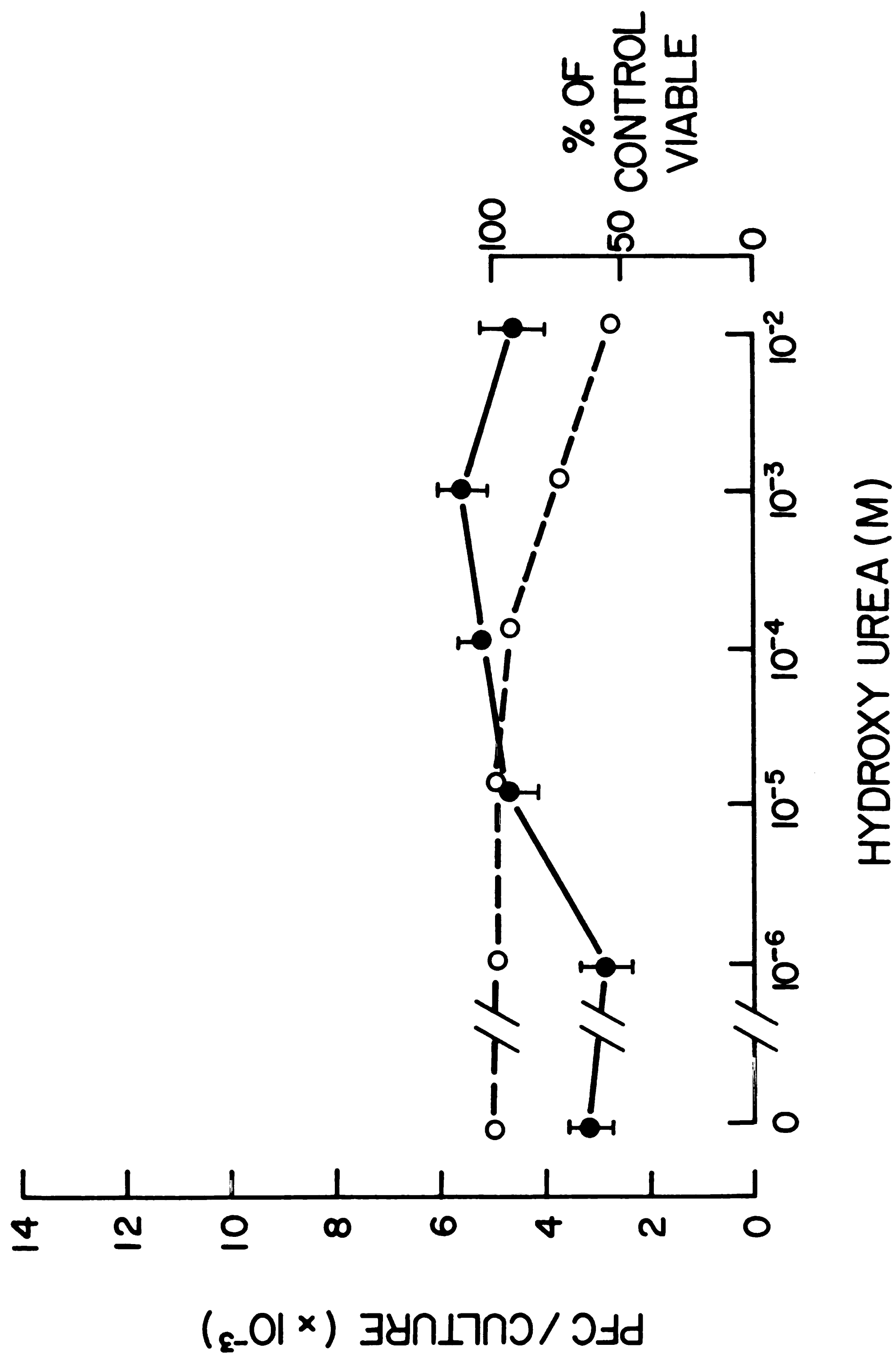

PFC / CULTURE (× 10⁻³)
12
10
8
6
4
2
TIME (HOURS)
0
4
8
12
16
20
24
(1·5)
(1·2)
(1·5)
(2·6)
(2·3)

produced a 50% increase in the number of SRBC-PFC, while producing a 40% decrease in the number of recovered viable cells. The dose-dependence for HU effects show that it can produce effects that are consistent with an anti-proliferative hypothesis for stimulation of humoral immunity.

Effects of ^{3}H-Thymidine on the Induction of Humoral Immunity to SRBC. Dutton and Mishell have shown that ^{3}H-thymidine inhibits the *in vitro* humoral immune response via a mechanism of inhibition of proliferation; therefore, the effects of this agent were tested for its effects on the induction of humoral immunity. When present at a concentration of 10 mcCi/ml in a 0-18 hour incubation, ^{3}H-thymidine produced a small dose-related increase in the number of SRBC-PFC. The doses of ^{3}H-thymidine that produced enhancement are similar to those that Dutton and Mishell showed that could inhibit proliferation (Dutton and Mishell, 1967). Thymidine (100 mcg/ml) was added to resuspended cultures following wash-out of ^{3}H-thymidine. Because thymidine can, on its own, exert effects on humoral immunity, the effects of ^{3}H-thymidine must be compared to an appropriate thymidine control.

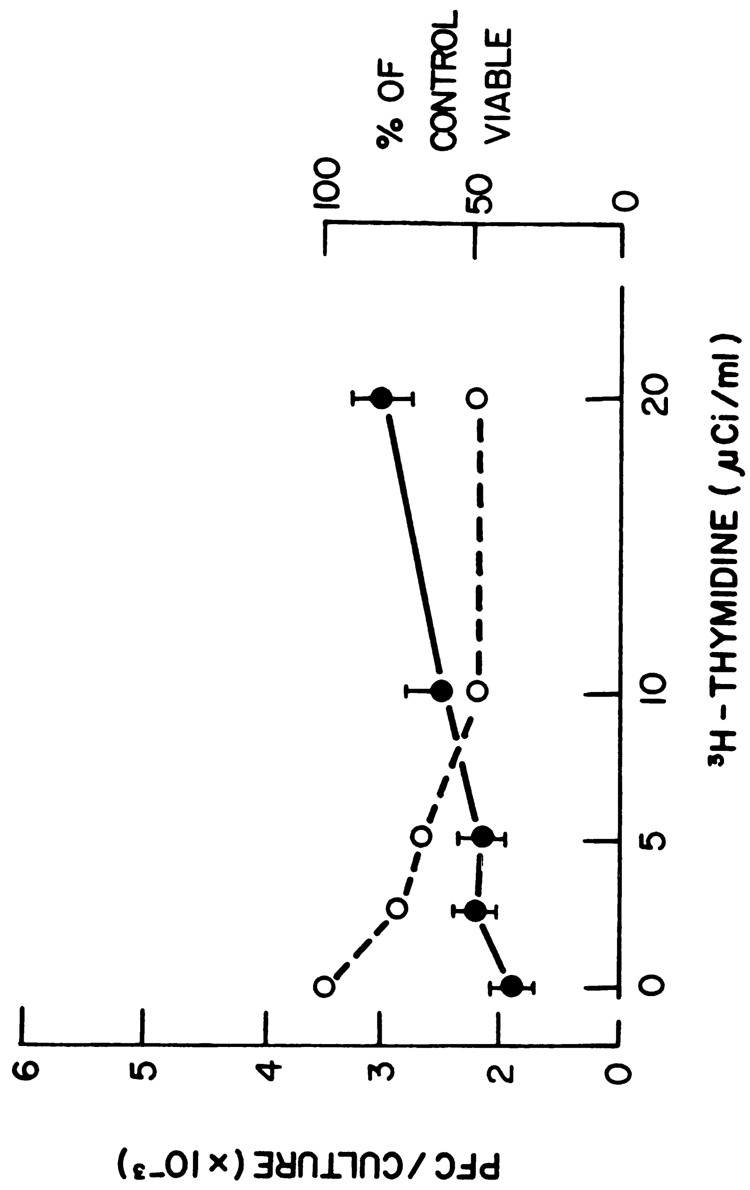
Time-Dependent Stimulation and Inhibition of the SRBC-PFC Response by Anti-Proliferative Agents. For the purpose of comparison of the effects of the various anti-proliferative agents on the induction of the SRBC response,

Figure 14. Dose-dependent effects of hydroxy urea on the SRBC-PFC response, when present in a 0-18 hour incubation (●——●). Effects of HU on the number of viable recovered cells compared to control (○--○). Viability data represent the average of duplicate cell counts not varying by more than 15%. Confidence intervals represent standard deviations for the PFC assay.



PFC / CULTURE (× 10⁻³)
0
2
4
6
8
10
12
14
% OF CONTROL VIABLE
0
50
100
HYDROXY UREA (M)
0
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
10⁻²

Figure 15. Dose-dependent effects of ^{3}H-thymidine, when present in a 0-18 hour incubation during the induction of humoral immunity to SRBC (●——●). Effects of ^{3}H-thymidine on the number of recovered viable cells, as compared to control cultures (○--○). Confidence intervals are $\pm$ standard deviations for the PFC assay. Viability data represents the average from duplicate cell counts not varying by more than 15%.


PFC / CULTURE (× 10⁻³)
0
2
3
4
5
6
0
5
10
20
³H - THYMIDINE (µCi/ml)
% OF
CONTROL
VIABLE
100
50
0

the effects of anti-proliferative agents were examined when they were present during the early (hour 0-18), late (hour 48-72), or the combined (hour 0-96) time periods (Table III). It is shown that anti-proliferative agents can stimulate the induction of the SRBC response, when they are present during the early stage of immune induction, whereas they inhibit this response if they are present during either the late or the combined stages. These results are consistent with the hypothesis that agents that elevate cyclic AMP can augment the humoral immune response via an anti-proliferative mechanism. However, the differential ability of these agents to produce augmentation suggests that other effects of these agents may also contribute to their effects on the induction of the SRBC response. It is notable that while colcemid, ARA-C, and ^{3}H-thymidine can all exert inhibitory effects on the PFC response that can be distinguished from their cytotoxic effects, such is not the case for DBcAMP.

Anti-Proliferative Agents Fail to Elevate Intracellular Levels of Cyclic AMP. In the preceding experiments, colcemid, cytosine-arabinoside (ARA-C), and hydroxy urea (HU) have all been used as putative anti-proliferative agents that can exert their effects independent of the cyclic AMP system. Support for this concept is shown in that these agents do not produce measurable changes in cyclic AMP in either a dose or time-dependent manner (Table IV).

TABLE III

Time-Dependent Stimulation and Inhibition of

SRBC-PFC by Anti-Proliferative Agents

Time (hours)	Viability (%)	PFC/CULTURE	S.I.[a]
0-18 (control)	100	1880 ± 400[b]	-
DBcAMP (5 x 10^{-4}M)	29	4560 ± 520	2.4
ARA-C (5 x 10^{-5}M)	67	12000 ± 1040	6.4
Colcemid (5 x 10^{-6}M)	59	3720 ± 200	2.0
Thymidine (100 mcg/ml)	91	1520 ± 360	0.8
^{3}H-thymidine(10 μCi/ml)	58	1720 ± 240	1.1[c]
48-72 (control)	100	5280 ± 560	-
DBcAMP	28	1400 ± 200	0.3
ARA-C	61	40 ± 40	0.007
Colcemid	40	40 ± 40	0.007
Thymidine	69	5160 ± 440	0.98
^{3}H-thymidine	48	760 ± 240	0.15[c]
0-96 (control)	100	1840 ± 200	-
DBcAMP	21	320 ± 200	0.17
ARA-C	51	40 ± 40	0.02
Colcemid	37	40 ± 40	0.02
Thymidine	70	1400 ± 80	0.76
^{3}H-thymidine	68	80 ± 40	0.04[c]
Thy-^{3}H-thymidine[d]	54	1320 ± 40	0.94[c]

[a] Stimulation Index

[b] Standard deviation for the PFC assay

[c] S.I. compared to the thymidine control

[d] Blocked-pulse, see text.

TABLE IV

Effects of Anti-Proliferative Agents on

Intracellular Concentrations of Cyclic AMP

Treatment		pmole cAMP/ 10^7 cells			
		15	30	60	120 minutes
Control (No drug)		6.8[a]	3.4	2.0	0.7
PGE_1 (5×10^{-6}M)		27.2	ND[b]	ND	ND
Colcemid	(10^{-7}M)	8.2	3.4	0.7	2.0
	(10^{-6}M)	5.4	4.1	1.4	2.4
	(10^{-5}M)	6.8	5.4	1.4	2.4
ARA-C	(10^{-6}M)	6.8	4.1	2.0	2.0
	(10^{-5}M)	6.8	3.4	1.4	1.4
	(10^{-4}M)	6.8	3.4	1.0	1.7
Hydroxy-	(10^{-5}M)	6.8	2.7	1.4	2.7
Urea	(10^{-4}M)	6.8	2.7	1.4	1.4
	(10^{-3}M)	6.1	3.4	2.0	2.0

[a] Values represent average determinations for duplicate samples not varying by more than 10%

[b] Not Determined

Control cultures do show a small increase in cyclic AMP that is time-dependent, probably resulting from the presence of fetal calf sera. A positive control for these experiments is shown by the ability of PGE_1 to produce its characteristic stimulation of cyclic AMP levels at the 15 minute time point. Though this experiment does not prove that these agents act independent of the cyclic AMP system in producing anti-proliferative effects, on the basis of what is known about their mechanisms of action, this experiment supports such a conclusion.

4. Discussion

The purpose of the experiments described in this section was to test the hypothesis that agents that have the ability to elevate intracellular levels of cyclic AMP may augment humoral immunity via an anti-proliferative mechanism. In support of this concept, several agents that produce inhibition of proliferation, but each via distinct biochemical mechanisms, have been shown to produce time-dependent stimulation of the humoral immune response to SRBC. All anti-proliferative agents tested produced dose-dependent stimulation of the SRBC response during time frames shown to produce stimulation with agents that elevate cyclic AMP.

Colchicine and colcemid both augmented the SRBC-PFC response, when they were present during the early stage of immune induction. However, because colcemid produced less cytotoxicity at higher concentrations than colchicine, this agent was selected for detailed study. Colcemid, a derivative of colchicine, inhibits proliferation of cultured cells via a mechanism of disruption of mitotic spindle microtubules (Wilson and Meza, 1973). Colcemid has proven to be useful in synchronizing cell cultures, because its effects are reversible following brief exposure (Stubblefield, et al, 1967; Romsdahl, et al, 1968). The stimulatory effects of colcemid on the induction of humoral immunity to SRBC in vitro in the present study are consistent with previous findings that, under appropriate conditions, colchicine can augment humoral immunity in vivo (Tanaka and Coons, 1954; Merritt, 1971; Shek and Coons, 1977).

Cytosine-arabinoside (ARA-C) and hydroxy urea (HU), agents that inhibit proliferation via effects on DNA synthesis (Yataganas and Clarkson, 1974), were also shown to be capable of augmentation of the SRBC response, when they were present during similar time frames as DBcAMP and colcemid. The mechanism by which ARA-C inhibits proliferation in believed to be via inhibition of DNA polymerase (Furth and Cohen, 1968), whereas HU probably acts by inhibiting the deoxyribonucleotide reductase reaction (Young, et al,

1967). It is unclear why HU exerts only marginal effects on the induction of humoral immunity in comparison to ARA-C. Data from previous investigators suggest that cells may be able to more easily escape proliferation control with HU than ARA-C (Yataganas and Clarkson, 1974). Such an effect might account for the variable ability found between these agents to augment humoral immunity.

Most investigators who have examined the effects of ARA-C on the induction of humoral immunity have reported immunosuppressant effects _in vivo_ (Harris and Hush, 1968) and _in vitro_ (Holtermann and Nordin, 1969). However, under appropriate conditions, augmentation of rat hemolysin titers have been described (Mitchell, et al, 1969). In the present study, the distinction between stimulatory and inhibitory effects of ARA-C is clearly based upon the time during which the agent is present. It is notable that, in other _in vitro_ humoral immune response systems, the inhibitory effects of ARA-C and HU have been observed in the absence of enhancing effects (Kishimoto, et al, 1975). Because these investigators also studied the effects of agents that elevate cyclic AMP in their system and described biphasic effects, this lack of effect of ARA-C and HU is notable (Kishimoto and Ishizaka, 1976). The system described by Kishimoto, et al measured secondary anti-hapten IgG responses; therefore, the different effects seen between their responses and those of the present study suggest that these two sys-

tems have different requirements for induction and/or modulation.

^{3}H-thymidine is an effective anti-proliferative agent, when it is used under the *in vitro* culture conditions described in this study (Dutton and Mishell, 1967). Because ^{3}H-thymidine is effective in inhibiting the immune response, when it is present during the late stages of immune induction, it is unclear why it does not produce stimulatory effects of a comparable magnitude to the other agents used in this study. A possible explanation for the decreased ability of ^{3}H-thymidine to produce augmenation of humoral immunity is that this agent is not effective in producing inhibition of proliferation during the early time period. This might be suspected because ^{3}H-thymidine produces inhibition of proliferation as a result of incorporation during DNA synthesis, but not necessarily at the time of DNA synthesis. Therefore, the effects of ^{3}H-thymidine may be sufficiently delayed to allow for critical proliferation to occur. Resolution of this point will be experimentally difficult.

The failure of ^{3}H-thymidine to produce marked enhancement of the SRBC response is not conclusive evidence that anti-proliferative agents cannot augment humoral immunity via a mechanism of inhibition of proliferation. In fact, the evidence from other investigators makes it seem almost certain that such a mechanism does exist.

In addition to those agents tested in this study, other investigators have examined the effects of other types of anti-proliferative agents for their effects on the induction of humoral immunity. Taliaferro and Taliaferra have reviewed the evidence that shows that low doses of X-irradiation can produce enhancement of humoral immune responses when they are given at the appropriate times (Taliaferro and Taliaferro, 1951). As the effects of X-irradiation are likely most pronounced in rapidly proliferating cells, these findings support the concept that anti-proliferative agents can augment humoral immunity.

More recently, it has been shown by several investigators that X-irradiation can augment humoral immunity. Chiorazzi, et al showed that X-irradiation and cyclophosphamide could increase the induction of IgE, when they were given at the appropriate times (Chiorazzi, et al, 1976). These investigators provided evidence that these agents augment humoral immunity via an effect of suppressor T cells (T_s). These results are consistent with those of several other investigators in showing enhancement of immune responses by X-irradiation (Gengozian and Makinoddi, 1958; Dixon and McConahey, 1963; Taliaferro and Taliaferro, 1969; Tada, et al, 1971; Schmidtke and Dixon, 1973).

Many investigators have shown increased immune responsiveness following certain administrations of cyclophosphamide (Lagrange and Mackaness, 1974; Polak and Turk,

1974; Marquet and Heystek, 1975; Bash, et al, 1976; Askenase, et al, 1975; Maguire and Ettore, 1967). Some of these studies have supported the notion that cyclophosphamide selectively inhibits suppressor T cells. However, on the basis of this present study, it is clear that if such selectivity exists, it probably has a temporal basis. Cyclophosphamide probably does not exert selective effects on cell types that have similar rates of proliferation. This has been shown by its ability to inhibit various cell types involved in immune responses, when it is present at appropriate times (Milton, et al, 1976; Martinez, et al, 1975; Schwartz, et al, 1976).

In summary, the results of previous investigations and those of the present study show that anti-proliferative agents can exert time-dependent stimulation of humoral immunity. These results are in distinct contrast to the immunosuppressive activity that each of these agents possess (Schwartz, 1965; Santos, 1967; Garielson and Good, 1967). On the basis of these studies, the hypothesis that agents that elevate cyclic AMP may exert stimulatory effects on the induction of humoral immunity via an anti-proliferative mechanism is supported. However, the results of following studies will show that an anti-proliferative mechanism is not sufficient to account for the effects of agents that elevate cyclic AMP. Further discussion and

and interpretation of the results obtained in this section will be deferred to the general discussion section.

C. Differential Effects of Anti-Proliferative Agents on the Induction of T-Dependent versus T-Independent PFC Responses

1. Introduction-Purpose

In the preceding section, it has been shown that agents that have the ability to elevate intracellular levels of cyclic AMP, and agents that can exert inhibition of proliferation, can augment the induction of the humoral immune response, when they are present during an early stage of immune induction. The results of these experiments are consistent with the hypothesis that agents that elevate cyclic AMP augment humoral immunity via a mechanism of inhibition of proliferation, but these results are not conclusive. The purpose of the experiments described in this section was to compare the effects of these agents for their effects on the induction of humoral responses to other antigens, in an attempt to determine whether these agents would always act in parallel.

DNP-Lysine-Ficoll (DLF) was chosen as an antigen to study because it is a putative T-independent antigen (Mosier, et al, 1974). Thus,DLF may have different requirements for activation than SRBC. Although T-independent antigens may not require T cells for the induction of humoral immunity, T cells may modulate these responses

(Baker, et al, 1973). Therefore, it cannot be claimed that if drug effects are exerted on the induction of humoral immune responses to T-independent antigens, that this shows a lack of drug effect on T cells.

For purpose of comparison, the examination of drug effects on the DLF response was done in the same manner as for the SRBC response. SRBC cultures were run in parallel with the DLF cultures to facilitate the comparison of drug effects.

2. Materials and Methods

Antigens. DNP-Lysine-Ficoll (DLF) was the generous gift of Dr. George K. Lewis (Department of Microbiology, University of California at San Francisco). DLF was stored in powder form in a light-shielded test tube, from which stock solutions of 1 mg/ml were prepared and then stored at -20 C. Stock solutions were periodically prepared, although no loss in antigenicity was ever noted. Immediately before each use, a 1 mg/ml stock solution was diluted to a 50X stock in SBSS to achieve a final concentration in culture of 3 ng/ml. For resuspension of cultures following drug incubations, DLF was re-added at this same concentration for the remainder of the culture period.

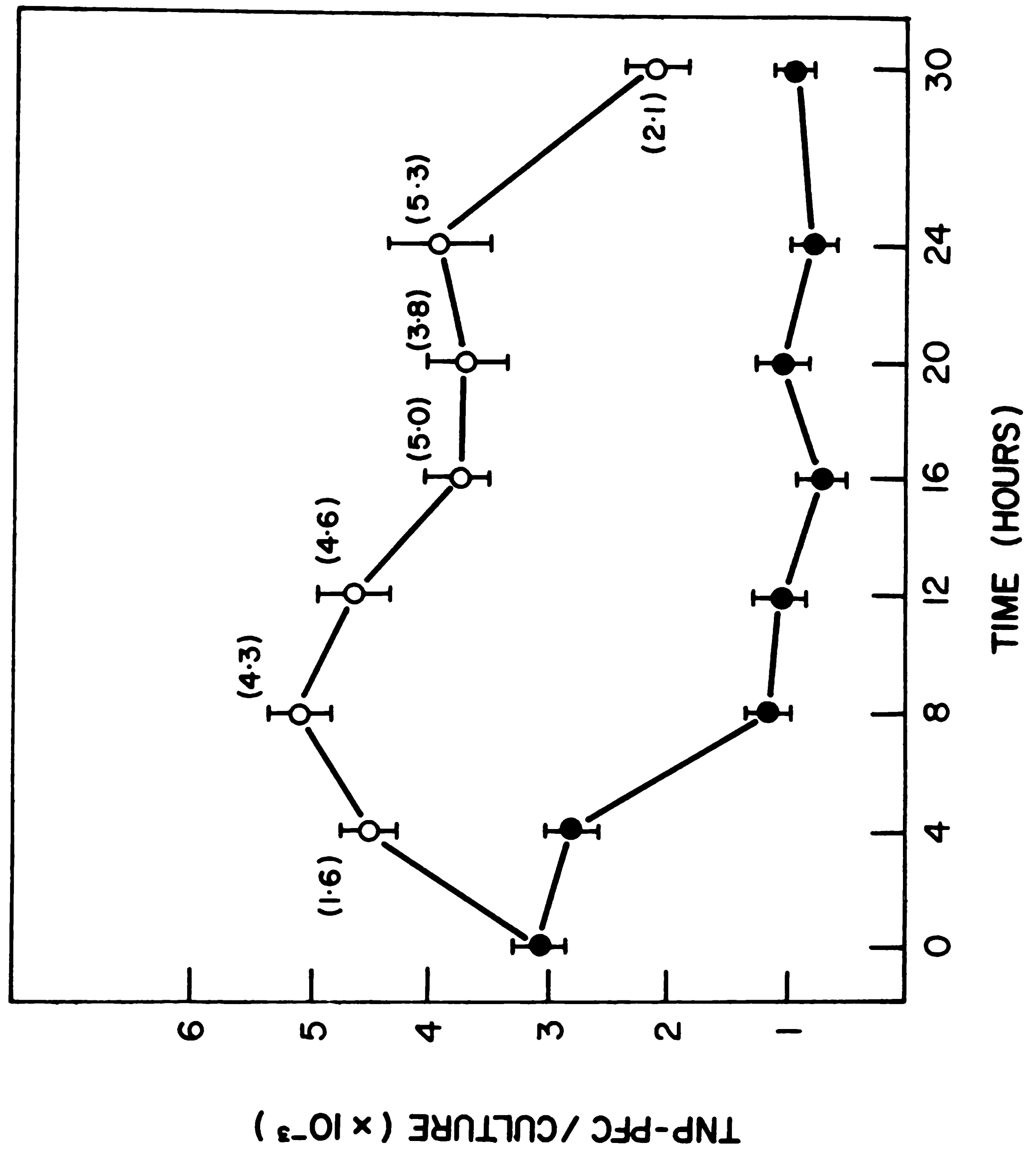
Assay of Plaque Forming Cells to DNP-Lysine-Ficoll. For the assay of plaque responses to DLF, or other DNP or

TNP haptenated antigens, trinitrophenylated horse red blood cells (TNP-HRBC), were used. TNP (Sigma, #P-5878) was coupled to the cell surface of HRBC by the method of Rittenberg and Pratt (Rittenberg and Pratt, 1969), as modified by Kettman and Dutton (Kettman and Dutton, 1971). TNP-HRBC were usually prepared immediately before each use, but they could be used for several days following conjugation if they were sufficiently washed with BSS. HRBC were received twice a month from Colorado Sera Inc. (#CS0003), and were stored at 4 C. Following conjugation, TNP-HRBC were washed three times in BSS and were then resuspended in a 1-15 vol/vol dilution in BSS. This suspension was then used in the PFC assay exactly as described for the SRBC assay.

3. Results

Time-Dependent Stimulation of the DLF Response by DBcAMP. When present at 5×10^{-4}M, DBcAMP produces time-dependent stimulation of the humoral immune response to DLF (Figure 16). When DBcAMP is added at the initiation of culture (time 0), maximal stimulation results from incubations of 0-16 to 0-24 hours. As previously noted for the SRBC response, cultures are sensitive to washing effects during intermediate times. DLF responses are, in general, more sensitive to washing effects during the

Figure 16. Time-dependent stimulation of the PFC response to DNP-Lysine-Ficoll by 5 x 10^{-4}M DBcAMP, when present in a 0-18 hour incubation (●——●). The effects of washing cultures at various times (○——○). Numbers in parenthesis indicate stimulation indexes for comparison of drug effects to appropriate wash control. Confidence intervals are ± standard deviations for the PFC assay.

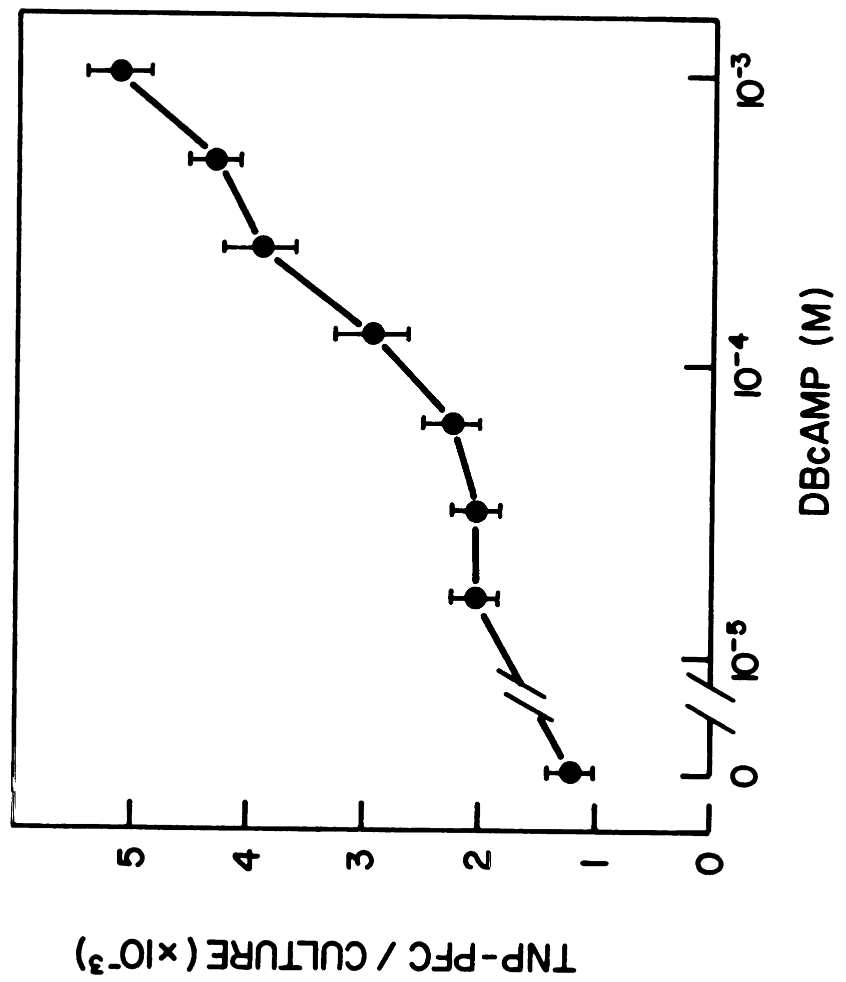

TNP-PFC / CULTURE (× 10⁻³)
1
2
3
4
5
6
0
4
8
12
16
20
24
30
TIME (HOURS)
(1·6)
(4·3)
(4·6)
(5·0)
(3·8)
(5·3)
(2·1)

early time periods (0-8, 0-12, and 0-16) than are responses to SRBC. The results from this experiment show that the time-dependence for stimulation of the DLF response is very similar to the time-dependence for stimulation of the SRBC response (Figure 1).

Dose-Dependent Stimulation of the DLF Response with DBcAMP. The dose-dependence for DBcAMP stimulation of the humoral immune response to DLF shows that maximal augmentation is achieved at 10^{-3}M DBcAMP, when it is present in a 0-18 hour incubation (Figure 17). Again, these results are very similar to those obtained with SRBC (Figure 2). Because the dose and time-dependence for DBcAMP effects are very similar for both the SRBC and the DLF response, it is likely that DBcAMP has a common component for the stimulation of both of these responses. These results do not prove that stimulation of both responses is via the same mechanism.

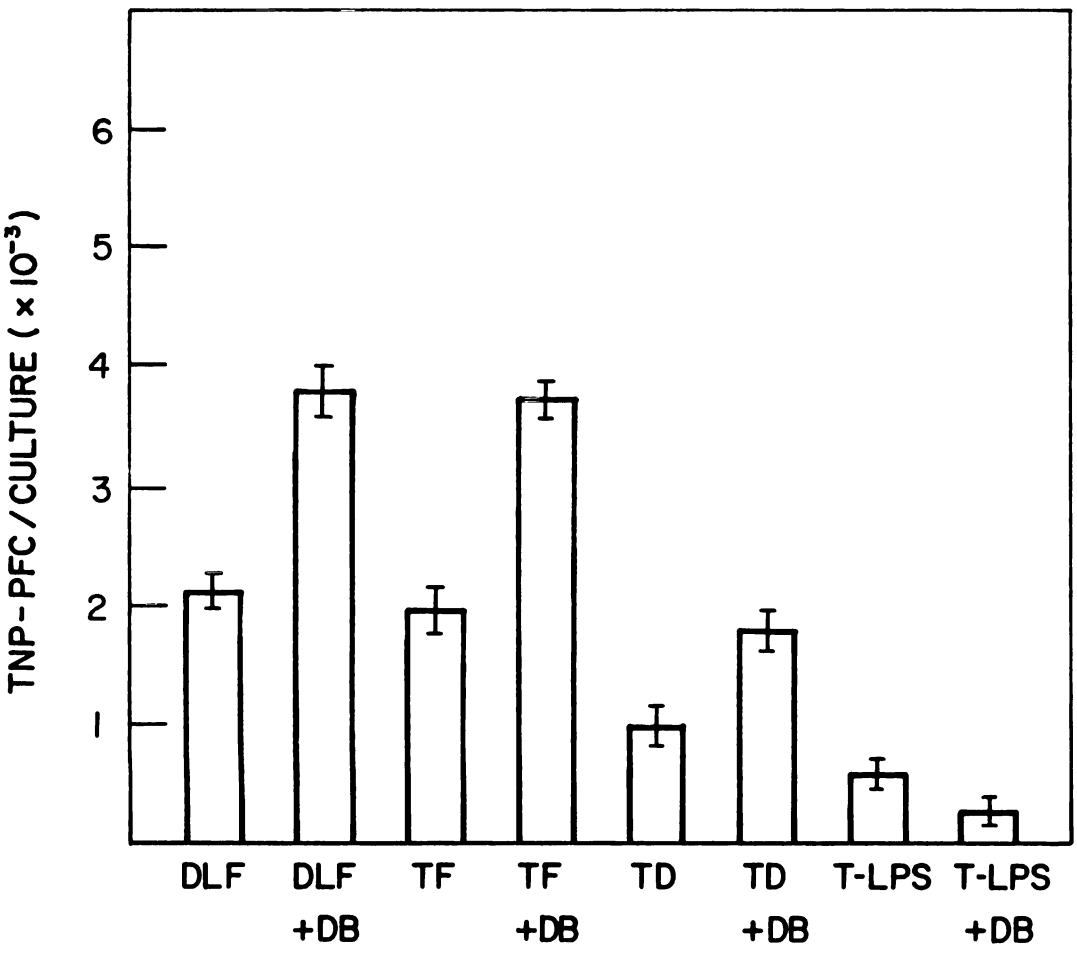
Differential Stimulation of T-Independent Responses by DBcAMP. It has been shown that DBcAMP augments the induction of humoral immunity to the SRBC and the DLF responses, when it is present during the early stages of immune induction, but DBcAMP is not capable of stimulating all antigenic responses. It has been found that DBcAMP can augment the response to DLF, TNP-Ficoll (TF), and TNP-Dextran (TD). DBcAMP cannot stimulate the induction of humoral immunity to the TNP-Lipopolysaccharide (TNP-LPS)

Figure 17. Dose-dependent stimulation of the DNP-Lysine-Ficoll response by DBcAMP (●——●), when present in a 0-18 hour incubation. Confidence intervals are ± standard deviations for the PFC assay.



TNP-PFC / CULTURE (×10⁻³)
0
1
2
3
4
5
DBcAMP (M)
0
10⁻⁵
10⁻⁴
10⁻³

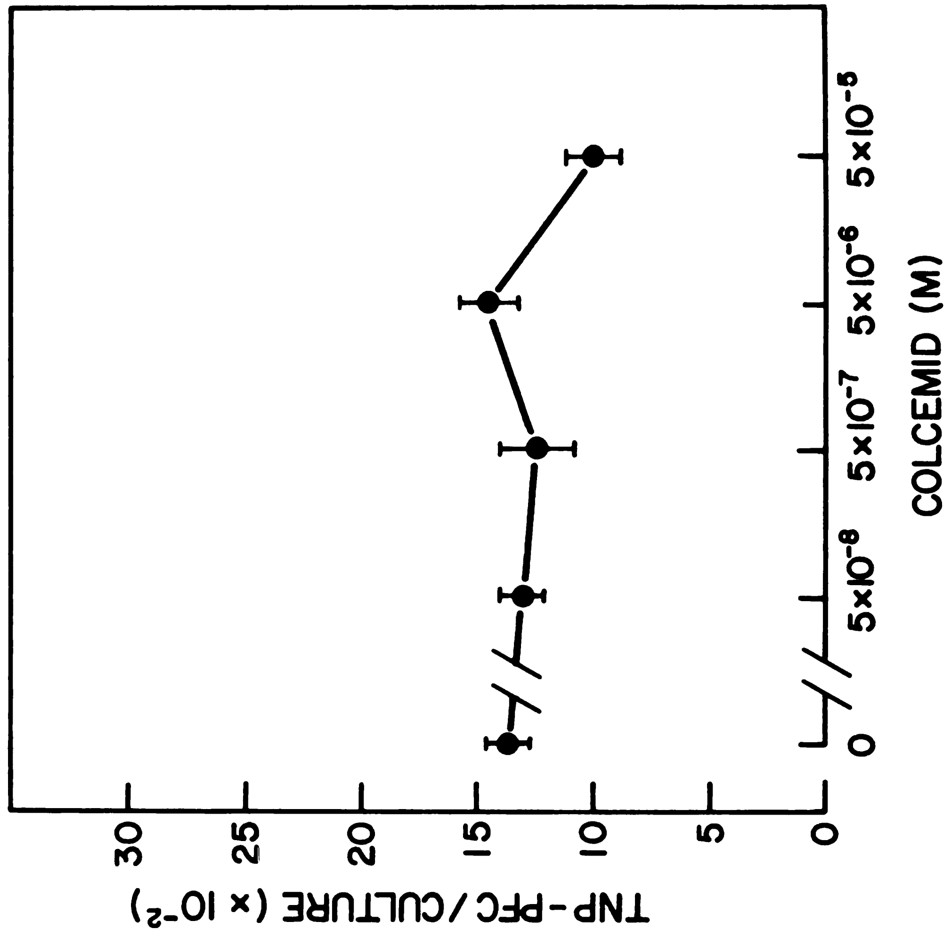
Figure 18. Differential stimulation of T-independent PFC responses by 5 x 10^{-4}M DBcAMP (DB), when present during a 0-18 hour incubation. DNP-Lysine-Ficoll (DLF), TNP-Ficoll (TF), and TNP-Dextran (TD) were present at a concentration of 250 ng/ml. Confidence intervals are $\pm$ standard deviations for the PFC assay.

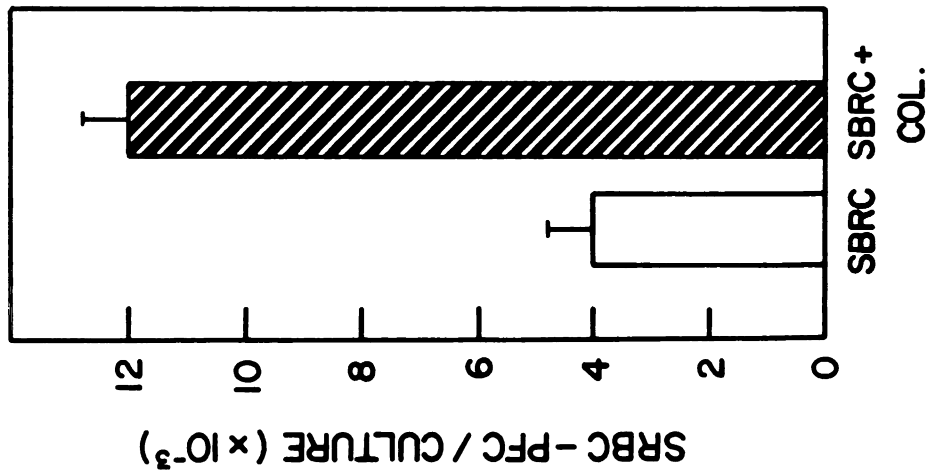

TNP-PFC/CULTURE (× 10⁻³)
6
5
4
3
2
1
DLF
DLF +DB
TF
TF +DB
TD
TD +DB
T-LPS
T-LPS +DB

response (Figure 18). This differential stimulation is reproducibly seen and suggests that the mechanism of activation of TNP-recognitive B cells may be different for these antigens.

Lack of Stimulation of the DLF Response with Colcemid. In section B, it has been shown that colcemid can exert both dose-dependent and time-dependent stimulation of the SRBC-PFC response. The time-dependence for colcemid effects were found to be very similar to the time-dependent effects of DBcAMP. Therefore, it was felt that colcemid and other anti-proliferative agents might help explain the mechanism by which cyclic AMP-elevating agents augment the humoral immune response. In this section, DBcAMP has been shown to exert similar effects on the induction of the DLF and the SRBC responses. When the effects of colcemid on the induction of the DLF response are examined, it is found that colcemid exerts no effect on the induction of humoral immunity (Figure 19). As a control for this experiment, the presence of 5×10^{-6}M colcemid in a SRBC-responding culture is found to produce characteristic augmentation, when the cultures are run in parallel with the DLF cultures. This experiment shows clearly the differential ability of colcemid to stimulate the humoral immune response to different antigens, and also shows that under appropriate conditions, the effects of DBcAMP and colcemid can be separated.

Figure 19. Differential stimulation of the SRBC versus DNP-Lysine-Ficoll (●——●) response by colcemid (COL.), when present in a 0-18 hour incubation. Colcemid was present at a concentration of 5×10^{-6}M in the SRBC-treated cultures. Confidence intervals are $\pm$ standard deviations for the PFC assay. SRBC and DLF cultures were run in parallel under identical conditions.

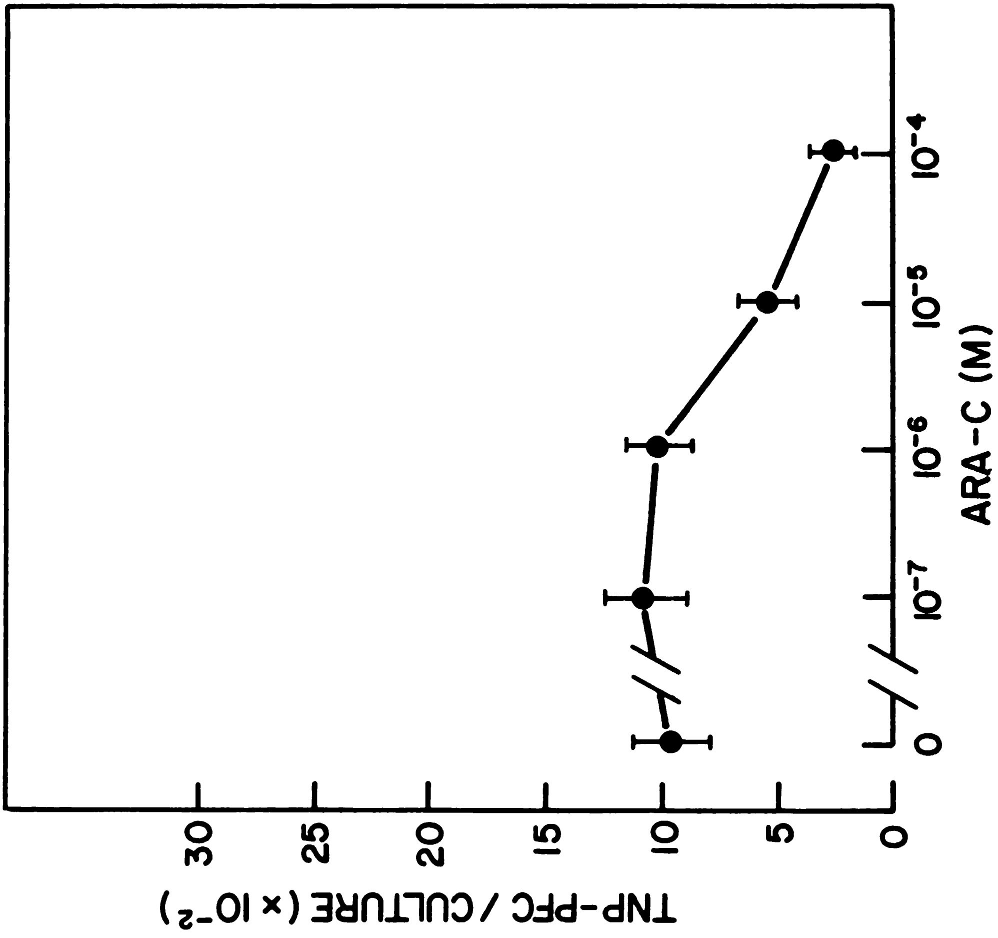

TNP-PFC/CULTURE (× 10^{-2})
30
25
20
15
10
5
0
0
5×10^{-8}
5×10^{-7}
5×10^{-6}
5×10^{-5}
COLCEMID (M)


SRBC-PFC / CULTURE (× 10^{-3})
12
10
8
6
4
2
0
SBRC
SBRC +
COL.

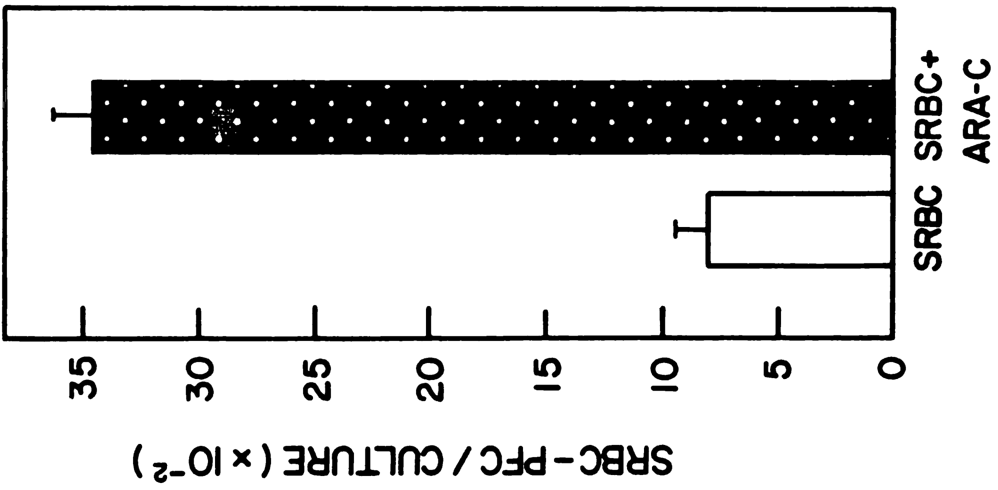
Lack of Stimulation of the DLF Response with ARA-C and Hydroxy Urea. Colcemid has been shown to have a differential ability to augment the humoral immune response to SRBC and DLF. If these differential effects result from anti-proliferative properties of this agent, then other agents which also produce inhibition of proliferation should show similar effects. This concept is supported by the observations that ARA-C (Figure 20) and hydroxy urea (Figure 21) exert stimulatory effects on the induction of the SRBC response, but cannot augment the DLF response, when present in a 0-18 hour incubation. These findings are consistent with those seen with colcemid and suggest that induction of humoral immunity to SRBC is qualitatively different.

Comparison of the Differential Effects of DBcAMP and Anti-Proliferative Agents on the Induction of Humoral Immunity. The experiments shown thus far have provided qualitative information concerning the differential effects of various agents on the induction of humoral immunity. For comparative purposes, the effects of DBcAMP (DB), colcemid (C), cytosine-arabinoside (AC), and hydroxy urea (HU) are tested for their effects on the induction of the SRBC and the DLF response, when these agents are present at concentrations that have been shown to produce optimal effects in separate experiments in a 0-18 hour incubation (Figure 22). Consistent with the findings of previous ex-

Figure 20. Differential stimulation of the SRBC versus the DNP-Lysine-Ficoll (●——●) response with cytosine-arabinoside (ARA-C), when present in a 0-18 hour incubation. ARA-C was present at 5×10^{-5}M in the SRBC drug-treated cultures. Confidence intervals are ± standard deviations for the PFC assay.

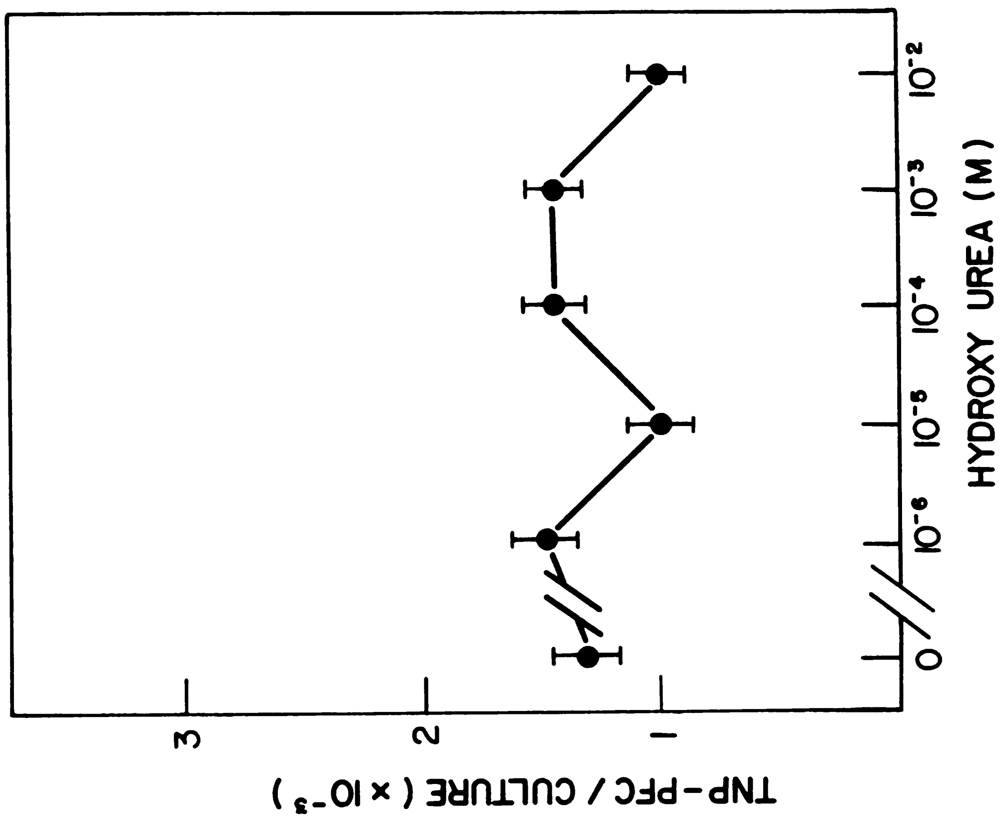


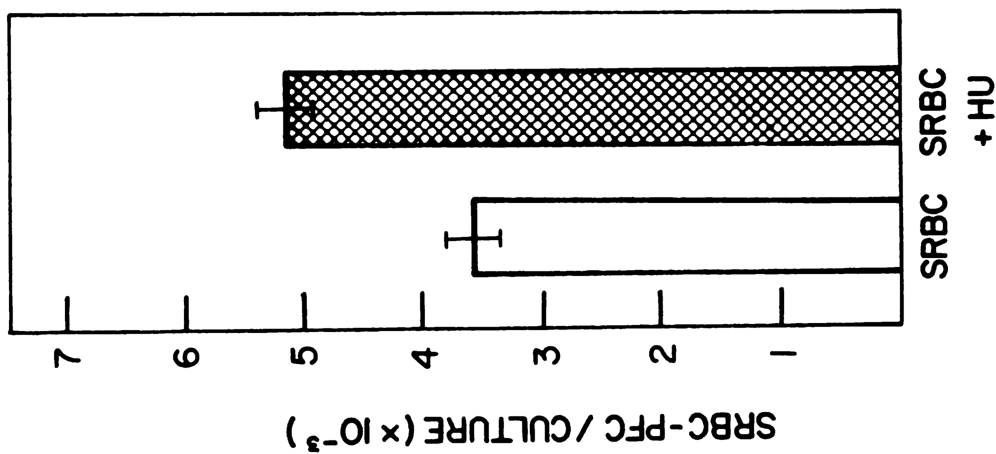
TNP-PFC / CULTURE (× 10⁻²)
0
5
10
15
20
25
30
ARA-C (M)
0
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴



SRBC-PFC / CULTURE (× 10⁻²)
0
5
10
15
20
25
30
35
SRBC
SRBC+ ARA-C

Figure 21. Differential stimulation of the SRBC versus the DNP-Lysine-Ficoll (●——●) response by hydroxy urea (HU), when present in a 0-18 hour incubation. HU was present at a concentration of 10^{-3}M in the SRBC drug-treated cultures. SRBC and DLF cultures were run in parallel under identical conditions. Confidence intervals are ± standard deviations for PFC assay.


TNP-PFC / CULTURE (× 10⁻³)
3
2
1
0
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
10⁻²
HYDROXY UREA (M)


SRBC-PFC / CULTURE (× 10⁻³)
7
6
5
4
3
2
1
SRBC
SRBC
+ HU

periment, DBcAMP is found to be most effective in augmenting the SRBC response, followed by ARA-C, colcemid, and HU. Although the effect of HU is small, its stimulatory effect is routinely seen.

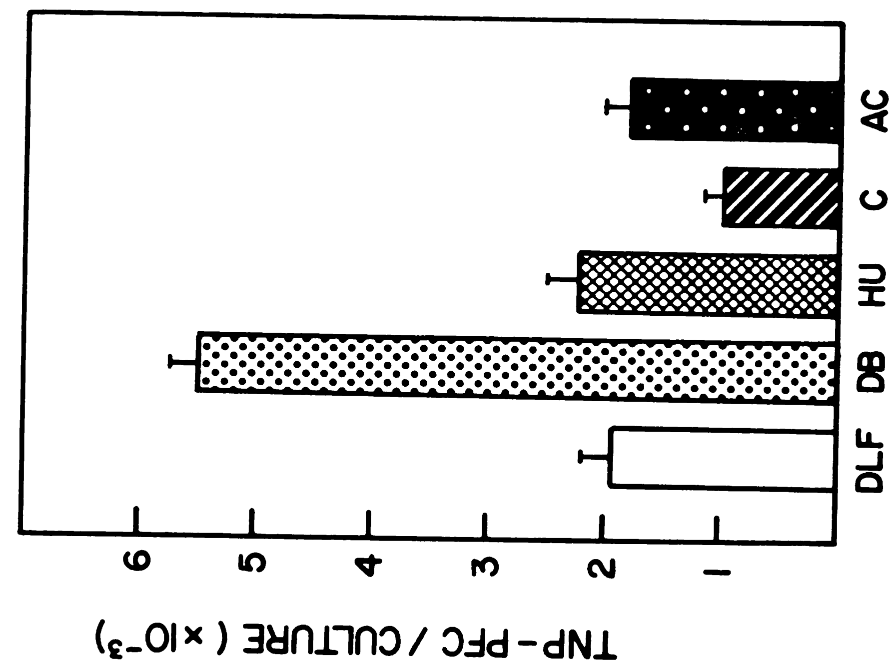
When these agents are tested for their effects on the DLF response, only DBcAMP is found to exert stimulatory effects. Colcemid, ARA-C, and HU exert either no effect or an inhibitory effect on the DLF response. The results of this experiment are consistent with the results of experiments where these agents were tested separately.

<u>^{3}H-thymidine Exerts Differential Stimulation of the SRBC versus the DNP-Lysine-Ficoll Response</u>. ^{3}H-thymidine has been shown to exert a small and variable stimulation of the induction of humoral immunity to SRBC. When it is tested for its effects on the SRBC versus the DLF response, it stimulates the SRBC response, but it has no effect on the induction of the DLF response (Figure 23). These results are consistent with the results of other experiments that show the differential ability of anti-proliferative agents to stimulate the SRBC versus the DNP-Lysine-Ficoll responses.

4. Discussion

The results of this section show clearly that DBcAMP

Figure 22. Comparison of the differential stimulation of the humoral immune response to SRBC and DNP-Lysine-Ficoll (DLF) by 5×10^{-4}M DBcAMP (DB), 10^{-3}M hydroxy urea (HU), 5×10^{-6}M colcemid (C), and 5×10^{-5}M cytosine-arabinoside (AC), when present in a 0-18 hour incubation. SRBC and DLF cultures were run in parallel under identical conditions. Confidence intervals are $\pm$ standard deviations for the PFC assay.


TNP - PFC / CULTURE (× 10⁻³)
1
2
3
4
5
6
DLF
DB
HU
C
AC

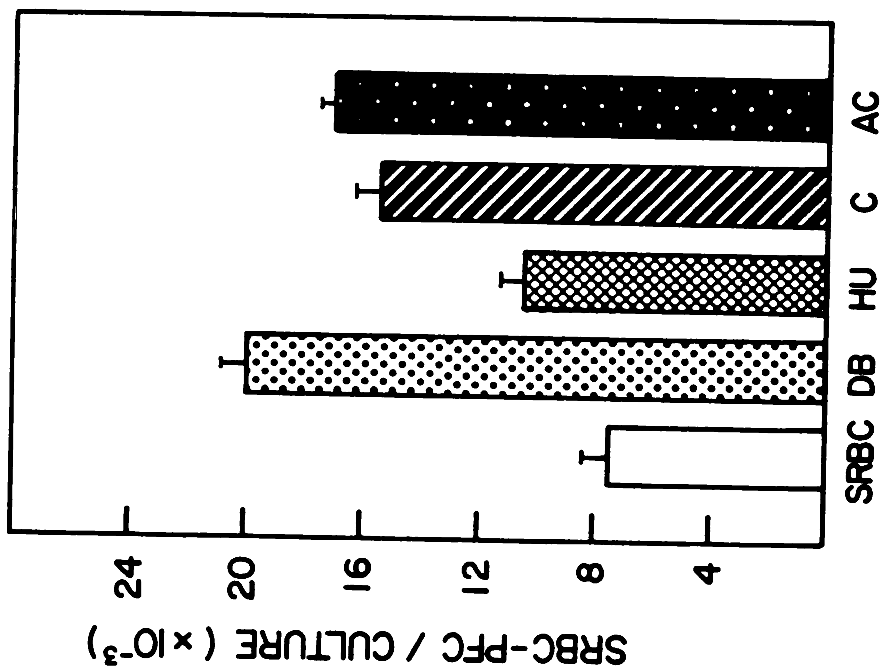
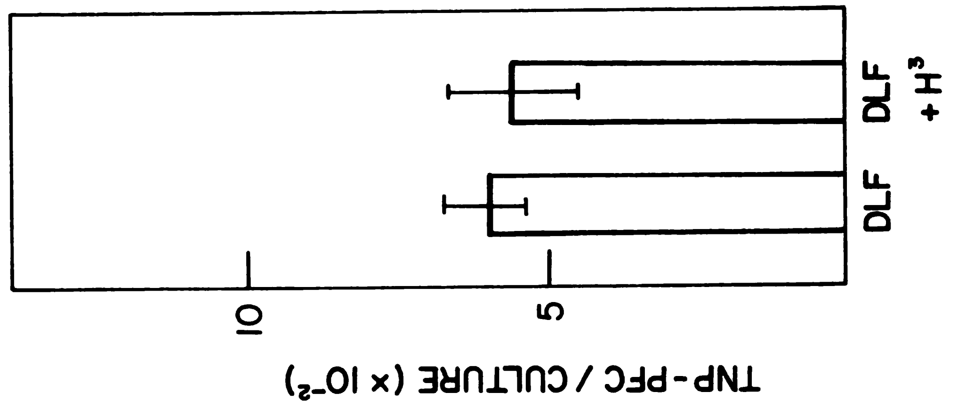
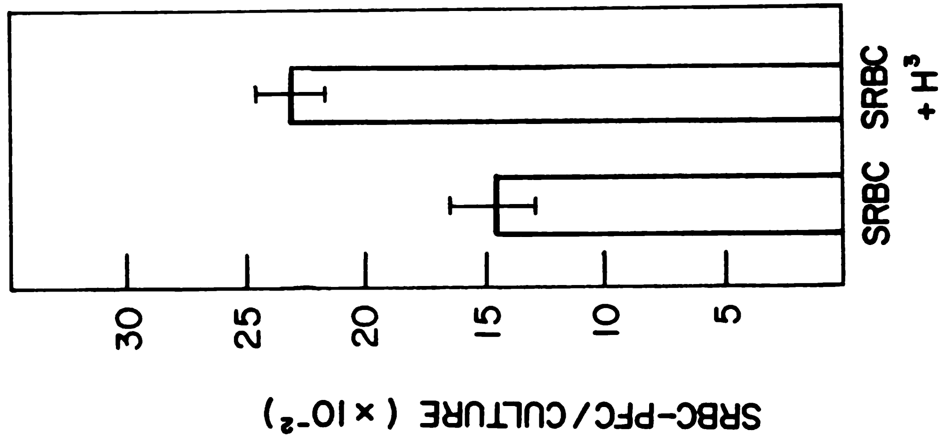

SRBC-PFC / CULTURE (× 10⁻³)
4
8
12
16
20
24
SRBC
DB
HU
C
AC

Figure 23. Differential stimulation of humoral immunity to SRBC versus DNP-Lysine-Ficoll (DLF) by ^{3}H-thymidine (H^3), when present at a dose of 10 mcCi/ml in a 0-18 hour incubation. Confidence intervals are $\pm$ standard deviations for the PFC assay.


TNP-PFC / CULTURE (× 10⁻²)
10
5
DLF
DLF
+H³


SRBC-PFC/CULTURE (× 10⁻²)
30
25
20
15
10
5
SRBC
SRBC
+H³

can stimulate the induction of humoral immunity to DNP-Lysine-Ficoll (DLF) in a manner very similar to that which it stimulates the SRBC response. As anti-proliferative agents have, in preceding sections, been shown to exert effects comparable to DBcAMP on the induction of humoral immunity to SRBC (suggesting a mechanism by which DBcAMP might augment humoral immunity) these agents were tested for their effects on the DLF response. In marked contrast to their effect on the SRBC response, anti-proliferative agents are found to be incapable of augmenting the humoral immune response to DLF. A preceding experiment has shown that the DLF response is sensitive to the inhibitory effects of colcemid (Figure 11); therefore, the hypothesis that the DLF-responding cells might not be sensitive to anti-proliferative agents cannot be invoked. These findings suggest that induction of the DLF response has different requirements than does the SRBC response, and that DBcAMP probably does not stimulate the induction of humoral immunity to the DLF response via a mechanism of inhibition of proliferation. However, as the DLF responsive-B cells are hypothesized to belong to a subset population that have different requirements for activation than the SRBC response (Lewis, et al, 1976), this evidence cannot be used to prove that inhibition of proliferation is not the mechanism by which DBcAMP augments the SRBC response. These results suggest that if such a mechanism does exist,

DBcAMP probably has an additional component for activation of the SRBC response.

The finding that DBcAMP has a differential ability to augment the humoral immune response to T-independent antigens is suggestive that within the T-independent compartment of B cells there may be heterogeneity. It has been postulated that T-independent antigens do not require T cell help, because they are mitogenic (Watson, et al, 1973), but this view has been contested by other investigators (Feldman, et al, 1975; Lewis, et al, 1976). The differential ability of DBcAMP to augment humoral immunity to T-independent antigens may relate to the ability of such antigens to provide a mitogenic signal to the B cell. Although this hypothesis is ammenable to experimental investigation, it was not within the scope of these studies to pursue such investigations.

In conclusion, the effects of DBcAMP and anti-proliferative agents can, under appropriate conditions, be separated. Resolution of the question of whether agents that elevate cyclic AMP augment the SRBC response via an anti-proliferative mechanism may be resolved on the basis of identifying which cells are responsible for the stimulation of humoral immunity. If the effects of DBcAMP can be separated on this basis, this will provide strong evidence that agents that elevate cyclic AMP act independent of an

anti-proliferative mechanism. These experiments are described in the following section.

D. A Cellular Basis for Stimulation of the Humoral Immune Response to SRBC by Cyclic AMP and Anti-Proliferative Agents

1. Introduction-Purpose

On the basis of preliminary studies that have been presented in this work, a possible correlation was shown to exist between the ability of agents that elevate cyclic AMP levels and agents that can inhibit proliferation to augment humoral immunity to SRBC. However, additional experiments showed that, under appropriate conditions, the effects of these two types of agents could be separated. Thus, although colchicine and colcemid, ARA-C, hydroxy urea, and ^{3}H-thymidine could stimulate the induction of humoral immunity to SRBC, only DBcAMP could stimulate both the SRBC and the DNP-Lysine-Ficoll (DLF) responses. These findings do not exclude the possibility that agents that elevate cyclic AMP can exert stimulatory effects on the induction of the SRBC response via an anti-proliferative mechanism. The SRBC response may have different requirements for the induction and modulation of humoral immunity than the DLF response.

If DLF is truly a T-independent antigen, it is possible that anti-proliferative agents that might work through a T cell-B cell interaction would be without effect, be-

cause T cells might be irrelevant in the induction of the DLF response. Caution must be exercised in attempting to make mechanistic interpretations on the basis of differences between the SRBC and the DLF response, as it is not completely clear what the involvement of T cells is in these two response. In addition, the role of macrophages is unclear, although these cells are required for the induction of these two responses (Unanue, 1972).

The purpose of the experiments described in this section was to attempt to identify the particular cell type involved in the stimulation of the SRBC response by agents that elevate cyclic AMP (DBcAMP) and antiproliferative agents (ARA-C is used as a prototype agent). The strategy employed was to use procedures for cell separation of various populations of spleen cells into highly enriched fractions; these populations were then separately treated with DBcAMP or ARA-C, and then the cell populations were reconstituted to study the antigenic response to SRBC. Before it was possible to conduct these experiments, it was necessay to establish the conditions under which cells could be separated and reconstituted to obtain humoral responses to SRBC. Having established such a system, it was then possible to ask whether the two agents tested were affecting the same population of cells. It should be noted, that the

inability of an agent to affect a particular cell type, under the conditions employed, does not constitute conclusive evidence that this agent cannot exert effects on that cell type. It is necessary to distinguish and to study specific functions of such cells in order to make such a conclusion. In addition, the separation of cell types prevents cell-cell interactions that might be necessary for the effects of certain agents to be seen. However, experiments showing positive drug effects can be taken as strong evidence for the localization of drug effects. These considerations and limitations must be remembered when interpreting the results of this data.

2. Materials and Methods

Preparation of T-Cell Depleted Populations of Cells. Because mature T cells express alloantigens on their cell surface that are specific for this type of lymphocyte, anti-sera can be prepared and used to specifically lyse these cells in the presence of complement. Murine brain homogenates have been shown to contain an antigen that cross-reacts with the T cell specific antigen; therefore, advantage of this property is taken in preparation of anti-T cell antisera in rabbits. Using the method of Golub, rabbit anti-mouse brain anti-sera (RAMB) was prepared, and

was the generous gift of Dr. George K. Lewis (University of California at San Francisco, Department of Microbiology) (Golub, 1971). A potential advantage in using RAMB anti-sera over other T cell anti-sera is that larger quantities can be made. In addition, it has been reported that RAMB has anti-T stem cell activity (Golub, 1972). As cyclic AMP has been shown to be capable of inducing the maturation of T cells from stem cells, this property might be important in removing possible effects of these agents on T cell differentiation that might have concomitant effects on the induction of humoral immunity (Scheid, et al, 1975).

Because the RAMB was prepared in rabbits using BDF_1 mouse brains, it will contain many anti-bodies that are not specifically directed against the T cell; therefore, this anti-sera must be absorbed to remove all non-specific antibody. In these experiments, RAMB was absorbed with BDF_1 erythrocytes prior to use. RAMB was then filter-sterilized (as described for sterilization of drugs), was aliquoted, and then stored at $-20^{o}C$. Guinea pig complement (C') was also the generous gift of Dr. George K. Lewis (as above). Non-specific cytotoxicity of the C' was reduced by an agarose (L' Industrie Biologique Francaise, Indubiose #A 37) absorption (80 mg/ml for 90 minutes at $4^{o}C$). The C' was then sterilized by filtration, was aliquoted, and then stored at $-70^{o}C$. The specificty

of RAMB was tested in a microcytotoxicity system (Dr. George K. Lewis, personal communication). This assay showed that at a 1-5 dilution (1-20 final) of the RAMB, in the presence of absorbed C', killed approximately 95% BDF_1 thymus cells and approximately 50% of spleen cells (Figure 24). This experiment shows that the RAMB has specificity for T cells; therefore, RAMB was used at this dilution in all subsequent experiments. A functional test for the specificity of RAMB was shown in that following this treatment, spleen cell could not mount humoral immune responses to SRBC unless a source of T cells was provided (Table V, to be discussed). Recovery of spleen cells, following RAMB treatment, was performed by washing these cells 3 times in SBSS, and then resuspending these cells in complete culture media for culture.

Preparation of T-Cell Enriched Populations of Lymphocytes. The method of Julius, et al was used to prepare T cell enriched populations of lymphocytes (Julius, et al, 1973). This method is based upon the observation that T cells do not adhere to nylon wool columns, whereas other spleen cells are retained under the appropriate conditions. The exact method of Julius was followed, except that larger (2X) columns were used, containing more (2X) of the nylon wool (Fenwall Laboratories), and that SBSS plus 5% fetal calf sera was used instead of the modi-

fied Eagle's media (MEM). The method used was found to be effective in enriching for T cells. Such cells were found to be incapable of mounting humoral immune responses to SRBC, but they could restore the response of RAMB-treated cells (Table V). Previous experiments showed that nylon wool-purified T cells were responsive to T cell mitogens and that they didn't react with a goat anti-mouse immunoglobulin reagent (In collaboration with Dr. Edith M. Lord and Dr. George K. Lewis).

Cultures. Because of the limitations in the number of cells that could be obatined from the preceding procedures, cultures were established as previously described, but on a smaller scale. For these experiments, whole spleen, T-deficient (BM), or T-enriched (T) cells were incubated with SRBC (approximately 2 x 10^6/culture) as 0.5 ml cultures in 24-well culture plates (Falcon, 3008) at a concentration of 10^7 cells/ml for the first 18 hours of culture in the presence of drug. Cultures were then harvested, as previously described, and were washed-resuspended to 70% of original volume in fresh complete media. For reconstituted cultures (ie. BM + T), cells were added in an equal volume of each population to achieve a final volume of 0.2 ml. All resuspended cells were recultured as 0.2 ml cultures in flat bottom microtiter culture plates (Linbro, IS-FB-96). The ratio of cells used for reconstitution was 1:1, as it was

found to give complete restoration of the SRBC-PFC response, with an excess of T cells (Table V).

3. Results

<u>Reconstitution of Humoral Immunity to SRBC Following Culture Separation</u>. The results of this experiment show that T cell deficient (BM) PFC responses to SRBC cannot be obtained unless T-enriched (T) cells are added to cultures (Table V). These experiments show that BM cells and T cells can be separately cultured for 18 hours and then can be combined to give responses of an equivalent magnitude to unseparated spleen cells. As both the BM and the T populations are unable to mount SRBC responses when present separately, these results are functional evidence that the methods used for fractionation and characterization of lymphocyte populations is efficacious. These findings also show that when equal numbers of BM and T cells are mixed, the cultures have an excess of T cells, as fewer T cells are actually needed to restore BM responses. T cells are added in excess so that cytotoxic effects of drugs on T cells do not make interpretation of these results difficult.

<u>Cellular Basis for the Stimulatory Effects of DB-cAMP on the Induction of SRBC-PFC Responses</u>. Having

Figure 24. The specificity of rabbit anti-mouse brain anti-sera (RAMB), when tested in a microcytotoxicity system. Each point represents the average of duplicate viable cell counts not varying by more than 15%. Thymus (▲—▲) and spleen (○—○) cells were obtained from female BDF_1 mice. RAMB was diluted 1-4 in the assay. Cytotoxicity control contains complement (de-toxified) only (C').

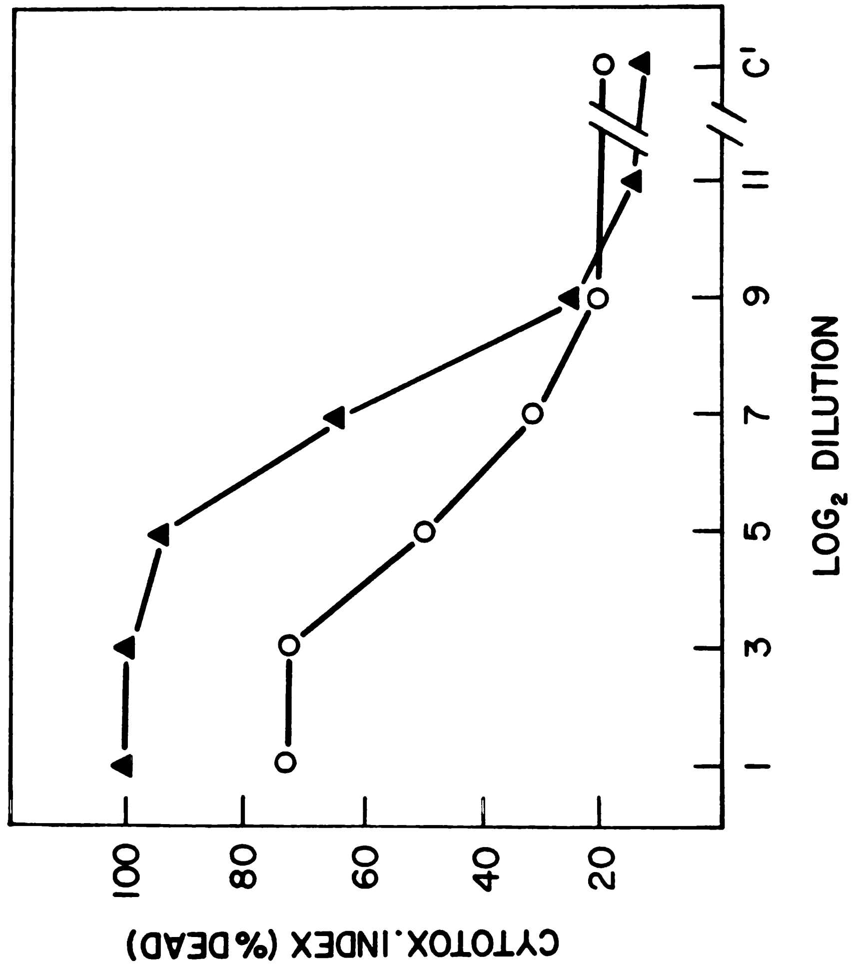

CYTOTOX. INDEX (% DEAD)
100
80
60
40
20
1
3
5
7
9
11
C'
LOG₂ DILUTION

TABLE V

Reconstitution of Humoral Immunity Following Culture-Separation[a]

Cell Type (Number of cells/culture)	PFC/CULTURE[b]
Whole Spleen (8×10^6)	670 ± 20[c]
" " "	470 ± 20
" " (4×10^6)	210 ± 30
BM[d] (4×10^6)	20 ± 10
" " + T[e] (10^6)	580 ± 70
" " + T (2×10^6)	420 ± 50
" " + T (4×10^6)	630 ± 50
BM (2×10^6) + T (5×10^5)	250 ± 20
" " + T (10^6)	200 ± 20
" " + T (2×10^6)	440 ± 20
" " + T (4×10^6)	450 ± 20
T[e] (4×10^6)	0 ± 0

[a] See text for culture conditions

[b] Assuming 10^7 cells/culture

[c] Standard deviation for the PFC assay

[d] RAMB + C' treated spleen cells

[e] Nylon wool non-adherent spleen cells

determined those conditions under which T-deficient (BM) and T-enriched (T) populations of spleen cells can be separately cultured during an early stage of immune induction, the effects of DBcAMP and ARA-C were then investigated. The results from two experiments are shown in Table VI and Table VII. Because both drugs were studied simultaneously in these experiments, the controls for Tables VI and VII are the same. Data has been separated for clarity.

The results of these experiments show that BM cells can have SRBC responses restored with T cells. The presence of 5×10^{-4}M DBcAMP in the 0-18 hour incubation, with either the BM or the T population, does not restore responsiveness. When BM cells are treated with DBcAMP and then T help is provided, marked stimulation of the PFC response is observed. These experiments show that DBcAMP can produce stimulation of humoral immunity by effects on the BM population of cells. When T cells were treated with DBcAMP, no stimulatory effects were seen, following reconstitution of culutres. These results show that DBcAMP probably does not augment humoral immunity to SRBC via an effect on T cells (See General Discussion).

The results of the experiments with ARA-C (Table VII) show that ARA-C can also exert effects on the BM population of cells and, in addition, it can also stimulate the SRBC response via an effect on T cells. The ability

TABLE VI

Cellular Basis for Stimulation of the SRBC-PFC Response by DBcAMP[a]

Cell Type (Treatment)	PFC/CULTURE Exp. 1	Exp. 2
Spleen	1690 ± 60[b]	430 ± 20
" + DBcAMP[a]	3740 ± 70	1870 ± 50
BM[c]	60 ± 10	30 ± 20
" + T[d]	1210 ± 50	390 ± 40
" + (T + DBcAMP)	1940 ± 60	230 ± 60
(BM + DBcAMP)	260 ± 20	40 ± 30
" + T	5890 ± 170	2690 ± 80
" + (T + DBcAMP)	4950 ± 170	2170 ± 100
T[d]	30 ± 10	0 ± 0
(T + DBcAMP)	10 ± 10	10 ± 10

[a] 5 x 10^{-4}M DBcAMP, see text for culture conditions

[b] Standard deviation for the PFC assay

[c] RAMB + C' treated spleen cells

[d] Nylon wool non-adherent spleen cells

TABLE VII

Cellular Basis for Stimulation of the SRBC-PFC Response by ARA-C[a]

Cell Type (Treatment)	PFC/CULTURE Exp. 1	Exp. 2
Spleen	1960 ± 60[b]	430 ± 20
" + ARA-C[a]	5330 ± 100	1970 ± 180
BM[c]	60 ± 10	30 ± 20
" + T[d]	1210 ± 50	390 ± 40
" + (T + ARA-C)	2370 ± 140	830 ± 70
(BM + ARA-C)	920 ± 50	440 ± 60
" + T	4530 ± 150	1170 ± 60
" + (T + ARA-C)	6770 ± 100	3180 ± 110
T[d]	30 ± 10	0 ± 0
(T + ARA-C)	10 ± 10	10 ± 10

[a] 5×10^{-5}M ARA-C, see text for conditions

[b] Standard deviation for the PFC assay

[c] RAMB + C' treated spleen cells

[d] Nylon wool non-adherent cells

of ARA-C to augment responses via an effect on T cells may support the concept that anti-proliferatives may augment humoral immunity by an effect on suppressor T cells. The ability of ARA-C to restore SRBC responses, in the absence of T cell help, was observed in these experiments. This effect was consistently seen and suggests that ARA-C and DBcAMP augment humoral immunity via different effects on the BM population of cells, as DBcAMP is incapable of restoring the SRBC response in the absence of T cells. That ARA-C is not simply providing a T cell signal is shown by the ability of T cells to further increase this response. The effect of ARA-C on the BM population of cells is very exciting, but it was not within the scope of this work to pursue these studies further.

4. Discussion

The results of this section clearly show that DBcAMP and ARA-C can both augment humoral immunity to SRBC via an effect on B cells or macrophages (BM), but evidence is presented that these agents may act via different mechanisms. In addition to the effect of ARA-C on BM cells, it is also found to have an effect on T cells. These results are consistent with those of preceding sections and will now be jointly considered in a general discussion section.

GENERAL DISCUSSION

The purpose of these studies has been to investigate the cellular mechanism(s) by which agents that elevate intracellular levels of cyclic AMP stimulate the induction of humoral immunity. An *in vitro* culture system was established that allowed for the examination of drug effects during various time frames of immune induction in semi-defined populations of spleen cells. In agreement with previous investigators, the results of this study show that various agents that increase cyclic AMP can augment humoral immunity when they are present during an early stage of immune induction. In addition, the present study has shown that these agents produce augmentation of the SRBC-PFC response in varying degrees. Evidence has been presented that those agents that are most capable of maintaining sustained elevation of intracellular levels of cyclic AMP are also most effective in augmenting humoral immunity.

In examining the mechanism(s) by which agents that elevate cyclic AMP augment humoral immunity, the consequences of sustained elevation of cyclic AMP in cultured lymphocytes were considered. A great deal of evidence has accumulated to show that elevation of cyclic AMP levels in cultured lymphocytes may produce inhibition of proliferation; therefore, effects of cyclic AMP on proliferation

during the induction of humoral immunity were considered.

Initial results of these studies showed that agents that have the ability to inhibit proliferation of cultured lymphocytes could produce augmentation of the humoral immune response to SRBC, when present during an early stage of immune induction. However, studies that followed revealed that the stimulatory effects of cyclic AMP could be separated from the effects of anti-proliferative agents when the induction of T-independent responses were examined. Thus, the data obtained from these studies could be used as evidence for or against the hypothesis that cyclic AMP augments humoral immunity to T-dependent responses via an anti-proliferative mechanism. Following discussion of the evidence that inhibition of proliferation is a mechanism by which T-dependent humoral immune responses can be augmented (a possible mechanism for cyclic AMP effects), alternative effects of the various agents tested will be taken into consideration.

That anti-proliferative agents can augment humoral immunity to T-dependent responses has been demonstrated by the ability of several agents that each produce inhibition of proliferation, but each via different biochemical mechanisms, to increase the PFC response to SRBC *in vitro*. Thus, colchicine and colcemid, cytosine-arabinoside (ARA-C), hydroxy urea (HU), and ^{3}H-thymidine all produced dose-dependent stimulation of the SRBC-PFC response.

The finding that ^{3}H-thymidine and HU produce less stimulation than other anti-proliferative agents tested may be explained on the basis of their variable ability to produce inhibition of proliferation. The reasons for suspecting such variation have been discussed in a preceding section. An alternative explanation is that these agents show that colchicine and colcemid, ARA-C, and agents that elevate cyclic AMP may act via mechanisms other than inhibition of proliferation in augmenting humoral immunity. However, in view of the observations of previous investigators that X-irradiation and cyclophosphamide also augment humoral immunity when they are present during an early stage, it seems probable that anti-proliferative effects are at least partially responsible for the stimulatory effects of these agents.

On the basis of this study and the work of other investigators, a mechanism for the time-dependent stimulation of T-dependent humoral immune responses by anti-proliferative agents is proposed (Figure 25). This model also encompasses the effects of other agents that may exert effects other than inhibition of proliferation. The effects of these agents will be considered in the discussion of alternative explanations for the results of this study.

Previous investigators have shown that cyclophosphamide and X-irradiation exert stimulatory effects on

the induction of humoral immunity to T-dependent antigens via an effect on suppressor T cells (Chiorazzi, et al, 1976; Polak and Turk, 1974). These investigators found enhancement when these agents were given at the time of immunization of mice. These results are consistent with the findings of the present study in showing enhancement when anti-proliferative agents are present during an early stage of immune induction. Cyclophosphamide produces irreversible inhibition of DNA synthesis via alkylation of DNA, whereas X-irradiation produces irreversible damage to DNA; therefore, these agents are irreversible inhibitors of cellular proliferation. Because the selectivity for the effects of these agents results only from the relative rates of cell proliferation, the enhancement of humoral immunity suggests that regulatory cells proliferate during an early stage of immune induction. Results from these studies showed that the regulatory cell involved was a T cell.

Taken collectively, substantial evidence has been provided to support the concept that inhibition of proliferation during an early stage of immune induction to T-dependent antigens will result in augmentation of humoral immunity. It is not clear whether cyclic AMP acts via this mechanism in stimulating the SRBC response. The findings that DBcAMP can augment T-independent responses in the absence of effects of anti-proliferative agents

suggests that cyclic AMP stimulates immune induction via a mechanism other than that of inhibition of proliferation. However, because the B cells that respond to T-independent antigens have been shown to have different requirements for activation than the B cells that respond to red blood cell antigens, it is not possible to make a direct comparison between the effects of various agents on the induction of these two types of responses (Lewis, et al, 1976). The lack of effect of anti-proliferative agents on the T-independent responses suggests that suppressor T cells are not involved in the induction of such responses. In addition, the results of these studies suggest that cyclic AMP exerts its stimulatory effects on either B cells or accessory cells (macrophages) for augmentation of T-independent responses.

Previous studies are consistent with the results discussed above that cyclic AMP can exert effects on either B cells or macrophages (Kishimoto and Ishizaka, 1976; Watson, et al, 1973). These investigators studied responses to T-dependent antigens by addition of T cells or T cell factors, following exposure of B cells and macrophages (BM) to cyclic AMP. Watson, et al found that cyclic AMP could not replace a T cell signal, a result confirmed in the present study, but that it could potentiate a T cell or a mitogenic (LPS) signal (Watson, et al, 1973 a,b). The finding that DBcAMP could potentiate

T-cell deficient SRBC responses that were restored with LPS is notable, as this present study has shown that DBcAMP is unable to augment responses when LPS is used as an antigen. The differential ability of DBcAMP to augment T-independent responses to TNP-Ficoll, TNP-Dextran, and TNP-LPS may prove useful in understanding the relationship between antigen and mitogen activation of lymphocytes.

In an attempt to distinguish between the effects of cyclic AMP on B cells versus T cells in induction of the SRBC response, separation-reconstitution experiments were performed. If cyclic AMP exerts an anti-proliferative effect on T cells as well as an effect on the BM population of cells, stimulation should be seen when DBcAMP is incubated with either population. Separate treatment of both populations of cells showed that the effects of cyclic AMP were limited to the BM population, but because ARA-C also exerted effects on this population of cells, as well as on the T cell population, it was still not possible to definitively separate the effects of these two agents. However, ARA-C was found to exert a qualitatively different effect than DBcAMP on the BM population of cells, exemplified by its ability to restore T-dependent responses in T-deficient cultures. These results suggest that ARA-C and DBcAMP augment humoral immunity via different biochemical mechanisms, but it is theoreti-

cally possible that they share a common component for stimulation.

Because both ARA-C and DBcAMP act upon the BM population of spleen cells in augmenting humoral immunity, it will be necessary to separate B cells and macrophages to determine which cells are responsive to the effects of cyclic AMP and anti-proliferative agents. Such studies may allow for the separation of the effects of these two types of agents, and may prove useful in determining if cyclic AMP can augment humoral immunity to T-dependent antigens via an anti-proliferative mechanism.

The effects of various agents tested in this study have been discussed in terms of their effects on cellular proliferation; however, it is clear that these agents can also exert effects on the induction of humoral immunity via other mechanisms. Because cyclic AMP, colchicine and colcemid, ARA-C, and HU may exert effects on lymphocyte activation, differentiation, and proliferation, as well as on the synthesis and secretion of antibody and humoral factors, such effects will be considered in conjunction with a proposed model for drug effects on humoral immunity (Figure 25). This model deals only with the effects of pharmacological agents on B cells and T cells, because of insufficient evidence to rationalize the role of macrophages in immune induction. It is realized that macrophages are a potential site for drug action.

Following discussion of the evidence to support the proposed model, drug effects will be considered in terms of possible experiments that may help resolve the mechanism(s) by which these agents modulate humoral immunity. The evidence to support this model is as follows:

1) B cells are cells that are precursors of antibody producing cells (APC). These cells are probably non-proliferating, G_0 growth-arrested, prior to antigen activation (Discussed in Watson, 1975).

2) The early stage of immune induction begins when antigen (Ag) triggers the B cell, but there is a 24 hour lag period until the B cell proliferates (Dutton and Mishell, 1967).

3) Ag triggers suppressor T cells (T_s) to proliferate during the early stage. T_s cells are either inactive or they are not present in sufficient numbers to exert their effects before they proliferate. This is a stage of T_s-cell expansion (This study). Other agents may by cytotoxic for these cells on the basis of their proliferation during this time period (Chiorazzi, et al 1976; Dixon and McConahey, 1963; Taliaferro and Taliaferro, 1951).

4) Active suppressor T cells (T_s) secrete suppressive factors which modulate B cell responsiveness (Tada, et al, 1975; Tadakum, et al, 1976; Feldman, et al, 1974; Thomas, et al, 1975; Kapp, et al, 1976). A

suppressive factor has been shown to be active only during the first 48 hours of culture (Kapp, et al, 1976); therefore, T_s may exert its effects during an early stage of immune induction. Agents that affect the secretion of suppressor factor(s) should be able to augment humoral immunity.

5) Agents that elevate cyclic AMP may act directly on B cells (Kishimoto and Ishizaka, 1976; Watson, et al, 1973 and 1975; this study). From these studies, drug effects on macrophages cannot be excluded.

6) Following antigen activation and lag phase, B cells start to proliferate; in this stage, B cells are sensitive to anti-proliferative agents (Dutton and Mishell, 1967; this study). B cells proliferate in the absence of T cell help (Dutton, 1975).

7) Helper T cells (T_H) exert their effects on B cells via secretion of helper factors (Dutton, et al, 1971; Schimpl and Wecker, 1972; Gorcynski, et al, 1973; Watson, et al, 1973; Armerding and Katz, 1974; Rubin and Coons, 1972; Harwell, et al, 1976; Feldmann and Basten, 1972; Munro, et al, 1974; Tada, et al, 1973). Inhibition of secretion of helper factors would be expected to result in inhibition of humoral immunity.

8) Helper T cells (T_H) proliferate during a late stage of immune induction (Araneo, et al, 1976 a,b).

9) Agents that inhibit secretion of antibody from APC result in inhibition of humoral immunity (Melmon, et al, 1974; Teplitz, et al, 1975).

The results of this study show that agents that have the ability to inhibit the proliferation of cultured lymphocytes produce augmentation when they are present only during an early stage of immune induction. Evidence has been presented that such augmentation may result from disinhibition of humoral immunity; that is, that these agents act by removal of a component in the induction of humoral immunity that normally limits the degree of responsiveness. It is suggested that these agents may act by removing the influence of suppressor T cells by virtue of the proliferation of such cells during the early time period. It is clear that disinhibition could be achieved by several different biochemical mechanisms. For instance, agents that are selectively cytotoxic for T_s cells or agents that inhibit the secretion of humoral suppressive factors could also account for the effects seen in the present study. Further experiments are needed to resolve whether effects other than simple inhibition of proliferation can account for the augmentation by various agents seen in these experiments.

I feel that two lines of investigation may prove lucrative in distinguishing the mechanism(s) by which

agents that elevate cyclic AMP and anti-proliferative agents augment humoral immunity to T-dependent antigens. These experiments are briefly summarized on the basis of the particular cell type that might be affected. The following experiments depend upon the availability of specialized methodologies and reagents that have been heretofore unavailable. Recent advances in cellular immunology make it likely that such experiments may be able to be performed in the near future, if not at the present time.

1) Can anti-proliferative agents exert augmentation of responses to T-dependent antigens via an effect on the induction or expression of suppressor T cells? The results of the present study suggest that the answer to this question will be affirmative. However, it is unclear whether agents that elevate cyclic AMP can act via this mechanism. The most conclusive experiments to resolve these points would be to specifically remove T_s cells and then to examine the effects of the various agents. Techniques are now available to perform such experiments. For instance, Cantor and Boyse have shown that T_s cells can be specifically lysed by an anti-Ly 2,3 antisera (Cantor and Boyse, 1975). Spleen cells that have been depleted of T_s cells are capable of mounting humoral immune re-

sponses to T-dependent antigens; therefore, drugs that act via a T_s cell mechanism would be found to be without activitiy in such an experiment.

If drugs are found to exert effects via a T_s cell mechanism, it would be interesting to know if their effects were exerted by disrupting the induction or the expression of such cells. The effects of drugs on induction could be tested in a transfer experiment of primed T_s cells. Thus, it might be shown that T_s cells can be induced to suppress humoral responses, and that this ability is diminished when an agent is present only during the priming stage of the T_s cells.

Examination of the effects of drugs on the expression of T_s might take advantage of the observations that T_s cells exert suppressive effects via the secretion of suppressor factors. Given sufficient methodology, agents might be tested for the ability to inhibit either the secretion or the activity of such factors.

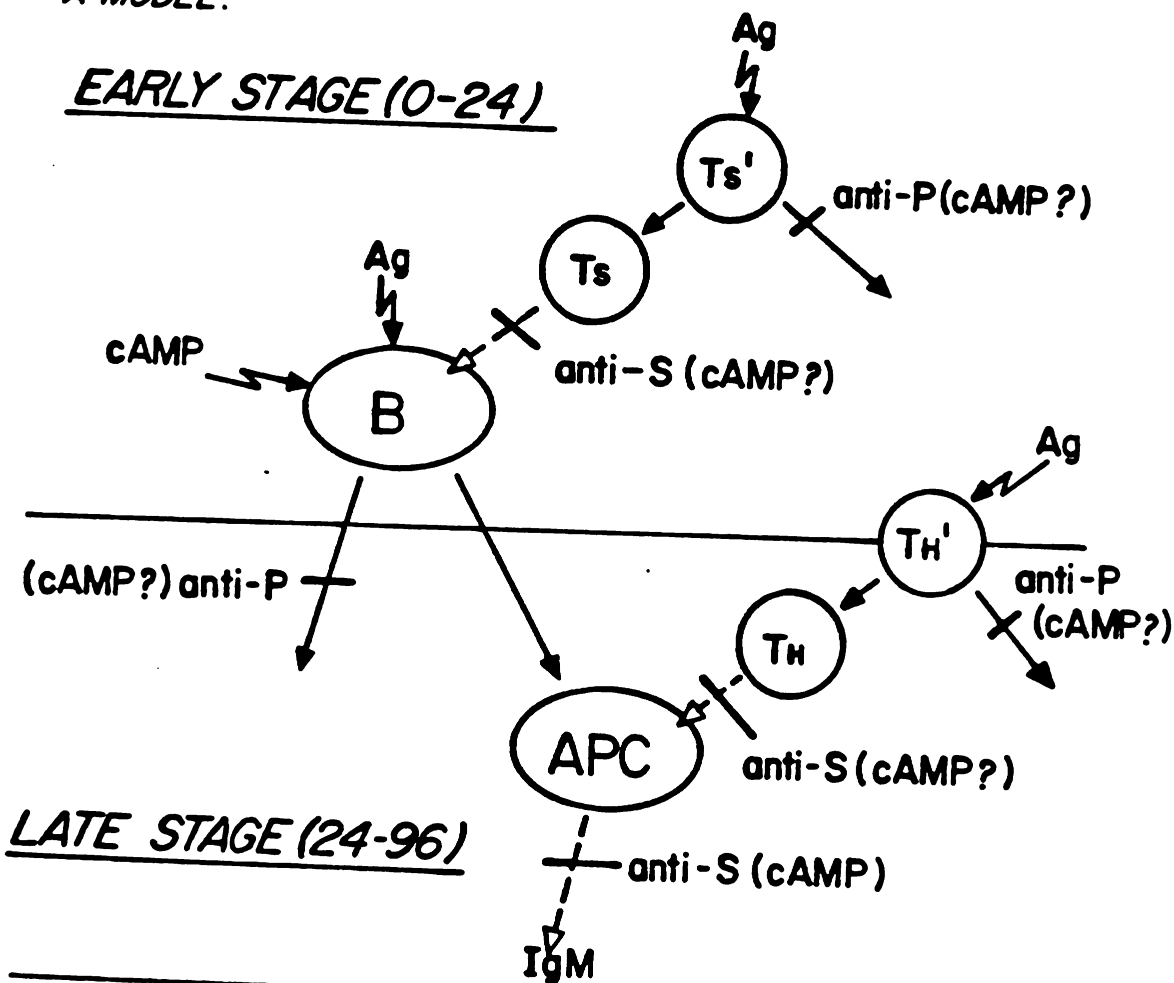
Similar experiments to those described for the resolution of drug effects on T_s cells could be used to determine if various agents can exert effects on the induction or expression of helper T cells.

2) Do anti-proliferative agents and/or agents that elevate cyclic AMP exert effects on the B cell or the macrophage population of spleen cells to augment humoral immunity? The results of the present study show that both ARA-C and DBcAMP can act on the T-cell deficient, B cells and macrophages (BM), population of spleen cells, yet ARA-C and DBcAMP exert apparently different effects. I feel that separation-reconstitution experiments of T and B cells with macrophages may help resolve whether anti- proliferative agents and agents that elevate cyclic AMP can augment humoral immunity via a common mechanism. Studies on the role of macrophages in immune induction have been hampered by the inavailability of sufficient methodology to specifically remove these cells from lymphocyte populations. If sufficient methodologies can be developed, these studies should prove to be most lucrative. Again, if drug effects can be localized to the macrophage population of cells, it would be of interest to determine if drugs were exerting effects on the induction or the expression of these cells.

I feel that resolution of the particular cell type involved in the stimulation of humoral immunity by various pharmacological agents will help define the roles of such

cells in the induction and the modulation of the humoral immune response. Such studies will also likely provide information concerning possible opportunities for the therapeutic use of pharmacological agents in modulation of the immune system.

PHARMACOLOGICAL MODULATION OF HUMORAL IMMUNITY: A MODEL.



PROLIFERATION (P)
SECRETION (S)
TRIGGERING (ACTIVATION)

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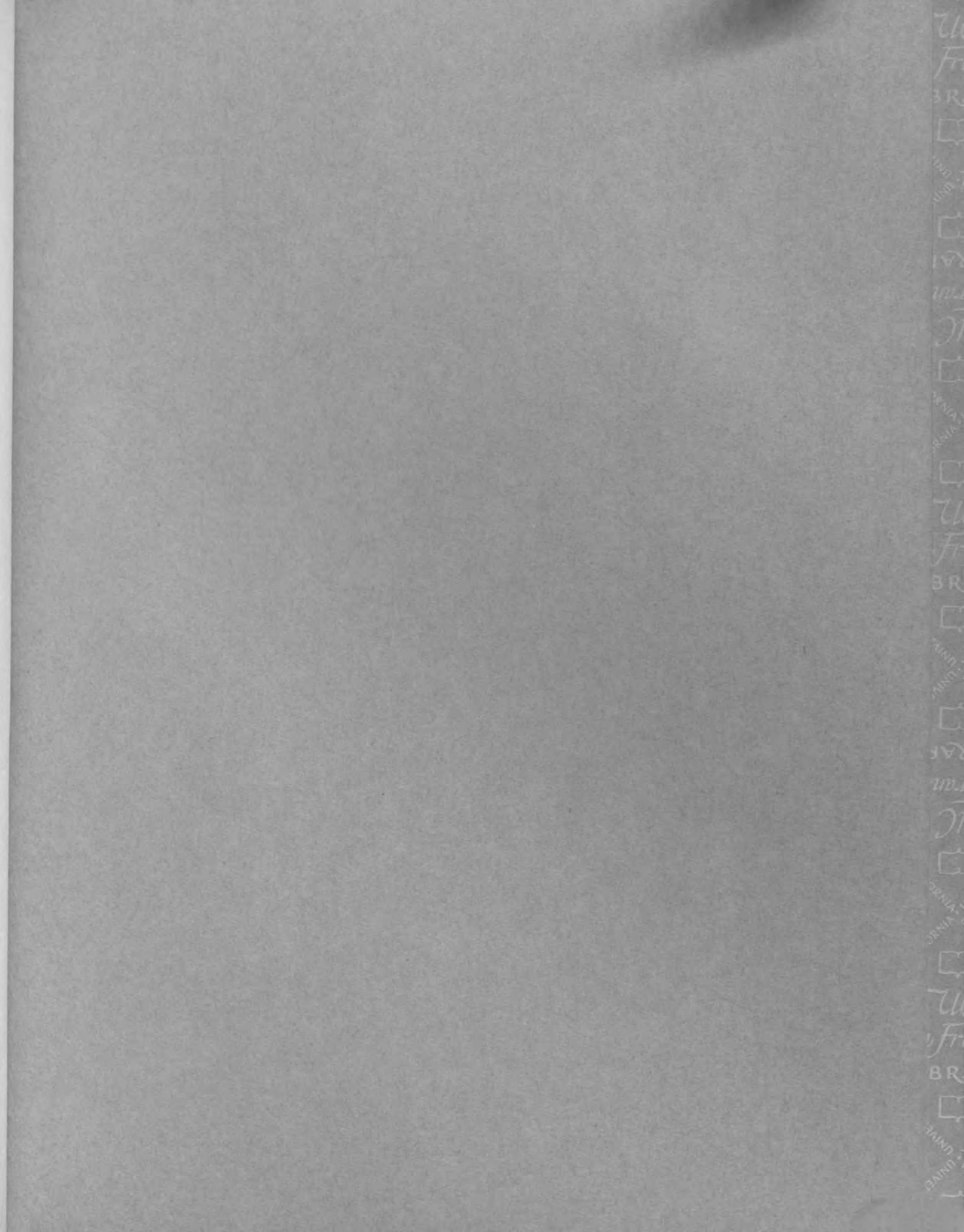

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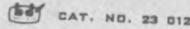

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