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LA THÈSE A ÉTÉ
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STIMULATION IN THE MURINE MIXED LEUKOCYTE REACTION

by

Chitra Roy

A thesis
submitted to the School of Graduate Studies and Research
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in
the Department of Biology

Ottawa, Canada, 1984



UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

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To Hiten, Arka and Kalapi

ACKNOWLEDGEMENTS

I thank Gordin Kaplan for giving me an opportunity to come back to scientific research and for guiding me throughout the course of this study. His inspiration and affectionate encouragement have made this work an enjoyable experience.

I am grateful to Dave Brown for making it possible for me to continue my research in his laboratory, in Ottawa, after Gordin left the city. His advice on the present project and on the preparation of this thesis is greatly appreciated.

I thank Mike McBurney for his suggestions and encouragement, which have been of great help, particularly during my "teething period". The discussions with Harold Rode of McGill University have been very valuable in formulating certain parts of this project. I thank Bruce Elliot of Queens University, Wayne Lapp of McGill University, Trevor Owens of University College London, Verne Paetkau of University of Alberta and George Setterfield of Carleton University for their interest in my work and for their gifts of technical facilities. The constant support of the members of the Biology Department, particularly that of John McNeill and Bernard Falloqene, is gratefully acknowledged.

It would have been difficult to accomplish this work without the goodwill and friendship of my colleagues in this department and that of Christine Bouzah and Wenda Greer in Gordia's laboratory in Edmonton.

I thank Connie Graveline and Judy Little for maintaining the general laboratory supplies and Nino Cavacciuti and Andre Richer for looking after my mice.

I thank Micheline Paulin-Levasseur for helping me with the French translation for the Resume in this thesis. I thank also Jacques Helie, George Ben-Tchavtchavadze and Paul Brunon for their expert help with the figures and plates. The efficient attention of the consultants in the Computer Centre helped me with the typing of this thesis.

I thank the Government of Ontario for awarding me the scholarships for this study.

ABSTRACT

In order to elucidate the stimulating mechanism in mixed leukocyte reaction (MLR), some of the features of the stimulatory population have been characterized. It has been established that a population of leukocytes can induce MLR optimally only when it is able to undergo activation in response to a stimulus, such as Con A. Since the stimulators are unable to undergo DNA replication (by operational definition), two of the relatively early events of activation, which precede DNA synthesis, were examined. One of these is the increase in potassium (K^+) influx that occurs about 6-8 hours post stimulation in murine lymphocytes (Owens and Kaplan, 1980) and the second is the formation of blasts, characterized by increase in RNA synthesis and in cell and nuclear volume (Setterfield et al, 1983).

On treating the stimulators with sublytic doses of a variety of agents such as ouabain, ultraviolet radiation (UV), 5-fluorouracil (5-FU) and colchicine, it was found that only those treatments which allowed the cells to demonstrate the initial (pre-S) responses of activation enabled the pre-treated cells to induce optimal proliferation in responders. It was noted that the process of activation has to proceed upto a stage where small blasts start to appear, when new

RNA is known to be transcribed (Mitchell, et al, 1978). The correlation observed between stimulating ability and Con A responsiveness was found to exist also in splenocytes immunosuppressed *in vivo* in mice induced with GVH reaction; these cells did not show increased K^+ transport in the presence of Con A and their stimulating capacity in MLR was inhibited. These results indicated that a population can stimulate in MLR optimally when they themselves can undergo activation.

That the stimulator cells do actually undergo activation in response to alloantigens of the responders was detected morphologically. This was shown by using fluorescein-labelled monoclonal antibody identifying the T cells of the stimulating population (primary target for the alloantigens of the responders) and demonstrating that these labelled cells showed increase in cell size and in RNA synthesis (incorporation of 3H -uridine). Up to one-third of the total blasts in the mixed leukocyte culture (MLC) were from the mitomycin C-treated stimulating population, when stimulator to responder ratio was at 2:1.

It was then shown that the presence of T cells in the stimulators is necessary for inducing optimal proliferation in the MLR. Depletion of T cells by treatment with monoclonal antibody and complement reduced the level of incorporation of 3H -thymidine in the responding population; the reaction could be restored by adding to the culture T cells from the stimulators but not from the responding population.

The stimulator-T cells have been found to regulate the level of interleukin 2 (IL 2) in the MLC. Mitomycin C-treated leukocytes themselves can produce IL 2 and they can affect the production of IL 2 in the responding population. The amount of IL 2 detected was reduced when the stimulating population was depleted of T cells or when its activation was prevented by treatment with UV. When the stimulating population lacked T cells, the culture supernatant contained a growth factor that had IL 2-like activity but which appeared to require DNA synthesizing responder cells for its detectable production; since IL 2 is known to be produced by cells in G1, this growth factor may not be IL 2.

It has been concluded that for optimal proliferation in MLR the stimulating population requires the presence of activated T cells which can produce IL 2 themselves and which also regulate the production of growth factors in the responders. These results are consistent with a model in which the metabolically active stimulators themselves undergo activation, leading to the amplification of the specific immune responses induced by the transplantation antigens.

RESUME

Afin d'expliquer le mécanisme de la stimulation au cours de la réaction leucocytaire mixte (MLR), nous avons caractérisé quelques unes des propriétés de la population stimulatrice. Nous avons établi qu'une population de leucocytes ne peut induire une MLR de façon optimale que lorsqu'elle peut être activée sous l'action d'un stimulus, tel que la ConA. Puisque la répllication d'ADN ne peut avoir lieu dans les stimulateurs (par définition opérationnelle), nous avons étudié deux des processus se produisant relativement tôt durant l'activation, précédant la synthèse d'ADN. Le premier processus est la hausse de l'influx du potassium (K^+) qui survient environ 6 à 8 heures après la stimulation dans les lymphocytes de souris (Owens et Kaplan, 1980) et le second est la formation de blastes, caractérisée par une augmentation de la synthèse d'ARN ainsi que du volume cellulaire et nucléaire (Setterfield et collab., 1983).

En traitant les stimulateurs avec des doses sublytiques de différents agents tels que la ouabaine, les radiations ultra-violet (UV), le 5-fluorouracil (5-FU) et la colchicine, nous avons trouvé que seuls les traitements laissant les cellules manifester les réactions initiales (pré-S) de l'activation permettaient aux cellules prétraitées d'induire une prolifération optimale chez les répondants. Nous avons noté que le processus d'activation doit se dérouler jusqu'au stade où de petites blastes commencent à apparaître

alors que, comme on le sait, le nouvel ARN est transcrit (Mitchell et collab., 1978). Nous avons également retrouvé cette corrélation entre la capacité à stimuler et l'activation par la ConA dans des splénocytes immunosupprimés in vivo de souris subissant une réaction GVH; ces cellules ne montraient pas de transport accru de K^+ en présence de ConA et leur capacité à stimuler lors d'une MLR était inhibée. Ces résultats ont montré qu'une population peut stimuler de façon optimale dans une MLR quand elle peut elle-même être activée.

Nous avons morphologiquement observé que les cellules stimulatrices sont en fait activées par les alloantigènes des répondeurs. Cela a été réalisé en utilisant des anticorps monoclonaux conjugués à la fluorescéine, pour identifier les cellules T de la population stimulatrice (première cible pour les alloantigènes des répondeurs), et en démontrant que ces cellules marquées ont des dimensions cellulaires et une synthèse d'ARN (incorporation de 3H -uridine) accrues. Jusqu'à un tiers de toutes les blastes dans la culture leucocytaire mixte (MLC) appartenaient à la population stimulatrice traitée à la mitomycine C, alors que le rapport stimulateur/répondeur était 2:1.

Nous avons ensuite démontré que la présence des cellules T parmi les stimulateurs est nécessaire pour induire une prolifération optimale dans la MLR. L'élimination des cellules T par traitement avec des anticorps monoclonaux et le complément réduisait le taux d'incorporation d' 3H thymidine

dans la population répondante; il était possible de rétablir la réaction en ajoutant, à la culture, des cellules T provenant des stimulateurs mais non pas de la population répondante.

Nous avons observé que les cellules T stimulatrices contrôlent le niveau d'interleubine 2 (IL2) dans la MLC. Les leucocytes traités à la mitomycine C peuvent-eux-mêmes produire IL2 et peuvent influencer la production d'IL2 dans la population répondante. La quantité d'IL2 détectée était réduite lorsque la population stimulatrice ne contenait pas de cellules T ou lorsque l'on en inhibait l'activation par traitement aux UV. En absence de cellules T dans la population stimulatrice, le surnageant de la culture contenait un facteur de croissance possédant une activité semblable à IL2 mais qui semblait requérir les cellules répondantes synthétisa l'ADN pour une production détectable; puisqu'il est reconnu qu'IL2 est produit par les cellules en G₁, ce facteur de croissance peut ne pas être IL2.

Nous avons conclu que, pour une prolifération optimale dans une MLR, la population stimulatrice requiert la présence de cellules T activées qui peuvent elles-mêmes produire IL2 et qui, également, contrôlent la production de facteurs de croissance chez les répondants. Ces résultats sont en accord avec un modèle où les stimulateurs métaboliquement actifs sont eux-mêmes activés, menant à l'amplification des réponses immunitaires spécifiques induites par les antigènes de la transplantation.

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Abbreviations

BSA	bovine serum albumin
Con A	concanavalin A
cpm	counts per minute
CTL	cytotoxic T lymphocyte (pCTL, pre-CTL)
EDTA	ethylenediaminetetraacetic acid
FITC	fluorescein isothiocyanate
5-FU	5-fluorouracil
GVH	graft-versus-host
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HTL	helper T lymphocyte (pHTL, pre-HTL)
IFN- γ	gamma (or immune) interferon
Ig	immunoglobulin
IL 1	Interleukin 1
IL 2	Interleukin 2
IL 3	Interleukin 3
MHC	major histocompatibility complex
MLR	mixed leukocyte reaction
Mls	minor lymphocyte stimulating locus
PBS	phosphate-buffered saline
PHA	phytohemagglutinin
SD	standard deviation
UV	ultraviolet radiation

Chapter I

INTRODUCTION

The allogeneic response is the process by which an animal recognizes and rejects foreign cells such as tumours and transplants. The invading cells which stimulate the generation of killer cells in the host are in turn lysed. This reaction, which is under the control of loci of the major histocompatibility complex (MHC), is mediated by leukocytes and constitutes one of the principal aspects of the immune response.

The mechanism of the allogeneic response can be better analyzed by studying its *in vitro* correlate, the mixed leukocyte reaction (MLR) (Bain *et al.*, 1964). Interaction *in vitro* of two populations of leukocytes bearing different histocompatibility antigens, or alloantigens, results in blast transformation and proliferation (MLR), accompanied by the generation of cytotoxic T cells (CTL) (Hayry and Defendi, 1970). The genetic aspect of the reaction is well established and has been reviewed (Klein, *et al.*, 1972; Klein, 1982; Dausset, 1981). In one-way MLR, one of the populations, the stimulators, is prevented from proliferating in culture by pre-treatment with mitomycin C (Bach and Voynow, 1966) or X-rays (Kazakura and Lowenstein, 1965). The cellu-

lar identity of the responding population has been clearly shown to be thymus derived (T) lymphocytes (Mosier and Cantor, 1971), which specifically recognize and respond to the stimulating cells.

It is commonly held that the stimulators are B cells (human) or macrophages and dendritic cells; the role of the T cells is controversial (Klein, 1982). However, the cellular requirement of the stimulating population and the mechanism by which it activates the cells in the responders is still not understood, nor in particular, why live cells are required, rather than metabolically inactive cells, or isolated membranes. Addressing these questions, in the murine system, forms the basis of this thesis.

This introduction will cover the subjects which are essential to the understanding of a study of murine MLR. It will consist of reviews on (1) the immune system, which includes the general components mediating MLR; (2) the immune response, elaborating the process of activation of T cells which is the consequence of MLR and which is implicated, in this thesis, to be involved in the stimulatory mechanism as well, and lastly (3) a composite picture of MLR.

1.1 IMMUNE SYSTEM

The immune system is responsible for defending an animal against foreign elements. It is a complex network of interacting cellular and soluble elements, the leukocytes and the cytokines, functioning under genetic control.

1.1.1 Leukocytes

Leukocytes, the white blood cells, constitute the cellular component of the body defence mechanism, both immune and non-immune. Functionally diverse cell populations of monocytes, granulocytes and lymphocytes comprise this group. Lymphocytes are the actual effectors in the immune system while the other leukocytes, particularly the macrophages and the dendritic cells are essential in the underlying reactions leading to the specific responses.

All vertebrates show immunologic reactivity. They have serum immunoglobulins, can immunologically recognize and destroy transplants of allogeneic tissues and can display some form of immunological memory.

Origin and differentiation: Leukocytes are found in large numbers in lymphoid organs and in smaller numbers in the stroma of many organs and in the blood. The pluripotent stem cells of the bone marrow differentiate into specialized or "committed" colony-forming units that are progenitors of the lymphoid and erythroid-myeloid series and these in turn give rise to the fully-differentiated circulating blood

cells, of which lymphocytes, macrophages and dendritic cells are of main interest to students of the mixed leukocyte reaction.

1.1.1.1 Lymphocytes

Burnet (1969) has said that the most important function of lymphocytes is to maintain the genetic integrity of the body; they constitute a part of the "immunological surveillance" mechanism the function of which is to eliminate antigenically distinct clones of cells before they can multiply and spread. The lymphocyte is responsible for the primary recognition of antigen as well as being an immunologically specific effector cell in the elimination of infectious agents, grafts and tumors (Murphy, 1926; Medawar, 1958). Making up 30% of the total white blood cell count, the lymphocytes are widely distributed in the body; in blood, peritoneal fluid, lymphatic channels, lymph nodes, gastrointestinal lymphoid tissues, thymus, spleen and bone marrow.

Morphology: All quiescent lymphocytes are morphologically homogeneous. These cells are small (7 to 8 um) and round, with a large nucleus to cytoplasm ratio. The cytoplasm is rich in free ribosomes and contains a number of mitochondria, a small Golgi zone, few lysosomes, a few microtubules and microfilaments and has but little endoplasmic reticulum and other organelles.

Specific antigens and non-specific mitogens can stimulate such quiescent lymphocytes to proliferate and differentiate to large blast cells which can further differentiate into effectors of cellular and humoral immunity.

Classification: Lymphocytes are very heterogeneous in their origin and function. The main functional groups are thymus-derived lymphocytes (T cells) and bone-marrow derived lymphocytes (B cells). This review will concentrate on T cells because of their essential role in MLE.

I. T cells

T cells are a highly diverse group of cells that function in the effector and regulatory phases of the immune response. Rapid progress in the understanding of this group of cells has been made possible because of the development of the monoclonal antibody techniques (Kohler and Milstein, 1975) and the development of the technology to propagate long-term lines of non-transformed antigen-specific T cells (Hengartner and Fathallah, 1978; Baker et al., 1979; Glasebrook and Fitch, 1980).

Origin and development:

The generation of T cells is thymus dependent. During ontogeny the earliest T cell precursors migrate to the thymus from the fetal liver. In the adult the committed stem cells of the bone-marrow proliferate in the thymus, resulting in the production of typical small lymphocytes which are immunocompetent (Beller and Unanue, 1978).

When the immunocompetent T-lymphocytes from the thymus are released into the circulation, they are localized in thymus-dependent areas of other lymphoid organs and they can then react with antigens, mitogens or other factors and undergo a second stage of differentiation and proliferation to form the functional populations of T cells responsible for various regulatory and effector functions (Sprent and Miller, 1971).

Surface structures: Since immune reactions are initiated at the cell surfaces of the leukocytes, the composition of cellular membrane has been quite extensively studied. Using monoclonal antibodies, several surface molecules have been identified and their corresponding genes have been discovered (Collins and Kinnon, 1984).

The T cells are characterized by a number of cell-surface markers which serve as identifying features of the various subpopulations showing functional specialities. Some of the more widely studied markers in the murine system have been described here.

(1) Theta antigen: The thymocyte and T cell phenotype are characterized by the Thy 1 (or theta) antigen which is a surface component of all subsets of T cells (as well as being present on some brain and epidermal cells) but is completely absent from the B cell line. Thy 1 appears after pro-lymphocytes encounter thymopoietin during the differentiation process in the thymus. Two allelic genes control-

ling this marker exist; AKB, RF and some other strains bear Thy 1.1. C3H and most of the other laboratory mouse strains used in research are Thy 1.2 (Reif and Allen, 1964; Raff, 1970). Mere presence of Thy 1 does not assure immunocompetence of T cells (Seddik et al., 1980).

(2) TL: Immature thymocytes and some leukemic cells but not mature peripheral T cells, carry a thymus leukaemia antigen (TL) that marks a transient phase of differentiation (Boyse and Old, 1969).

(3) Lyt series: The Lyt series, as identified by alloantisera, define functional subsets of the T cell components. A Lyt-1,2,3⁺ phenotype, that also expresses Thy-1 and receptors for specific antigen, becomes "committed" to its specific T cell function following the loss of either Lyt-1 to become a cytotoxic or suppressor T cell (Lyt-2,3⁺) or the loss of Lyt-2 and Lyt-3 to become a helper T cell (Lyt-1⁺) (Cantor and Boyse, 1978). It has, however, been shown recently that suppressor T cells do not form a homogeneous group (Hodes, 1982). Okada and Henney (1980) and Swain et al. (1981) have proposed that Lyt phenotype may correlate more directly with the antigen specificity of the T cells than with their class of function.

(4) Mitogen receptors: There is evidence that separate populations of cells may respond to the mitogens Con A and PHA (Stobo et al., 1972). Only Con A but not PHA can activate splenic helper T cells directly, as shown by anti-Lyt

sera and complement treatments. Con A binds glucose, mannose, glucosamine and other structurally related sugar residues of membrane glycoproteins (Lis and Sharon, 1973) whilst other lectins have different binding specificities. PHA binding, for instance, is specific for N-acetylgalactosamine.

(5) Antigen receptor: The mystery of the T cell receptor has at last yielded to experimental attack after many inconclusive attempts to characterize them. The gene for the T cell receptor has now been discovered (Williams, 1984; Yanagii *et al.*, 1984). T cells, functioning either as helpers, suppressors or killers, appear to show clonal recognition of specific antigens (Klein, 1982). T cells respond exclusively to antigens present on the surface of other cells, they cannot directly bind soluble or particulate antigen that has not been processed by macrophages. Using monoclonal antibodies, it has been found that the antigen receptor consists of two polypeptide chains linked together by disulfide bonds (Reinherz *et al.*, 1983). The molecules vary from clone to clone and this peptide variability may account for the antigenic specificity. The recognition of foreign antigens by T cells is MHC restricted (Zinkernagel and Doherty, 1979); simultaneous recognition of the antigen and self MHC antigen on the antigen presenting cell is required (Zinkernagel and Doherty 1979; Klein, 1982).

FUNCTIONS: T cells are of crucial importance in the immune system. They serve not only as effectors against foreign cells, they regulate the immune network. Three major subclasses of T cells are recognized, each programmed to express a characteristic set of immunological responses.

(1) Helper T cells (HTL): Lyt 1⁺ cells, comprising one-third of the peripheral T cell population are programmed to help or amplify functional activities of other cells after stimulation by antigens associated with class II MHC antigens (I-region in mouse) (Cantor and Boyse, 1978). Activated Lyt 1⁺ cells mediate their help through factors which they have been shown to release in the MLC or on stimulation with mitogens. Their amplifying effects have been demonstrated in relation to three target cells: (1) B cells, for antibody production; (2) cytotoxic T cells, for initiating DNA synthesis (3) macrophages and monocytes, for inducing activation (Larsson, 1982).

(2) Cytotoxic T cells (CTL): They arise during the course of cell-mediated immune responses to allografts and to certain virus-infected cells. Generation of CTL was first described *in vivo* where mice were immunized with allogeneic tumour cells or in graft-versus-host (GVH) reactions (Cerottini and Brunner, 1974). (GVH reaction is induced when immunocompetent lymphoid cells are inoculated into a host incapable of reacting against them; the grafted cells proliferate and produce CTLs in response to antigens on the

host's cells which they recognize as being foreign). The differentiation pathway of CTLs has been elucidated mainly from *in vitro* studies of mixed leukocyte reactions (MLR) (This has been described in section 1.3 of this Introduction).

CTLs are effective in tumour and allograft rejection *in vivo* (Cerottini and Brunner, 1974) and in the lysis of allogeneic (Engers and MacDonald, 1976) and virus- or hapten-modified syngeneic target cells *in vitro* (Doherty and Zinkernagel, 1975).

(3) Suppressor T cells: They constitute one of the major cellular components of the immune network. They are induced as a direct or indirect consequence of antigenic stimulation and exert their functions by reducing the amplification signal provided by helper T cells or by acting directly on effector B cells or CTLs (Cantor and Gershon, 1979). These suppressor cells are composed of subpopulations bearing different Lyt phenotypes which are functionally distinct from one another and which interact with one another in highly ordered and complex fashion (Hodes, 1982).

Genetic restriction of T cell functions: All MHC restricted reactions are T cell dependent responses and cell interactions involving functionally different T cell subclasses are restricted by various elements in the MHC. This raises the possibility that T cell functions are mediated via MHC products. Restrictions are controlled by the I-region gene products (Klein, 1982).

Zinkernagel and Doherty (1979) have demonstrated the importance of the thymus in conferring on T cells the ability to recognize antigens in association with particular MHC products. Thymic selection is necessary but not sufficient for full development of MHC recognition capabilities.

II. B cells

B cells are the effectors of humoral immunity; mediating protection through antibody functions. Characterized by surface immunoglobulins (Ig) which can be readily detected by immunofluorescence (Greaves, 1970; Raff, 1970), they are the precursors of immunoglobulin-secreting plasma cells. Surface Ig bearing cells comprise 10 to 20% of lymph node and 25 to 35% of spleen; there are none in the thymus. They develop from stem cells that originate in the fetal bone marrow or the liver. The site of B-cell differentiation may be in fetal bone liver, the gastrointestinal lymphoid tissue or in peripheral lymph node; in birds it is the bursa of Fabricius (Abney et al., 1978). B cell maturation is antigen-driven (Kishimoto and Ishizaka, 1972); they recognize antigens through the surface Ig molecules. The particular antibody produced in response to the antigen reflects the antigenic binding specificity of the original B cell receptor (Paul, 1970). B cells have been implicated in antigen-presentation functions (Chestnut et al., 1982) and are thought to act as stimulators in an MHC, particularly in the human system (Klein, 1982).

1.1.1.2 Macrophages

Structure: The macrophage is the largest cell amongst the leukocytes, ranging from 12 to 20 μm in diameter. The nucleus is centrally located and has a bilobed kidney shape with considerable peripheral condensation of nuclear chromatin. The cytoplasm contains a great variety of organelles, including endoplasmic reticulum, a Golgi complex, mitochondria, ribosomes and phagocytic vacuoles.

Origin and differentiation: A specific colony-stimulating factor induces myelopoiesis from bone marrow stem cells; 73 distinct protein changes occur during the sequence of differentiation (Hume and Weideman, 1980).

Function: The primary function of the macrophages is phagocytosis. However, they have an essential role in the immune response (Moller, 1978). The following immunological functions have been recognized:

(1) Antigen presentation: The uptake of antigen by the macrophage is the usual first step in the chain of immune reactions. The antigen is then processed metabolically (Germain, 1981) and presented to a specific antigen recognizing cell.

(2) Regulation of the immunoregulatory circuit: This is achieved through the release of over 60 different substances (Adams and Nathau, 1983). These products perform a variety of biological functions. One of primary importance is interleukin 1 (see subsection 1.2.1).

1.1.1.3 Dendritic cells

They are irregularly shaped cells that adhere to glass and plastic and found in spleens and various organs in mouse, rat and man so far (Steinman and Nussenzweig, 1980; Van Voorhis *et al.*, 1982). They are Ia⁺, Ig⁻, Thy 1⁻ bone marrow derived elements that show little endocytic activity. They occur in low frequency accounting for less than 1% of the cells in all organs studied. Effective in very small numbers, they stimulate allogeneic and syngeneic MLR and serve as accessory cells for the development of *in vitro* immune responses: Steinman and Nussenzweig (1980) found that their removal by monoclonal antibody from stimulators in MLC reduced the stimulating capacity by 75 to 90% and the function could be restored by adding them back (0.5% of total cells).

1.1.2 Cytokines

Cytokines are a group of regulatory and effector substances, usually glycoproteins, which exhibit a variety of biological activities at low concentrations, in the 10^{-10} to 10^{-15} M range (Oppenheim *et al.*, 1981). They are produced by a wide variety of cell types including lymphocytes, macrophages, platelets, fibroblasts and keratinocytes. They are hormone-like. The biological activities of these factors are immunologically nonspecific and genetically unrestricted. They act on leukocytes, osteoclasts and endothelial cells.

The factors most extensively encountered in immune responses are interleukins 1 and 2.

1.1.2.1 Interleukin 1

Macrophages can produce a factor known as interleukin 1 (IL 1) (Aarden et al., 1979) when activated by a wide variety of stimulants. The biological role of this monokine, cytokine released by monocytes, consists of: (1) enhancement of proliferation and differentiation of T lymphocytes (including induction of receptors); (2) production of antibody by B lymphocytes and (3) production of lymphokines, soluble products released by lymphocytes. There are additional roles of IL 1 associated with fever centres. It is a polypeptide of M.W. 12,000 which has biological activity at 10^{-11} M (Mizel and Mizel, 1981).

1.1.2.2 Interleukin 2

Interleukin 2 (IL 2) (Aarden et al., 1979) is a genetically unrestricted, soluble immunoenhancing factor which is produced by helper T cells following stimulation with T cell mitogens (Baetkau et al., 1976; Watson et al., 1979) allogeneic cells (Gillis et al., 1978; Shaw et al., 1978; Wagner et al., 1980), tumour cells (Baker et al., 1978) and in syngeneic MLR (Lattime et al., 1981). It has not yet been shown if IL 2 is produced and secreted following specific antigen stimulation.

The generally accepted biological assay for IL 2 measures the factor's ability to maintain the growth of factor-dependent cytotoxic cell lines (Gillis and Smith, 1977). Several other biological activities have been ascribed to IL 2 (Primi *et al.*, 1979; Watson *et al.*, 1979). It has been reported to (1) induce thymocyte proliferation either in the presence (Chen and DiSabato, 1976; Paetkau *et al.*, 1976) or absence (Farrar *et al.*, 1978) of a suboptimal concentration of T cell mitogen; (2) augment the proliferation and generation of cytotoxic cells by alloantigen-stimulated T cell population (Wagner and Rollinghoff, 1978; Farrar *et al.*, 1978) and in the process, induce the synthesis of gamma interferon (IFN- γ) by the alloantigen-stimulated T cells (Farrar *et al.*, 1981); (3) act as a second signal in the activation of antigen-specific helper T cells and maintain these cells in long-term culture (Watson *et al.*, 1979); (4) augment the plaque-forming cell responses of nude mouse spleen cells to sheep erythrocytes (Farrar *et al.*, 1978; Watson *et al.*, 1979).

IL 2 is a glycopeptide of M.W. of 15,000 in man and 30,000 in mice (Shaw *et al.*, 1978; Watson *et al.*, 1979); the essential component of murine IL 2, however, is a peptide of M.W. 16,000 (Caplan *et al.*, 1981).

A study of the mechanism of IL 2 production shows that IL 2 is produced through an interaction of sensitized T-helper cells with activated macrophages (Larsson *et al.*, 1980; Shaw *et al.*, 1980; Palacios, 1982) or with IL 1 (Farrar *et al.*,

1980). Activated helper T cells produce IL 2 when they are in late G1, reaching a peak at 30 to 50 hours of mitogen stimulation in human cultures (Stadler *et al.*, 1980; 1981). Palacios (1982) noted its production at 6 hours when human leukocytes were stimulated with Con A.

IL 2 is absorbed and used not by resting cells but only by activated T cells in late G1, with maximal utilization in S phase (Bonnard *et al.*, 1979; Stadler *et al.*, 1981).

Additional lymphokines which are biochemically and functionally distinct from IL 2 but which perform similar immunoenhancing activities have been described (Farrar *et al.*, 1982). Among this group of related lymphokines are interleukin 1, colony stimulating factor, gamma interferon, B cell growth factor and T cell replacing factor (Farrar *et al.*, 1982; Prystowski *et al.*, 1982).

1.1.3 Genetic control

The immune system functions under the guidance of various genetic factors, particularly the major histocompatibility complex (MHC), a multigene complex (Benacerraf and McDevitt, 1972; Klein, 1982; Thorsby, 1983).

Minor histocompatibility antigens evoke comparatively weak transplantation reactions (Klein, 1982). They can stimulate the production of cytotoxic T cells and serve as their targets, provided the responding T cells and targets are syngeneic in expression of Class I MHC antigens.

1.1.3.1 Classification of MHC

The two most extensively studied MHCs are that of man, called HLA and that of the laboratory mouse, called H-2. In mice, the H-2 complex is located on chromosome 17 and in man it is on chromosome 6.

There are three broad classes of genes and molecules. Class I products, expressed on the membranes of all nucleated cells, are glycoproteins that serve as the principal targets of rejection and mediate the recognition and destruction of transplants, virus infected or neoplastic cells. They are the gene products of K, D and L loci in mice. Class II products are expressed principally on the membranes of lymphoreticular cells. To some extent they appear to determine whether an external stimulus is "self" or not. They are encoded by the I-region and the products are Ia antigens. Ia molecules mediate cellular interactions among T and B lymphocytes and macrophages by displaying the property of allelic restriction, that is only cells identical at the MHC interact successfully. Class III genes determine the structures of at least three discrete components of the complement system (a series of interacting serum factors instrumental in destroying and eliminating bacteria and other foreign cells).

In mouse, a number of additional genes, called Qa and Tla have been found. These are related in basic structure to Class I genes, and code for antigens of unknown function.

1.1.3.2 Products of MHC

The Class I gene products of man are membrane integrated glycoproteins of about 45000 molecular weight (MW) existing in situ in noncovalent association with the 12000 MW non-MHC encoded B2-microglobulin. They have been sequenced. In Class II antigens two types of Ia molecules are found on cells of most mouse strains. They consist of three polypeptide chains encoded by I-A and I-E subregions. The proteins encoded by the Class III antigens are C2, C4 and Bf, the early components of the complement system, which promote the uptake of foreign material by macrophages.

1.2 IMMUNE RESPONSE: T CELL ACTIVATION

The immune system responds to virtually any foreign molecular configuration in one or more of a series of specific ways. Different stimuli activate different classes of lymphocytes, activating only T- or only B- cells or both. The responses to the stimuli often require interactions of different subsets of lymphocytes. Activation leads to clonal expansion of T and or B cells, followed by differentiative processes which generate effector cells, the antibody secreting B cells and the cytotoxic T cells. It is possible that most of the T cell functions can be carried out by cells which have undergone the early stages of activation, a shift from G0 to G1, and that the synthesis of DNA leads to amplification of these responses. This has been shown with

suppressor cells (Shand et al., 1980). T-helper cells in G1 produce augmenting factors (Stadler et al., 1981; Palacios, 1982; Prystowski et al., 1982). MacDonald (1982) hypothesizes that the CTL may be produced by activation of its precursor by a process independent of DNA synthesis. Making a distinction between early activation and DNA synthesis is important in the context of the work presented in this thesis, where the term 'activation' will be used for early activation (G0 - G1 shift) and the term 'proliferation' for DNA synthesis (G1 - S shift). Kaplan (1983) has described the parameters characterizing these different stages of the cell cycle.

This introduction will discuss the processes of activation and proliferation of T cells; an understanding of these processes is essential to a discussion of MLP.

1.2.1 Components

1.2.1.1 T cells

All subpopulations of T cells are required to undergo activation for participating in immune functions. They respond to specific stimuli, increase in cell size, remodel their chromatin, synthesize DNA and undergo cell division. Each clone, constituting initially less than 0.1% of total lymphocytes, may be raised to 1-3% through cell proliferation (McConnel, 1975).

1.2.1.2 Stimulus

T cells are activated by antigens, alloantigens or modified syngeneic cells and polyclonal activators. They respond specifically to antigens and the antigen-specific receptors are clonally distributed (Mitchison, 1970; Reinherz et al., 1983). When T cells are activated by allogeneic cells, the process is called the mixed leukocyte reaction; this is described in a later section (section 1.3). The process of activation by the modified syngeneic cells is the same as that by allogeneic cells.

Polyclonal activators are widely used for *in vitro* studies of leukocyte culture which form a crucial aspect of immunological studies (Kaplan, 1979A). Even though the activation produced by polyclonal activators is non-specific and the recognition is independent of Ia restriction, the activation process constitutes the same series of events that leads to clonal expansion *in vivo* (Palacios, 1982). Several clones of T cells, 70 to 80% of all lymphocytes, irrespective of their antigen specificity can be activated (Ling and Kay, 1975). A large variety of agents is known to cause mitogenic activation *in vitro* (Moller, 1972); the most commonly used mitogens for T cell activation are the lectins Con A and PHA. In the murine system Con A is most extensively used. It is a plant lectin isolated from jack bean. It is a tetrameric protein with 4 binding sites. It binds to B and T cells but triggers only the T cells into activation (Greaves

and Janossy, 1972); B cells may be secondarily activated (Owens and Kaplan, 1982).

1.2.2 Genetic control of T cell activation

The genes in the MHC guide the functions of T cells, which can recognize antigens and respond to them by undergoing activation or they can interact with targets and exert effector function by simultaneous recognition of the antigen and I-region determinants on the same cells which present the antigen (Rosenthal and Shevach, 1973; Katz and Benacerraf, 1975; Zinkernagel and Doherty, 1979).

1.2.3 Changes associated with cell activation

The lymphocytes which respond to mitogenic or antigenic stimulus are in the quiescent or G0 stage of the cell cycle. They are characterized by small amounts of cytoplasm, a compact nucleus containing mostly heterochromatin and a low level of metabolic activity. Activation induces a train of events leading to morphological and biochemical changes which culminate in cellular proliferation and expression of differentiated properties.

1.2.3.1 Morphological changes

Some of the easily recognized changes are the following:

(1) Cell size: The cell diameter increases considerably within 24 hours; this may lead up to a three-fold increase during the maximum growth period.

(2) Nuclear changes: Setterfield *et al* (1983) have classified nuclei of resting and Con A stimulated human and murine cells into three morphotypes: I, unstimulated; II, partially-stimulated and III, fully stimulated. Between morphotypes I and III, the nuclear volume increases upto 6 fold, interchromatinic volume rising 10-fold and the chromatinic volume only 1.5 fold. The chromatin becomes disaggregated into small clumps from membrane associated aggregates. The change from morphotype I to III is dependent on RNA synthesis through the early part of the stimulation period (0-16 hour) and protein synthesis is also required. Morphotype II nuclei appear at around 13 hour when poly A(+) transcripts are first detected (Mitchell *et al*, 1978; Schaffer and Mitchell, 1979). The Con A stimulated change in nuclear structure precedes the onset of DNA replication and is independent of the effects of the drugs that inhibit G1/S transition (Setterfield *et al*, 1983).

(3) Cytoplasmic changes: A considerable increase in volume is seen so that increased ratio of cytoplasmic to nuclear volume is a distinguishing feature. The cytoplasm is richer in organelles, compared to the resting cells. There is an increase in microtubular components of the cytoskeleton (Brown *et al*, 1983; Waterhouse *et al*, 1983).

(4) Surface markers: During lymphocyte mitogenesis surface receptor repertoire is altered radically. Biochemical analysis of the cell surface antigens of murine T lympho-

cytes has revealed that several new proteins appear after activation of T lymphocytes in MLC or by stimulation with lectins such as Con A (for review see Bonavida *et al.*, 1982). A role for any of these proteins has not been clearly demonstrated. A change that might be of particular significance in immune functions is the change in Ia antigens; Letarte *et al.* (1980) have reported an increase of this antigen in Con A stimulated mouse lymphocytes and there are several reports of increased Ia expression on activated human T lymphocytes (Indiveri *et al.*, 1980; Schuurman *et al.*, 1980; Wollman *et al.*, 1980; Forre *et al.*, 1982).

1.2.3.2 Biochemical changes

They can be broadly classified into (A) membrane events, (B) intracellular changes, (C) initiation of DNA synthesis.

A. Membrane events:

(1) Transport of ions : It is one of the earliest detectable changes after stimulation.

(a) Divalent cations: Calcium plays a controlling role in the activation of lymphocytes (Bard, 1978; Whitfield *et al.*, 1982). Influx of Ca^{++} into lymphocytes has been noted within an hour of the addition of mitogen (Allwood *et al.*, 1971; Hesketh, 1979). A small, prolonged rise in the Ca^{++} level of the cytoplasm appears to be required for initiation and sustenance of lymphocyte activation (Hesketh, 1979). Involvement of calmodulin has been indicated by an early

doubling or tripling of the calmodulin level in the stimulated lymphocytes (Veitch et al., 1982). There is some evidence that calcium is needed as a cofactor for some critical enzymes in the cell membrane (Whitfield et al., 1982). It has been difficult to define the role of calcium in the activation process because the changes in the cytoplasmic free calcium are transient, due to its rapid sequestration by organelles, because of the multiplicity of Ca^{++} binding sites on the cell surface and because of the Ca^{++} exchangeable pools within the cell (Buzsaki and Weideman, 1980). Heterogeneity of the cells studied adds to the difficulty in the interpretation (Whitfield et al., 1982).

(b) Monovalent cations: The role of monovalent cations on cell activation has been reviewed by Kaplan (1978). One of the earliest signals of lymphocyte activation is the transport of monovalent cations across the membrane. PHA, at mitogenic concentrations, cause K^+ channels to open more rapidly, within one minute of addition of the mitogen (DeCoursey et al., 1984). In human lymphocytes a 2-fold increase in K^+ influx has been conclusively demonstrated within 30 minutes (Quastel and Kaplan, 19703; Averdunk and Lauf, 1975; Matteson and Deutsch, 1984). In Con A stimulated mouse splenocytes a 1.75-fold increase has been detected in 6 hours (Owens and Kaplan, 1980). This increase, which is maintained through the process of activation, is characterized by an increased V_{max} with no change in K_m . The flux

activation is sensitive neither to actinomycin D nor to cycloheximide (Quastel and Kaplan, 1970A). Potassium efflux has also been found to be doubled (Segal and Lichtman, 1976; Hamilton and Kaplan, 1977). To maintain the same intracellular K^+ concentration, in the enlarged lymphoblasts, which are seen at 16-20 hours post stimulation, and in the unstimulated lymphocytes, an increase in K^+ influx compared to the efflux should occur; however that has not been found. This paradox has been discussed by Kaplan (1978; 1979B).

The sodium pump, the enzyme that transports K^+ , is a magnesium-dependent $Na^+K^+ATPase$ (Skou, 1975), located in the plasma membrane. Its function is inhibited by the cardiotonic steroid, ouabain. It translocates across the plasma membrane 3 Na^+ ions from inside to outside and 2 K^+ ions from outside to inside, against electrochemical gradients at the expense of one molecule of ATP (Glynn and Karlish, 1975).

(2) Transport of other metabolites: Increases in transport of sugars, uridine and amino acids have been noted in the first 6 hours (O'Brien *et al.*, 1978). Protein synthesis is not required for the transport of ions, sugars or uridine; the already existing sites are activated by the mitogens (Mandelsohn *et al.*, 1971).

(3) Phospholipid metabolism: Altered lipid composition and altered membrane fluidity are among the early changes detected in the activation process (Ferber and Resch, 1977).

Activation of the enzyme lysolecithin acyltransferase is associated with enhanced turnover of membrane phospholipids (Kay, 1968; Resch et al., 1976).

(4) Polarization: There is an early (within 2-4 hours of mitogen addition) drop in membrane potential, which is followed by repolarization and hyperpolarization over the next 48 hours. The depolarization is due to binding of mitogen. The repolarization seems to be correlated with a secondary activation and possibly due to IL 2 action (Kiefer et al., 1983).

B. Cytoplasmic changes

(1) Protein and RNA metabolism: Protein synthesis is activated within 4 hours and continues to increase until 48 to 72 hours (Kay, 1968). It results from the translation of preexisting mRNAs (Jaques-Smith and Kay, 1976). Inhibitors of translation, present in the resting cells, are themselves inactivated immediately after mitogen binding. Increase in size of the activated lymphocytes, the blast, is dependent on this early protein synthesis (Schafer and Mitchell, 1979). Although no new RNA is formed until about 11 hours, there is processing of preformed mRNAs in the first 10 hours and they are exported to the cytoplasm (Mitchell et al., 1973). Transcription of new RNA starts at 12-14 hours. Concomitant with this is the formation of blasts which display the morphological features characteristic of activated cells (Setterfield et al., 1983).

(2) Intracellular second messenger: A second messenger is required to transmit the signal generated at the plasma membrane to the cytoplasm; cyclic AMP (cAMP), in conjunction with Ca^{++} uptake has been considered for this role (Whitfield et al, 1976). There is a transient rise in cAMP concentration in the the first 30 minutes, returning to control levels by 2 hours (O'Brien et al, 1978). Another increase in level occurs in late G1 and persists until mid-S phase (Wang et al, 1978; Foker et al, 1979). cAMP is thought to act as both negative and positive regulator of activation (Huxle and Weideman, 1980).

(3) Other changes: These include enhanced aerobic glycolysis and accumulation of polyamines and of biologically active products such as the cytokines.

The cytoplasmic changes described above lead to the formation of blasts and constitute the period of growth. This process differentiates the cell so that it can respond to a signal(s) initiating proliferation. Blast formation may be uncoupled from proliferation although the former is a prerequisite of the latter; inhibitors of DNA synthesis do not affect the integrity of the blast (Setterfield et al, 1983).

C. DNA synthesis

(1) DNA replication: DNA synthesis commences approximately 30 hours after stimulation as the cells enter S-phase. Maximum incorporation of 3H -thymidine occurs after 48-60 hours and falls rapidly thereafter. The increase in

DNA is preceded by increased activity of DNA replicating enzymes such as DNA polymerase, thymidine kinase, thymidilate kinase (Loeb et al., 1970; O'Brien et al., 1978).

(2) DNA repair: This is an early nuclear event that has been recently reported. Activation of lymphocytes induces repair of strand breaks that are present in the DNA of resting cells. This occurs within 2 hours of Con A stimulation in mice (Greer and Kaplan, 1983) and 8 hours in human (Johnstone and Williams, 1983). It is not yet known how this event is causally related to the mechanism of proliferative response. It is a necessary step; its inhibition suppresses proliferation.

1.2.4 Mechanism of T cell activation

The pathway of T cell activation has been illustrated in Fig. 1. Initiation of activation takes place at the membrane. Con A, which is not required to enter the cells (Bettel and van den Berg, 1972), can trigger stimulation by binding directly to the membrane, independently of Ia positive cells (Resch and Gensa, 1979; Larsson and Coutinho, 1979; Palacios, 1982). The antigens can initiate this step only in association with Ia bearing syngeneic accessory cells. All antigens can directly trigger responding T cells (Klein, 1982). All these stimulants arouse two populations of T cells, IL 2 acceptors and IL 2 producers. Both the populations follow two distinct sequential chains of events;

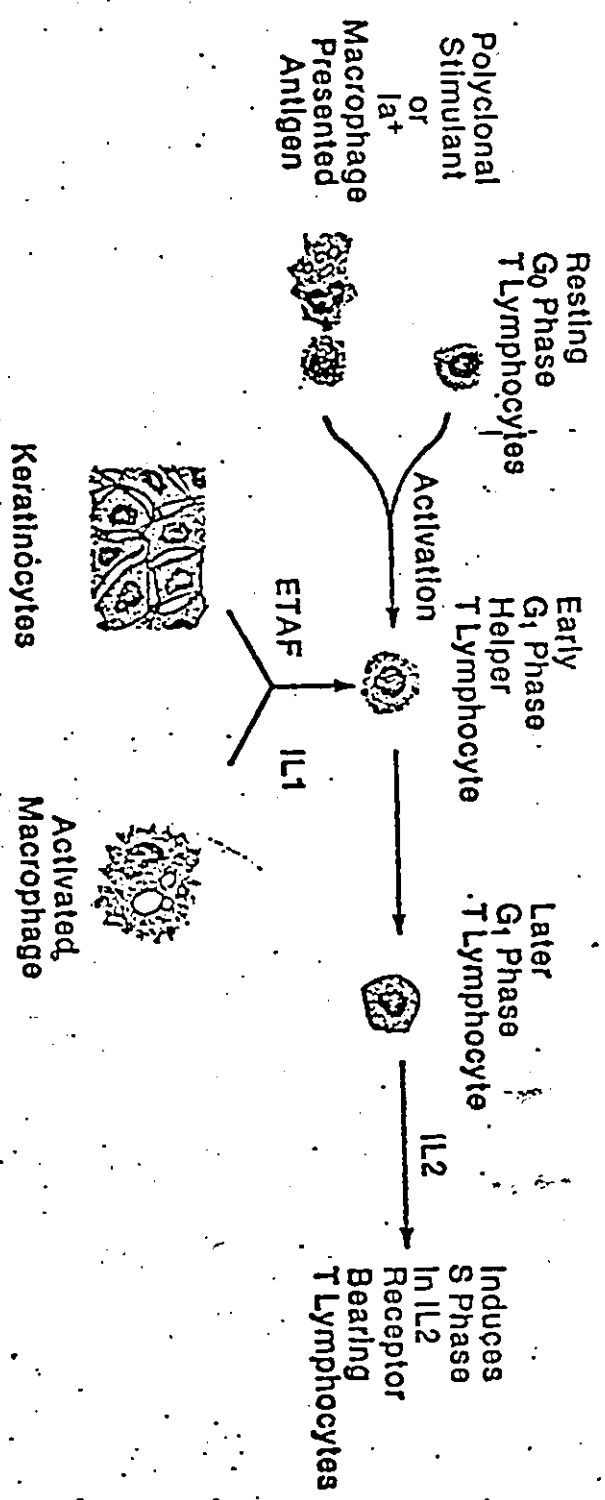


Figure 1. Scheme showing sequences of activation in T Lymphocytes (Oppenheim and Gery, 1982).

growth cycle (step 1), initiated by signal 1 and DNA-division cycle (step 2), initiated by signal 2.

Step 1: It is initiated by a recognition event that is clonally distributed and provides the basis for immunological specificity (Larsson *et al.*, 1980). It involves a shift from G0 to G1, accompanied by synthesis of RNA (Bettens *et al.*, 1982; Stadler *et al.*, 1981) in IL 2 accepting and IL 2 producing cells.

A. IL 2 acceptors: They are mainly CTLs, Lyt 2⁺ in mice and OKT8⁺ in human (Larsson *et al.*, 1982; Palacios, 1982). The activated T cells acquire responsiveness to IL 2 (Baker *et al.*, 1978; Bonnard *et al.*, 1979), bearing 1000 to 2000 more IL 2 receptors than the resting cells (Robb *et al.*, 1981; Moretta *et al.*, 1982; Malek *et al.*, 1983). Gamma interferon plays an important role in the expression of IL 2 receptors (Johnson and Farrar, 1983).

B. IL 2 producers: They are mainly HTLs, Lyt 1⁺ in mice (Larsson, 1982; Palacios, 1982) and OKT4⁺ in human (Reinherz *et al.*, 1981). The triggered cells move from early G1 to late G1 after interacting with Ia bearing cells, (Shaw *et al.*, 1980; Oppenheim and Gery, 1982), requiring cell-cell interaction (Akiyama *et al.*, 1983). IL 1 may substitute for the Ia positive cells (Farrar *et al.*, 1980; Gillis and Mizel, 1981; Palacios, 1982). IL 2 receptors have not been found in activated IL 2 producers.

Step 2: It initiates the proliferative or S-phase in the cell cycle through an interaction between a growth factor and its receptor (Stadler *et al.*, 1981).

A. IL 2 acceptors: Using radiolabelled IL 2 Robb *et al.* (1981) have shown that IL 2 binds to the IL 2 receptor of CTLs: this happens rapidly (15 min at 37°C). IL 2 binding is specific for activated T cells. This is followed by a receptor- and lysosome-dependent degradation of IL 2 culminating in DNA synthesis; the actual steps involved are not known.

B. IL 2 producers: The step 2 for IL 2 producer T cells is not clear. They can proliferate without the addition of exogenous factors such as IL 2, IL 1 or interferon (Palacios, 1982). The IL 2 producer clones retain their responsiveness to T cell mitogens. IL 3 induces differentiation of precursor cells into IL 2 producer mature T cells (Ihle *et al.*, 1982). IL 3 is a lymphokine whose biological activity is measured by its capacity to induce the expression of 20 α -hydroxysteroid dehydrogenase in lymphocytes of nude and normal mice. It is produced constitutively by Lyt1⁺ mouse T cells.

1.2.5 Regulation of T cell activation

Regulation of activation, which is genetically determined and controlled by the immunoregulatory network, may be induced as well, *in vivo* and *in vitro*.

1.2.5.1 Natural immune regulation

(1) Genetic control: Initiation and extent of immune response is controlled genetically (Benacerraf and Germain, 1973). I-region genes within MHC are responsible for this.

(2) Immunoregulatory network: T cell proliferation does not continue indefinitely, cell division ceases after a few rounds and the quiescent state returns. This is controlled by the immunoregulatory network, primarily through the regulation of IL 2 availability (Palacios, 1982). Suppressor T cells, bearing IL 2 receptors, absorb the factor (Sasportes et al, 1981). They also exert their inhibitory effect through the suppressor factor they release which contains I-J molecules. Macrophages produce an enhancing effect when present in low concentrations and an inhibitory effect when they are in higher concentrations. Prostaglandins have been shown to inhibit the production of IL 2 (Walker et al, 1983). Certain serum factors in mice and human may also show inhibitory effects.

1.2.5.2 Induced immunosuppression

(1) In vivo: The graft-versus-host (GVH) reaction, induced by injection of parental strain lymphoid cells into F1 mice causes a state of permanent immunosuppression, both cell mediated and humoral (Lapp and Moller, 1969). The suppression found in the early period of the GVH reaction is mediated by adherent cells which suppress helper T- and B-cell function (Elie and Lapp, 1977). It has been suggested that suppressor T cells are generated during the course of a GVH reaction (Byfield et al 1973) but it has not been confirmed (Parthenais and Lapp, 1978). It has been found by passive transfer experiments that GVH induced immunosuppression is caused, at least in part, by the functional depletion of a thymic mediator (Lapp et al, 1974). In mice, in the late phase of GVH reaction (days 19 or more post GVH induction) immunosuppression is not due to the presence of suppressor cells but to the inability of the T cells to show immunocompetence (Seddik et al, 1980). They fail to show responsiveness to PHA or Con A (Lapp and Kirschner, 1979).

(2) In vitro regulation: The agents that inhibit or stimulate mitogenesis may directly interfere with the proliferative capacity of the responding cell or their effects may be exerted on cell-interactions that make up the regulatory network.

Some of the physical and chemical agents that have been shown to interfere with the cell activation process are

ionizing radiation, ultraviolet radiation, colchicine, 5-fluorouracil (5-FU), mitomycin C and ouabain. The following aspects are known regarding the mode of action of these agents:

Ionizing radiation: Treatment of IL 2 producing T cells with 2000 rads of X-ray, a dose which inhibits DNA synthesis, does not modify their capacity to synthesize growth-factors (Kasakura, 1971; Gordon *et al.*, 1979; Palacios, 1982). Cells irradiated with X-rays (1500 rads) have been shown to be capable of delivering helper factors (Pilariski, 1977). Presumably these treatments do not inhibit the early stages of activation, which is a prerequisite for IL 2 production. Higher doses of X-ray, however, significantly inhibit IL 2 production (Gordon *et al.*, 1979; Palacios, 1982).

Ultraviolet radiation: It causes dimerization of pyrimidines within the nuclear DNA. This lesion is recognized and reversed by photoreactive enzymes (Banavalt *et al.*, 1979). Castellanos *et al.* (1982) showed severe inhibition of the proliferation of human lymphocytes *in vitro* particularly when resting cells were irradiated immediately prior to stimulation with Con A. Principal effects of UV were shown to be on early events of the activation sequence and some of these effects appeared to be independent of whatever damage the radiant energy caused to nuclear DNA.

Colchicine: This plant alkaloid that specifically binds to the microtubule protein tubulin and results in microtu-

bule disassembly (Margulis, 1973), has been a subject of controversy regarding its effect on lymphocyte activation. The ability of colchicine to inhibit mitogen stimulated DNA synthesis has been reported by several workers (Greene et al., 1976; Resch et al., 1977 and Rudd et al., 1979); there have been some negative reports too (Betel and Martijne, 1976; Staen and Lidno, 1978). There are conflicting reports with respect to the early events of lymphocyte activation also. Sherline and Mundy, (1977) and Resch et al., (1977) found no inhibition of PNA and protein synthesis. In contrast, inhibition was observed by Hauser et al. 1976; Greene et al. (1976) and Rudd et al. (1979). Studies with colchicine and with taxol, the microtubule stabilizing drug, suggest that normal microtubule function is necessary only for cell division and that drug effects on mitogen-induced blast transformation and initial DNA synthesis are unrelated to microtubules (Brown et al., 1983; Cuthbert and Shay, 1983).

5-Fluorouracil (5-FU): It is a pyrimidine analogue which is utilized for nucleotide synthesis by the same pathway as uracil but is not converted to cytosine or thymine nucleotides (Heidelberg, 1964). Bouwan et al. (1984) found that when 5-FU was added as a pulse early after mitogen stimulation, a very severe and irreversible inhibition of subsequent DNA synthesis occurred without affecting the earlier phases of activation such as K^+ transport and blast formation. The extent of inhibition of RNA synthesis depended on

when during the activation process the drug was present. Greatest inhibition of RNA synthesis (87%) was obtained with treatment in the first 6 hours of activation and the minimum inhibition (30%) with the exposure in S-phase. 5-FU inhibits the processing of ribosomal RNA (Wilkinson *et al.*, 1975). The most fundamental effect of 5-FU has recently been reported by Greer and Kaplan (1983); this drug has been found to cause irreparable strand breaks in the DNA of mouse lymphocytes and this was the basis of the irreversible inhibition noted by Boumah *et al.* (1984).

Mitomycin C: Bach and Voynov (1966) described the use of this drug for the study of one-way MLR. It prevents replication of DNA by cross-linking its complementary strands (Szybalski and Iyer, 1964). Mitomycin C-treated lymphocytes are capable of producing IL 2 under the stimulation of PBA (Palacios, 1982).

Quabain: ouabain, a cardiotonic steroid, is a specific inhibitor of Na^+ and K^+ transport acting via the membrane $\text{Na}^+\text{K}^+\text{ATPase}$ (Post *et al.*, 1960; Skou, 1975). Its effect on lymphocyte activation has been reviewed (Kaplan, 1978). It inhibits reversibly all parameters of stimulation of lymphocytes no matter how the stimulation is performed, in human (Quistel and Kaplan, 1968) and in mice (Owens and Kaplan, 1980; Kaplan and Owens, 1980). In mice, the effect of this drug was irreversible when a concentration of $1 \times 10^{-3} \text{M}$ or more was used (Owens, 1981). Rodent cells are about 4 or-

ders of magnitude less sensitive to ouabain than are human cells (Quastel and Vogelfanger, 1971) although the sodium pump isolated from membranes of most species are essentially biochemically identical (Skou, 1975). There are certain persistent effects of ouabain on human lymphocytes (Wright and Kaplan, 1976). Prolonged inactivation of cation transport, for example, incubation of human lymphocytes with ouabain for 48 hours caused certain effects which persisted after removal of the drug. These effects were exerted on the stimulating cells in an MLR and on the proliferative response to antigens, but not to mitogens.

Thus T cell activation, which is a multi-step process, may be affected by various elements although the underlying processes may not be obvious.

1.3 MIXED LEUKOCYTE REACTION

Medawar (1958) demonstrated that the allogeneic or allograft response, the process involved in rejection of tumours and transplants, is an immune function: recognition of the non-self antigen induces proliferation of lymphocytes and generation of killer cells in the host to lyse the foreign cells. Normal tissues that carry allogeneic histocompatibility antigens are attacked probably because they are mistaken for tumour cells by the immune surveillance system (Burnet, 1969) where T-cells are particularly well equipped to recognize and respond to altered self or allogeneic antigens (Doherty and Zinkernagel, 1975).

Mixed leukocyte reaction (MLR), an *in vitro* model of allograft reaction, expresses immune response reaction against genetically determined incompatibilities. It was first observed by Bain *et al* (1963) when they mixed two populations of leukocytes bearing different alloantigens. (Alloantigens are strain specific antigens, representing alternate forms of alleles within a single species.) MLR results in a blastogenic response and proliferation (Bain *et al*, 1964; Bach and Hirschhorn 1964), followed by differentiation into cytotoxic lymphocytes which specifically lyse cells bearing the alloantigens against which the response is directed (Hayry and Defendi, 1970). During the course of MLR a series of events takes place, including the production of a number of cytokines (Rocklin *et al*, 1980) and suppressor cells which play a wide variety of roles in the immunoregulatory circuit. MLR is thus very useful to an immunologist as an *in vitro* model for studying the immune response. An important clinical application of this reaction is that it reflects an individual's capacity to identify foreign cells and to initiate specific immunological reactions against them (Bach and Aros, 1967).

1.3.1 Various kinds of MLR

1.3.1.1 One-way MLR

One-way MLR was devised to enable one to study the response of one allogeneic cell population to the stimulus

given by the other set of leukocytes. Techniques were developed to abolish proliferation without damaging the stimulating capacity; treatment of the stimulating population with mitomycin C (Bach and Voynow, 1966) or X-rays (Kasakura and Lowenstein, 1965) could achieve this; such agents cross-link or damage the DNA and prevent its replication. When the stimulators and the responders differ in their MHC loci, untreated responder lymphocytes proliferate; this is detected by incorporation of tritiated thymidine into the DNA synthesizing cells. This is the one-way mixed leukocyte reaction or MLR. The cytotoxic cells generated in the culture are assessed by cell-mediated lympholysis (CML) assay, where one measures their ability to lyse radioactive sodium chromate (^{51}Cr) labelled target cells bearing the same antigens as the stimulator in MLC (Cerottini and Brunner, 1974).

1.3.1.2 Primary and secondary MLR

In the primary MLC, when two populations of freshly isolated cells are mixed, proliferation reaches peak activity around day 3-4, in mice and day 5 in human cells, and then declines, the cells reverting to small non-dividing cells (Andersson and Hayry, 1973). A secondary response, secondary MLR, may then be elicited with an accelerated rate and increased CTL response, suggesting the presence of increased numbers of memory cells derived from CTL-precursors. This may be induced with fresh or metabolically inactive stimulator

cells (Pilarski, 1977) or by the addition of T-cell growth factor (Uotila et al, 1978). In the murine system, leukocytes immunized *in vivo* may be induced to produce secondary MLR *in vitro*.

1.3.1.3 Autologous MLR

Autologous MLR was first reported in mice (Scwe, 1973) and then in human (Opelz et al, 1975) and guinea pig (Yamashita and Shevach, 1980). It is defined as a T-cell proliferative response that occurs upon *in vitro* stimulation by syngeneic non T-cells. Although the physiological role of such T-cells remains unclear, this system has been useful in dissecting the nature of the stimulator cell type and other aspects of MLR. The responder cells are Lyt 1⁺ and the stimulator cells are Ia⁺ macrophage-like adherent cells (Yamashita et al, 1982) or dendritic cells (Steinman and Witmer, 1978). Dos Reis and Shevach (1981) suggest that autologous MLR represents antigen-independent polyclonal activation of antigen-specific T cells through their receptors for self-Ia. It exhibits both memory and specificity (Yamashita and Shevach, 1980) and is under the control of the antigens encoded by the MHC (Hausman and Stobo, 1979).

1.3.2 Immunogenetics of MHC

The role of the MHC in graft rejection was discovered by Gorär, Snell, Dausset and Benacerraf (Snell *et al.*, 1976). T-cells recognize conventional antigens only in association with "self" MHC gene products (Zinkernagel and Doherty, 1979; Klein, 1982). An exception to this restriction is the recognition of alloantigens, which are recognized directly. There is a high frequency of T-cells with clonally distributed specific receptors for these antigens (Bach *et al.*, 1977). The antigens coded by MHC activate allogeneic T lymphocytes very efficiently (Matzinger and Bevan, 1977), however, they are functionally competent only when presented on metabolically active stimulator cells (Lafferty and Woolnough, 1977).

A non-H-2 linked minor lymphocyte-stimulating (Mls) locus encodes alloantigens that provoke a strong primary T-cell proliferative response (Festenstein, 1973). These Mls antigens are recognized in association with "self" H-2 determinants (Gress *et al.*, 1981). Viral antigens, haptens or minor histocompatibility antigens are also recognized by allogeneic cells in association with self H-2 K or D antigens (Zinkernagel and Doherty, 1979).

1.3.3 Components

1.3.3.1 Responders

It has been established unequivocally that the responding cells in MLR are T cells (Mosier and Cantor, 1971). B cells have been reported to divide in a secondary way (Piquet and Vassali, 1972; Perkins *et al.*, 1975). Natural killer cells (Ortaido and Bonnard, 1977; Zarling *et al.*, 1981) and anomalous killer (AK) cells (Karre *et al.*, 1983) have also been reported to be generated. During the period of the response an array of cytokines are produced (Rocklin *et al.*, 1980).

A large fraction of T lymphocytes respond vigorously to alloantigens, especially to the antigens encoded by the MHC. One to 12% of T lymphocytes proliferate in response to alloantigens of a single MHC haplotype (Bach *et al.*, 1977). Different alloantigens of MHC appear to stimulate preferentially functionally distinct subsets of T lymphocytes, namely the T-helper, T-suppressor and cytotoxic T cells.

T-helper cells (HTL): In man, HLA-D/DP antigens induces the production of helper cells in the allogeneic responder. In mouse, the helper cells arise when large numbers of T lymphocytes bearing the Lyt 1⁺ phenotype proliferate but generate no cytotoxic activity in response to H-2, I-A/E, and to non-H-2 MHC alloantigens (Scherdel and Bach, 1975; and Festenstein, 1973; Benacerraf and Germain, 1978). K/D difference may also induce the production of helper activity in Lyt 2⁺ cells (Okada and Henney, 1980; Swain *et al.*, 1981).

Approximately 1 in 330 splenic T lymphocytes develop helper activity in response to alloantigen of a single haplotype (Lutz *et al.*, 1981). Most HTL appear to cooperate nonspecifically with sensitized cytolytic cells (Lutz *et al.*, 1981) to yield functionally active CTLs (Larsson *et al.*, 1980; Paetkau *et al.*, 1980). The activated T cells release an array of lymphokines (Rocklin *et al.*, 1980; Prystowski *et al.*, 1982) which serve as essential growth factors for lymphocyte proliferation and differentiation and as regulators of the immune network. Allosensitization produces two types of HTLs: one induces the macrophages to produce IL 1 and the other one receives the IL1 and produces IL 2 (Larsson, 1982).

T-suppressor cells: They arise in response to I-J regions of the stimulator in mice (Okumura *et al.*, 1976; Mitchison, 1981) and in human to the D-region of the MHC (Mitchison, 1981). The suppressive factor(s) released by them selectively acts on T cells and they are involved in the regulation of the immune response by modulating early events in T-cell activation (Rode *et al.*, 1978; Kramer and Koszinowski, 1982); suppression of activation of CTL-precursors (pCTL) (Susskind *et al.*, 1981) and of T cell growth factor producing cell (Gullberg and Larsson, 1982) has been shown. In the murine system suppressor cells arise through an interaction between Lyt 2⁻ and Lyt 2⁺ cells (Chacuat *et al.*, 1982).

Cytotoxic T-cells: The pre-killer cells (Lyt 2⁺ phenotype in the mouse) are stimulated by Class I (K and D subregions) of MHC (Cantor and Boyse, 1978; Nagy *et al.*, 1976). In man HLA-A/B elicit cytotoxic cells. The sensitized cells, which have been shown to bind vesicles containing allogeneic K and D antigens (Elliot *et al.*, 1980), are further activated by IL 2. (Paetkau *et al.*, 1980) resulting in effective killer cells which specifically recognize target cells bearing the same K and D antigens as the stimulators (Zinkernagel and Doherty, 1979). Various groups have estimated the precursor frequencies of alloreactive CTLs (Miller *et al.*, 1977; Ryser and MacDonald, 1979). Miller *et al.* (1977) have shown that pCTL are highly specific and are precommitted to respond to particular alloantigens but under certain conditions upto 20% of the entire pool of pCTL can respond and generate CTL specific for the same alloantigen.

1.3.3.2 Stimulators

Cellular requirement

There is no conclusive knowledge regarding the cellular nature of the stimulator cells. Cell fractionation studies had at earlier times, indicated the B lymphocytes to be the most effective stimulating cells (Plate and McKenzie, 1973; von Boehmer, 1974). More recent literature has assigned the stimulatory role to macrophages (Fode and Gordon, 1974; Ahman *et al.*, 1979; Sunshine *et al.*, 1982.), the dendritic cells

(Steinman and Witmer, 1978; Sunshine et al., 1982; Steinman et al. 1983), and a radio-resistant Ia-bearing, non-T and non-adherent cell (Reme et al., 1981). Sunshine et al. (1982) showed heterogeneity in the stimulating capacity of the various cell types; dendritic and adherent macrophages being most effective. Very little potential was found in T or B cells alone. A number of workers in human MLR have found a strong stimulatory activity in activated Ia-bearing T-cells (Indiveri et al., 1980; Schuurman et al., 1980; Wollman et al., 1980; Forre et al., 1982).

Whatever the nature of the cell, the stimulator is required to be Ia bearing (Accolla et al., 1981; Sunshine et al., 1982). Treatment with monoclonal anti-Ia antibody and complement totally abrogated the stimulating capacity in their studies; even K/D difference was effective only when borne on Ia bearing cells.

All these findings are based on the stimulatory function of fractionated cell populations. It is not known which cells in the splenocytes participate or collaborate in delivering the stimulus when they are present in the unfractionated population.

Cells other than leukocytes can also stimulate allogeneic reactions. Ia-bearing endothelial cells and Langerhans cells are capable of stimulating allogeneic cells (Hirschberg et al., 1982).

Some non-Ia bearing cells are very effective in inducing CTL production (Davidson, 1977). Neoplastic cell lines such as P815, EL-4 or L-cells are potent stimulators (Lafferty and Woolnough, 1977).

By separating subpopulations of the responders using anti-Lyt-2 antibody and flow microfluorometry, Czitrom *et al* (1983) have shown heterogeneity in the requirement of stimulator cells for eliciting response in the different subgroups of the responders. They have shown that either macrophages or dendritic cells are required to stimulate proliferating Lyt 2⁻ cells. On the other hand, the Lyt 2⁺ population, which showed little proliferation and which contained all the precursors of CTLs, differentiated into alloantigen-specific cytotoxic cells in response to any alloantigen-bearing stimulator cell provided IL 2 was present. Davidson (1977) has also found that Ia removal reduces proliferative response but not CTL activity. She found that expansion of Lyt 1⁺ cells, in response to Ia difference, is necessary when Lyt 23⁺ cells are in limiting numbers.

Metabolic activity

Unlike mitogens or specific antigens, alloantigens can induce lymphocyte activation only when borne on metabolically active cells (Hardy and Lin, 1969). Leukocytes would not function as stimulators if they were killed or disrupted (Hardy and Lin, 1969) or inactivated by heating or iodoacetic acid (Schellekens and Eijsvogel, 1970) or by UV

(Lindahl-Kiessling and Safwenberg 1971). It has been found that UV irradiation does not affect alloantigens on the surface, as determined by serological methods (Lindahl-Kiessling and Safwenberg, 1971; Lafferty *et al.*, 1974); such treated cells could function as stimulators if a third party of γ -irradiated cells were added to the MLC (Lafferty *et al.*, 1974). Pilarski (1979) found that the same was true with glutaraldehyde-fixed stimulators. Lafferty and Cunningham (1975) suggested that in addition to the transplantation antigens the stimulators are required to provide an "allogeneic stimulus" which required metabolic activity. When the metabolic activity of the stimulators is compromised, their immunogenicity can be restored by soluble mediators. This was shown when the stimulators were soluble MHC antigens (Uctila *et al.*, 1978), or when they were inactivated by UV (Paetkau *et al.*, 1976; Okada *et al.*, 1979; Wagner *et al.*, 1980); or heat (Scott *et al.*, 1980) or glutaraldehyde (Wagner *et al.*, 1980).

1.3.4 Mechanism of CTL production

Three major steps can be distinguished in the production of CTLs: (1) induction, (2) proliferation, (3) differentiation.

1.3.4.1 Induction

In the murine MLR, in which the strains differ at H-2, activation is induced when specific pCTLs bearing Lyt 2⁺ cells in the responding population recognize H-2K and/or D molecules (Signal 1) on the cells of the stimulator; a 4 to 12 hour exposure of pCTL to alloantigens is sufficient for optimum signal 1 delivery (Lalonde *et al.*, 1980; Larsson *et al.*, 1980; Hermann *et al.*, 1982). This process does not need Ia bearing cells (Davidson, 1977; Schnagl and Boyle, 1981; Weinberger *et al.*, 1982; Czitrom *et al.*, 1983). Lafferty *et al.* (1980) have suggested that induction of activation in all responding T cells involves a simultaneous recognition of MHC antigens and a soluble product released by the stimulating cells. Activation by alloantigens leads to the induction of receptors for growth factors on the pCTLs (Robb *et al.*, 1981; McCreeta *et al.*, 1982; Malek *et al.*, 1983).

1.3.4.2 Proliferation

DNA synthesis is initiated when a second signal, IL 2, interacts with its receptor on the activated pCTL (Robb *et al.*, 1981). For optimal effect, IL 2 must be present at 12 to 24 hours after interaction of pCTL with allogeneic stimulators (Waquer *et al.*, 1980; Hermann *et al.*, 1982). The biochemical pathway of IL 2 driven T cell proliferation has not been completely elucidated.

The source of the second signal is a matter of debate. A second subset within the responder cell population, usually consisting of T-helper cells, has been widely implicated as the source of the second signal (Bach et al., 1977; Okada et al., 1979; Pilarski, 1977). Alternatively, the second signal may be provided by all cells or some subset of cells within the stimulator population (Lafferty and Woolnough, 1977).

Helper activity is generated when precursors of helper T lymphocytes (pHTL) within the responder recognize alloantigens; this is achieved only when these antigens are processed and presented by Ia⁺ splenic adherent cells (Weinberger et al., 1981) which may be syngeneic to the pHTL (Schnagl and Boyle, 1980), or allogeneic (Czitrom et al., 1983) or both (Weinberger et al., 1982). Elliot et al. (1980) showed that pHTL can bind vesicles containing both self and allo Ia. HTL sensitized to alloantigens can produce IL 2 after interacting with IL 1 (Farrar et al., 1980; Oppenheim and Gary, 1982). The T-helper cells that induce the macrophages to produce IL 1, have been identified as distinct from the IL 2 producing cell (Larsson, 1982).

That the activated pCTL interacts with activated HTL or its soluble growth product, to be able to proliferate actively, has been shown by Lalande et al. (1980), Teh and Teh (1980), Wagner et al. (1980) and Herzann et al. (1982). They were able to dissect in time the two events: (1) activation of the pCTL by the first signal (alloantigens) and (2) in-

teraction of these activated cells with the second signal, delivered by the activated HTL. A direct proof for a two-signal mechanism of cytotoxic T-lymphocyte proliferation has come from the development of T-cell cloning technology which has allowed the isolation of alloreactive cloned helper and cytotoxic T-cells and the study of the collaboration between them (Glasebrook and Fitch, 1979; 1980). Amplification of CTL activity could be achieved when the two types of clones were physically separated, showing that the signal 2 function lies in a soluble factor.

There are some CTL clones which can proliferate independently of T-helper cells, just by responding to allogeneic stimulation (Widmer and Bach, 1981; Glasebrook et al, 1983). They can produce growth factors themselves.

1.3.4.3 Differentiation

There is some evidence that CTL proliferation and differentiation are controlled by separate factors. Mannel et al (1983) have shown that three different factors are sequentially required in distinct phases of cytotoxic response. One of them is IL 2 and of the other two, one is required for the first 48 hours and has been called cytotoxicity inducing factor 1 (TCF1) and the other one, TCF2, is required in the last 48 hours. TCF1 is similar to the differentiating factor described by Raulet and Savan (1982) and Wagner et al (1982). TCF2 is provided by irradiated I-region incompatible

stimulating population (Reddehase et al, 1982). Finke et al (1983) have found that IL 2 is not a sufficient signal to generate CTL from splenic T cells stimulated with heat or UV treated cells; CTL could develop in response to a factor generated by macrophages when they interact with activated T-cells. Since the requirement of this macrophage-derived factor becomes apparent when the stimulators are metabolically inactivated, it is quite probable that normally its function is carried out by some product generated by the stimulators; this factor may be the same as TCF2 of Mannel et al (1983).

It has been suggested by MacDonald (1982) that the process of differentiation may precede the stage of proliferation. It is possible that the actual differentiation step from pCTL to CTL does not depend upon soluble factors and that the role of such factors is merely to increase the number of CTLs to detectable levels.

1.3.5 Modulation of MLR

The MLR may be modulated by treating either the responder or the stimulator or the reaction mixture.

Several laboratories have treated the stimulators with various physical and chemical agents. Most of these treatments do not affect the antigenicity, that is the antigens are serologically detectable; their immunogenicity, however is lost.

Christen *et al* (1975) reported dual effects produced by ouabain, a specific inhibitor of $\text{Na}^+\text{K}^+\text{ATPase}$; a 48 hour pretreatment with $2 \times 10^{-6}\text{M}$ totally inhibited the stimulating capacity of human lymphocytes but $1 \times 10^{-7}\text{M}$ enhanced it 2-fold. Similar effects were seen in the rhesus system (Keller, cited in Kaplan and Owens, 1982).

Dual effects on the MLR by pretreatment of the stimulating population have been reported by other workers also. Colchicine has been reported both to suppress (Ranney and Pincus, 1976) or augment (Stenzel *et al*, 1978) the reaction. Suthanthiran *et al* (1980) noticed in human MLC opposite effects depending upon the colchicine treatment protocol; there was an enhancement when the stimulators or the responders were pretreated with $1 \times 10^{-6}\text{M}$ for a brief period (30 minutes) but total inhibition occurred when either of these populations was pretreated with 10^{-2}M or $1 \times 10^{-6}\text{M}$ was added to the MLC. They suggest that the state of the microtubular assemblies may modulate immune responses involving cell-cell interaction.

Suppression of MLR and of generation of CTL has been observed when the cell surface of the responding or stimulating cells was modified by oxidizing agents such as tandem treatment with neuraminidase and galactose oxidase or with sodium periodate; this effect could be reversed by reduction of the cell surface with sodium borohydride (Suthanthiran *et al*, 1982).

Suppression of stimulating capacity up to 25 fold was observed by Wagner (1973) when the stimulators were pretreated with actinomycin D or antimycin, inhibitors of RNA and protein synthesis respectively. He suggested that the suppression could be due to diminution of surface transplantation antigens or due to a possible reduction of turnover of surface proteins. However, Uotila *et al* (1978) found that the presence of soluble antigens, up to any concentration, could not initiate MLR unless growth factors were added.

Enhancement of MLR and resulting cytotoxic activity have been reported when the stimulators were treated with inhibitors of protein synthesis, such as cycloheximide (Folch and Waksman, 1974) or pactamycin (Metzler *et al*, 1976); increase of 2 to 3 fold was recorded. In both cases the authors speculated that suppressive factors were inhibited. Pretreatment of the stimulators with interferon has been found to cause enhancement (Finhorn and Blomgren, 1980). Expression of H-2 and HLA-determined antigens is increased by interferons though I-region determined antigens, as assessed serologically, has not been found to change (Lonai and Steinman, 1977). Nakamura *et al* (1984), however, have reported that IFN- γ causes a 10-fold increase in the level of RNA corresponding to the I-region encoded surface molecules in macrophages. Uotila *et al* (1978) could increase the peak and duration of the proliferative response in MLC by the daily addition of growth factors.

All in all it would appear that an agent can regulate the MLR response if it can affect either the surface antigens or the metabolic activity of the stimulators and the responders or if it can influence the augmenting or suppressing factors generated during the process of MLR.

1.4 THE THESIS PROJECT

1.4.1 The question

The project of this thesis was undertaken to elucidate the stimulatory mechanism in the allogeneic response, in particular to characterize the functions of the stimulating cells in an MLR and specify if the stimulators produce a second signal, IL 2.

1.4.2 Assumption

The stimulating population arouses reaction in the responding population by virtue of its transplantation (Class I) antigens and by expressing another function that requires metabolically active cells. Lafferty and his colleagues (1975, 1977) suggested that the metabolic activity of the stimulators provides an "allogeneic stimulus" or a second signal for the induction of proliferation in transplantation antigen-triggered responding cells. Since IL 2 acts as the second signal and since its production can satisfy the metabolic requirement of the stimulators in an MLR it can be postulated that the stimulators themselves produce IL 2 or

that they induce the responders to produce it. Since IL 2 is produced by activated T cells, it was considered reasonable to assume that the stimulators themselves would undergo activation if they were to produce IL 2 themselves.

1.4.3 Approach

I approached this problem by examining the stimulators for their ability to respond to an immunological stimulus, namely Con A, under the effects of a variety of agents that have been reported to inhibit stimulating capacity of leukocytes in an MLR. Since the stimulators, by operational definition, are incapable of synthesizing DNA, their response to Con A was assessed by measuring the parameters characteristic of pre-S phase activation, namely, increases in K^+ influx and cell size. On testing the effects of ouabain, UV, 5-FU and colchicine, a correlation was found between the ability of leukocytes to respond to Con A and their ability to stimulate allogeneic cells in an MLR.

1.4.4 Hypothesis

The hypothesis formulated from the above observation was that the stimulators themselves are required to undergo an allogeneic activation (pre-S) in order to induce optimal proliferation in MLR. The T cells in the stimulating population would then be activated.

1.4.5 - Experiments

Experiments were conducted to see if cultures showing optimal proliferation did actually contain and require activated T cells in the stimulating population and if so, what their role was.

1.4.5.1 Activation of T cells in the stimulators

Using a monoclonal antibody identifying the stimulator-T cells, it was shown that these cells underwent pre-S activation, showing increase in K^+ influx and in RNA synthesis (incorporation of 3H -uridine).

1.4.5.2 Requirement of T cells in stimulators

Depleting the stimulators of T cells caused a significant reduction in MLR. Optimal proliferation could be restored by adding to the culture T-enriched cells from the stimulators but not the responders.

1.4.5.3 Role of the stimulator T cells

The stimulating population itself was capable of producing IL 2 and it regulated the amount of IL 2 produced by the responders. Depletion of T cells from the stimulators reduced the level of IL 2 in the culture and involved the participation of DNA synthesizing cells in the responders to produce it.

1.4.6 Conclusion

The experimental results supported my hypothesis that optimal proliferation requires the presence of activated T cells among the stimulating population. The stimulator T cells can produce IL 2 themselves and regulate the level of IL 2 in the culture. Growth factors, however, may be produced independently of stimulator T cells as well. It is suggested that activation of T cells in the stimulators raises the the stimulating potential and constitutes a component of the network which enhances the specific response induced by the transplantation antigens.

Chapter II

MATERIALS AND METHODS

2.1 MICE

Male C57BL/6J, CBA/J, Balb/cByJ and AKR/J mice (8 to 10 weeks old) were obtained from the Jackson Laboratories, Bar Harbor, ME. Some of the earlier experiments were conducted with mice bought from Charles River Breeding Colonies, Wilmington, MA. The strains A/J and (CBA x A)F1, used for graft-versus-host reactions, were bred in the laboratory of Dr. W.S.Lapp, in the Department of Physiology, McGill University, Montreal. Some of the genetic characteristics of these mice are described in Table 1.

2.2 THE SPLENOCYTES

All experiments were conducted with mouse splenocytes which were either unfractionated, or fractionated or treated with various inhibitory agents.

2.2.1 Preparation of leukocytes

Mice were killed by cervical dislocation. Spleens removed aseptically were disrupted on a wire mesh screen and the cells suspended in RPMI (Flow).

Table 1
Genetic markers of the mice strains^a.

Strain	H-2 ^b	Mis ^c	Thy ^d
A/J	a	c	1.2
AKR/J	k	a	1.1
Balb/c By J	d	b	1.2
C57Bl/6J	b	b	1:2
CBA/J	k	d	1.2

^aThe strains varied in genetic markers relevant in MLC. The information was derived from Festenstein (1974) and Klein (1982).

^bHaplotype (Major histocompatibility complex).

^cMinor lymphocyte stimulating locus (M locus).

^dThy differentiation marker.

After centrifugation, the cell pellet was treated with ammonium chloride (0.83% in distilled water) for 5-7 minutes at room temperature to lyse erythrocytes. The resultant cells (95% lymphocytes) were washed with RPMI once before use. Unless otherwise stated, the cells were then resuspended in complete medium which consisted of RPMI (Flow) fortified with 2mM L-glutamine (Sigma), 6% fetal calf serum (Flow), penicillin (100 U/ml) - streptomycin (100 ug/ml) (Difco) and 5×10^{-5} M 2-mercaptoethanol (Sigma) and buffered with HEPES (20 mM) (pH 7.2).

2.2.2 Fractionation and characterization of lymphocytes

T-depleted and T-enriched leukocytes were prepared from unfractionated cell population. After removing the T or the Ia-bearing cells, the remaining cells were suspended in complete medium, in the same volume as the corresponding unfractionated culture; that is, the volume required to obtain a cell density of 3×10^6 cells/ml of unfractionated cells. This way the density of all the cells, other than those removed, remained the same in unfractionated and fractionated leukocyte suspensions. The cultures with fractionated cell populations contained approximately half the number of lymphocytes as the whole populations.

2.2.2.1 T-depletion

Spleen-leukocytes (1×10^6 cells in 1 ml of serum-free medium) were incubated with monoclonal anti-Thy 1.2 (New England Nuclear, AKR anti-C3H) at a final titer of 1/800 on ice for 1 hour. The cells were washed once, then incubated with guinea-pig complement (kindly supplied by Dr. J. Lainq, Agriculture Canada), at a dilution of 1/20 for 1 hour at 37°C. After two washes, the entire process was repeated. This fraction contained no fluorescent cells when tested by fluorescein isothiocyanate (FITC)-conjugated anti-Thy 1.2 (New England Nuclear).

2.2.2.2 T-enrichment

Leukocytes were enriched for T cells on anti-Ig coated-dishes as described by Wysocki and Sato, 1975. These dishes, which could be stored in the freezer, were prepared by pouring into each plate 100 ul of anti-mouse immunoglobulins (rabbit) (Cedarlane) diluted in 10 ml 0.05M Tris, pH 9.5 and leaving the plates for 40 minutes at room temperature, followed by 4 washes with PBS. $2 - 3 \times 10^7$ splenic leukocytes suspended in 3 ml phosphate buffered saline (PBS) containing 5% FCS, were incubated in anti-Ig coated 100 x 15 mm polystyrene petri dishes (Fisher Cat. No. 8-757-12) at 4 C for 110 minutes, swirling once after 70 minutes. The non-adherent cells which contained the T-enriched cells were collected by decanting the supernatant. Fewer than 3% Ig-bearing

cells remained after the treatment as assessed by fluorescein labelled goat-antimouse anti-Ig (Cappel).

2.2.3 Treatment of leukocytes with inhibiting agents

A. Ultraviolet light

3 ml. of leukocytes suspended in PBS at $5 \times 10^6/\text{ml}$ were plated in siliconized glass petri dishes (60 mm) and the open dishes were exposed for varying lengths of time to a 15-W ultraviolet light source whose calibration has been described by Castellanos et al (1982): the final dose ranged from 120 to 360 ergs/mm².

B. Chemical agents.

The chemical agents used were colchicine, 5-fluorouracil (5-FU), and ouabain, all obtained from Sigma. They were dissolved in RPMI 1640, sterile filtered and stored in the freezer in aliquots of 200 to 500 ul. They were added at various concentrations, in volumes of 100 to 300 ul, to cultures of splenocytes in complete medium in 75 cm² flasks (Corning). The cultures were incubated at 37°C for various lengths of time. Ouabain was added at $2 \times 10^{-6}\text{M}$ or $1 \times 10^{-2}\text{M}$ to mouse leukocytes at $5 \times 10^6/\text{ml}$ and cultured for 48 hours. The rest of the chemical agents were added to cultures at 10×10^6 cells/ml. 5-FU was added at 3.3 and $6.6 \times 10^{-5}\text{M}$ for 6 hours, colchicine at $1 \times 10^{-5}\text{M}$ and $1 \times 10^{-6}\text{M}$ for 4 hours.

After each of these treatments with the physical and chemical agents, the cells were further treated with mitomycin C (Sigma) (40 ug/ml), washed twice in medium and then two cultures were set up: (1) cells cocultured with allogeneic responder cells and (2) cells incubated with Con A.

2.2.4 Glutaraldehyde-fixed cells

These cells were used where non-functional lymphocytes were required; for example, for replenishing the number of deficient lymphocytes in cultures containing fractionated cell populations. Unfractionated leukocytes were incubated with 0.1% glutaraldehyde (J.B.E.M Services) at 37°C for 3 minutes and the cells were then washed 3 times in RPMI containing 10% calf serum (Flow).

2.2.5 Leukocytes from mice undergoing GVH reaction

GVH reactions were induced by injecting 7 to 8 week old (CBAXA)F1 mice with 50 to 75 x 10⁶ A strain spleen and lymph node cells as described by Elie and Lapp (1977). The splenocytes in these mice were found to be immunosuppressed (Table 2), using an assay for plaque forming cells (Lapp et al, 1974). This work was done in the laboratory of Dr. W.S. Lapp, McGill University. The spleens from these mice were processed for obtaining leukocytes, as described above in section 2.2.1.

Table 2

Ability of splenocytes from GVH-mouse to form plaques to sheep erythrocytes^a.

Culture	Number of plaques ^b
Control	187950 ± 10656
GVH	200 ± 100

^aNormal CBA x AF₁ and GVH (CBA x AF₁ injected with parental A strain mice were immunized with SRBC on day 7 post GVH and their splenocytes tested for their ability to form plaques to SRBC. For each culture 5 Cunningham chambers were used for detecting the plaques.

^bThe number of plaques were counted macro - and microscopically. Absolute numbers are given.

2.3. CELL CULTURE

Cells were cultured in complete medium, usually at a density of $2 - 4 \times 10^6$ cells/ml in either microplates (Linbro), at 200 μ l/well (flat- or round-bottomed) or in 25 cm^2 flasks (Corning), at 10 μ l/flask, which were sealed and incubated in an upright position at 37°C in a humidified atmosphere. For better cell survival, the cultures were supplemented with complete medium, 10% of the total volume, every day.

2.3.1 Mixed leukocyte reaction

Leukocytes prepared from two sets of mice differing in their haplotypes (see Table 1) were used as the responding and stimulating populations respectively. The responders (R) were adjusted to 4×10^6 per ml. The stimulators (S) were prepared by treating the leukocytes with 40 μ g/ml mitomycin C (m) for 45 minutes at 37°C and washed three times. With this treatment with mitomycin C, incorporation of ^3H -thymidine was effectively reduced in the stimulators. In control cultures where both the allogeneic populations were treated with mitomycin C, incorporation of ^3H -thymidine peaked on day 2 and was reduced by 88 to 90% compared to that in the cultures where the responders were uninhibited and where the peak was on day 3. The responders were incubated with stimulators at 1:2 and 1:1 ratio in a total volume of 8 μ l in 25 cm^2 flasks (Corning). Proliferative response in MLR was measured over a 5-day period in a flat

bottom microculture plate; incorporation of ^3H -thymidine, expressed as counts per minute (cpm), was taken to represent this response. At the initiation of culture, 200 μl of cells were placed per well, and each day, cells in three wells were pulsed with ^3H -thymidine for 6 hours and harvested with Titertek cell harvester. The cell culture in the flask was used for microscopic studies.

In cultures where fractionated leukocyte populations (T-depleted and T-enriched) were used, the total number of cells (6×10^6 cells/ml) was kept constant by adding the required number of glutaraldehyde fixed cells from the responding population.

2.3.2 Con A culture

Con A (Calbiochem) was made up at 10 mg/ml in saline and kept frozen in 200 μl aliquots. It was thawed out immediately prior to use. Leukocytes, $2 - 3 \times 10^6/\text{ml}$, were incubated with Con A (Calbiochem) (3 $\mu\text{g/ml}$) in flasks at 37°C . Aliquots were removed at various times for assessing different parameters of cell activation.

2.4 ASSESSMENT OF CON A RESPONSE

Three parameters of lymphocyte activation were measured:

2.4.1. Potassium influx

Activation of potassium influx was measured according to the procedure described by Owens and Kaplan (1980), who established that ^{86}Rb is transported identically to ^{42}K . ^{86}Rb was substituted for K^+ in all experiments due to a very short half-life of the latter. Briefly, at 8 - 10 hours after Con A stimulation, approximately 40 μCi (in 25 μl) of $^{86}\text{RbCl}$ (New England Nuclear) was added to each ml culture in Falcon tubes. After a 30 minute incubation at 37°C , 200 μl aliquots were centrifuged through 100 μl of silicon oil (Dow Corning 550 and 560 at 12:13 ratio), using a Beckman microfuge 152. The tips of the tubes were cut off and the radioactivity in the pellet was determined by using a gamma counter. Counts due to trapped medium, which invariably remained associated with the cells, were accounted for by a zero time uptake at 4°C . This involved adding ^{86}Rb to 0.8 ml cells cooled to 4°C and sampling 200 μl of it for radioactivity counts within 30 seconds. Counts were converted to potassium uptake measurement according to equations (1) and (2).

$$(1) \text{SRM} (\text{Counts/fmol } \text{K}^+) = (\text{counts/ml}) / (z (\text{fmol/ml})),$$

where SRM was the specific radioactivity of the medium and z the concentration of K^+ in the supernatant (5.4×10^9 fmol/ml).

$$(2) \text{K}^+ \text{ uptake (fmol/cell)} = ((\text{counts/pellet}) - (\text{trapped counts})) / (\text{SRM} \times (\text{viable cells/pellet})).$$

2.4.2 Classification

After 24 hours of adding Con A to a leukocyte culture, cells were aliquoted into 1.5 ml micro test tubes (Bic-rad) and adjusted to $10 - 15 \times 10^6/ml$, 25 ul of which were layered on poly-L-lysine-coated cover-slips and fixed in 3% paraformaldehyde. After three washes in phosphate buffered saline (PBS) (pH 7.2), they were fixed again in cold ($-5^{\circ}C$) ethanol. Two fixations were used in order to be consistent with certain methods used in related experiments described in section 2.5. The coverslips were mounted on slides, viewed and photographed, using a Zeiss Universal photomicroscope. The cell size was determined with an ocular micrometer. The mean diameter of resting cells was 6.25 ± 0.81 um; these were classified as type 1. Activated cells or blasts were grouped into types 2 and 3, the former being 8 to 10 um (mean diameter being 8.87 ± 1.04) and the latter measuring 10 um or more. These three groups of cells correspond to morphotypes 1, 2 and 3, in Con A-stimulated lymphocyte categories, describing the sizes of nuclei and the associated state of aggregation of their chromatin (Setterfield et al, 1983).

2.4.3 DNA synthesis

At 42 hours after the initiation of Con A culture, cells were aliquoted into 3 wells of a microcytotoxicity plate, 200 ul/well (flat or round bottomed). 3H -thymidine, at 2

UCI/ml, was added into each well and the plates incubated for 6 hours. The cells were then harvested onto glass-fiber filters and radioactivity determined by scintillation counting. ^3H -thymidine incorporation, expressed as counts per minute, (cpm) was correlated with the extent of DNA synthesis.

2.5 DEMONSTRATION OF ACTIVATION IN STIMULATOR-T CELLS

MLR cultures were set up using leukocytes of C57Bl/6 (Thy 1.2) as stimulators and AKR (Thy 1.1) as responders at stimulator (S) to responder (R) ratio of 2:1. FITC-conjugated monoclonal anti-Thy 1.2 was used for identifying the T cells of the stimulating population of C57Bl/6 and pre-S phase activation of these cells was assessed by measuring increases in cell size and in RNA synthesis.

2.5.1 Cell size

Cells from a 24 hour MLR culture (C57Bl/6(*) + AKR) (n-methylmann C-treated) were processed for immunofluorescence. FITC-conjugated anti-Thy 1.2 (New England Nuclear) was diluted in PBS containing 5% bovine serum albumin (BSA) at 1/10 dilution and 5 ul of it was then added to 25 ul cells ($20 \times 10^6/\text{ml}$) in a microfuge tube so that the final dilution of the antibody was 1/50. This cell suspension was then layered onto a poly-L-lysine coated area on a slide (using a glass marking pencil, a rectangle of about 1×0.5

cm² was marked out on the slide, on the side opposite to the one where cells were to be placed). After 10 minutes, the slide was washed once in PBS (30 seconds) and then dipped in p-formaldehyde for 30 minutes. After three washes in PBS, it was immersed in cold (-5°C) ethanol for 15 minutes and then air-dried. 10 ul of mounting medium (0.2% p-phenylene diamine in 50% glycerol-PBS (v/v), pH 7.0) were placed on the cells on the slide which were then covered with a coverslip and sealed with nail polish. The cells were viewed under a microscope equipped with epifluorescence and phase contrast optics. The stained cells were measured using a calibrated eye-piece and those larger than 8 um were considered to be blasts. They were then photographed.

2.5.2 RNA synthesis

24 hour cultures containing C57Bl/6(m) and AKR were examined. To measure RNA synthesis in the stimulator-T lymphocytes, cells were double-labelled with fluoresceinated anti-Thy 1.2 and ³H-uridine (New England Nuclear) based on the procedure described by Landreth *et al.*, 1981. To 1 ml aliquots of a 20 hour MIR culture, ³H-uridine was added at 8 uCi/ml. After a 4 hour incubation the cells were washed 3 times and processed for immunofluorescence as described above (Section 2.7.1). After the ethanol fixation the slides were dried and then processed for autoradiography. The slides were dipped in Ilford L4 bulk emulsion for 2 seconds,

drained and placed in a slide box containing the dessicant Drierite. The box, protected from light, was stored in the cold (5°C) for 36 hours. The slides were then developed in Kodak Dektol/water, 1/1, for 2 minutes and fixed in Kodak Rapid Fix for 4 minutes. After rinsing in water for 30 minutes, the slides were air-dried and mounted as described above. The cells viewed under UV and by phase-contrast microscopy were examined for immunofluorescence and for the presence of silver grains. Those showing more than 10 silver grains were considered activated. The mean number of grains in the resting population was 7 ± 3 . Micrographs of these cells were prepared.

2.6. ASSESSMENT OF IL 2 ACTIVITY

2.6.1 Interleukin 2

IL 2 (Fr. 3, G VII.40): This was a generous gift from Dr. V. Paetkau (University of Alberta, Edmonton, Alberta). The procedure for its preparation has been described by Blackley et al., 1982. This IL 2 was used for restoring IL 2-deficient cultures, as a standard control in IL 2 bioassays and for maintaining the IL 2-dependent cell line MTL (see section 2.6.2.1).

Rat Con A supernatant: In later experiments, the MTL cell line was maintained with IL 2-containing rat Con A supernatant. This was a generous gift from Dr. B.E. Elliot (Queens University, Kingston).

2.6.2 Bioassays for IL 2

2.6.2.1 MTL assay

MTL cell line: The cell line MTL 2.8.2 was a gift from Dr. V. Paetkau (Edmonton). It is an IL 2-dependent cytotoxic T cell clone, the generation and characterization of which has been described (Bleackley *et al.*, 1982). It was maintained in the laboratory, using tissue culture techniques. Briefly, the cells grown in a tissue culture flask were removed with 0.02 M EDTA in Mg⁺⁺Ca⁺⁺-free PBS. About 10⁶ cells were suspended in 25 ml of complete medium containing IL 2 (1:250 dilution) in a 75 cm² flask (Corning). These were incubated at 37°C, humidified atmosphere containing 5% CO₂, for 3 - 4 days, after which they were repassaged.

Microassay: Measurement of growth of the IL 2-dependent clone MTL 2.8.2 was used as an assay for detecting IL 2 in supernatants of MLR cultures (Bleackley *et al.*, 1981). 10⁴ cells (100 ul) per well were cultured in a flat bottom microcytotoxicity plate (Linbro) along with 100 ul of the test material for 24 hours. Each plate contained controls consisting of IL 2-free medium (negative control), and purified IL 2 (Fr. 3, G VII.40) (positive control) instead of the test supernatant. For the final 6 hours of incubation the cells were pulsed with 2 uCi/ml of ³H-thymidine contained in a labelling mixture. At the end of the labelling time, the culture supernatant in each well was replaced with 200 ul of EDTA. After 5 minutes the cells were harvested onto glass

fiber filters. Incorporation of radioactivity determined by scintillation counting was expressed as counts per minute (cpm) and used as a measure of proliferation and thus of the intensity of IL 2 activity.

2.6.2.2 Thymocyte bioassay

Thymocytes: Thymuses were removed from 3 to 6 weeks old CBA mice and processed the same way as the splenocytes (section 2.2.1) for obtaining thymocytes.

Microassay: The enhancement of mitogen-stimulated thymocyte proliferation was used for assessing IL 2 activity (T. Owens, University College, London, England, personal communication). 5×10^5 thymocytes/well (150 μ l) were seeded in microculture plates (flat bottom). Purified PHA (Burroughs Wellcome, BA 16) was added at a final concentration of 0.4 μ g/ μ l (1/2500). After adding 50 μ l of the test samples the trays were incubated for 48 hours at 37°C. Appropriate controls were included in each tray. They consisted of thymocytes/no PHA, thymocytes/PHA/no supernatant, and thymocytes/supernatant/no PHA. The cells were pulsed with 2 μ Ci/ μ l of 3 H-thymidine for the final 6 hours and harvested using a Titertek cell harvester. Incorporation of radioactivity (cpm) was used as measure of thymocyte proliferation and related directly to IL 2 activity.

Chapter III

RESULTS

In order to understand the stimulatory mechanism in an MLR, an attempt was made to characterize the functions of the stimulating population, specifically to find out if in addition to the transplantation antigens a second signal is delivered by the stimulators, as suggested by Lafferty and his colleagues (1975;1977). I have hypothesized that in order to produce a second signal, the stimulators would have to undergo activation, a metabolically active process, and that it would be the stimulator-T cells presumably, the primary target of the alloantigens of the responders, that would be activated. Experiments were conducted to test this hypothesis in general and in particular to find out (1) if the stimulating population is able to undergo activation; (2) if it is necessary for stimulators to undergo activation; (3) if the stimulator-T cells do actually undergo activation during MLR (4) if the presence of T cells in the stimulating population is required and (5) what role the activated stimulator-T cells play during MLR. This chapter describes these experiments.

3.1 ACTIVATION IN STIMULATING CELLS

According to my hypothesis the mitomycin C-treated stimulating cells should themselves be able to undergo activation. To test the feasibility of this idea, an experiment was set up to determine if mitomycin C-treatment allowed the pretreated cells to undergo activation in response to Con A.

3.1.1 Effect of mitomycin C on Con A response

C57Bl/6 splenocytes were incubated with 40 ug/ml of mitomycin C for 45 minutes, the minimal dose that inhibited DNA replication in the presence of Con A; this is the standard method of preparing the stimulators for one-way MIR. Con A response of mitomycin C-treated cells was determined by measuring K^+ influx, blast formation and tritiated thymidine incorporation (Table 3). In both control and treated cultures K^+ influx increased about 1.7 - 1.8 fold in response to Con A. In the presence of Con A, mitomycin C-treated cultures showed the same pattern of blast formation as in the control; small blasts (type 2) and fully formed blasts (type 3) were seen (Figure 2). (For definition of 'type' see 'Materials and Methods', section 2.4.2). Percentages of blasts in the two cultures (50% in the control and 47% in the mitomycin C-treated culture) were not significantly different. Thus measurements of these parameters of pre-S phase cell activation show that mitomycin C treatment did not interfere with the initiation of activation although it inhibited DNA replication (Table 3).

Table 3

Effect of mitomycin C on the various stages of Con A activation^a

Treatment	Early events in Con A responses		Blast formation	DNA synthesis ³ H-Thymidine Incorporation (cpm) ^b
	K ⁺ influx fmoles/cell/hr ^b	Ratio		
Control	3.32 ± 0.15 (100) ^d	1.75	8	3022 ± 190 (100) ^d
Control + Con A	5.80 ± 0.4 (175)		50	148161 ± 4180 (4902)
Mitomycin C	3.41 ± 0.13 (103)	1.68	6	340 ± 10 (11)
Mitomycin C + Con A	5.74 ± 0.12 (173)		47	18914 ± 571 (625)

^aC5781/6 splenocytes were incubated with 40 µg/ml of mitomycin C for 45 min., at 37°C, washed and then resuspended in medium containing Con A. K⁺ influx was measured at 8 hr, cell size at 12 hr and ³H-thymidine incorporation at 48 hr. At least 5 experiments were done; the range of individual values did not differ from the mean by more than 10%. Values from one experiment are given.

^bValues for K⁺ influx and ³H-thymidine incorporation are means of triplicates ± S.D.

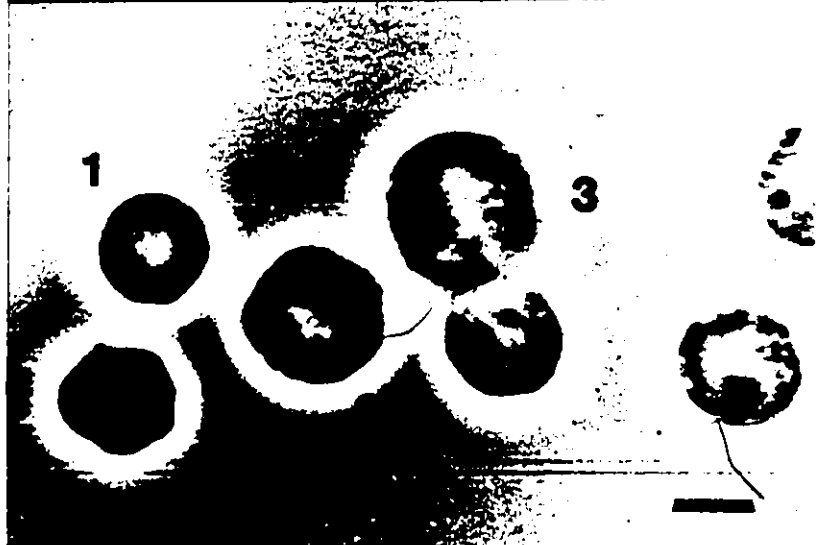
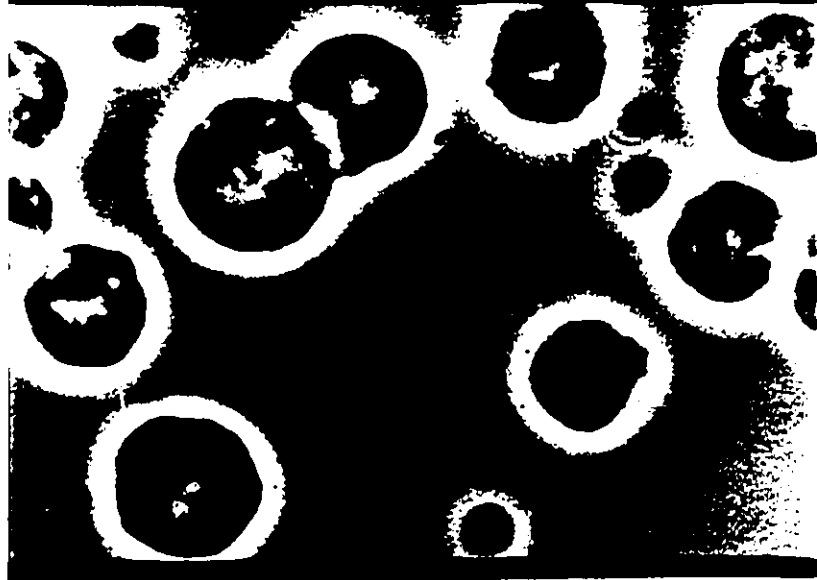
^cFor estimating cell size over 300 cells were measured for each treatment; blasts were defined as those cells whose diameter was greater than 8 µm.

^dPercent of control values, given within brackets, were calculated on the basis of assuming that the control value was 100%.

Figure 2. Light micrographs showing Con A response of untreated and mitomycin C-pre-treated lymphocytes.

C57Bl/6 splenocytes were pretreated with 40 μ g/ml of mitomycin C for 45 minutes, washed and then cocultured with Con A. Panel a shows small lymphocytes, under 8 μ m, in an untreated population not incubated with Con A. In panel b, untreated, Con A stimulated cells show a full range of sizes from unstimulated small cells (type 1) (1) to cells showing intermediate degree of stimulation (type 2) (2) and large blasts (type 3) (3). Mitomycin C treated cells, when cultured with Con A (panel c) show a pattern similar to that seen in panel b.

Bar is 5 μ m.



3.1.2 Effect of inhibiting agents on Con A response

A variety of physical and chemical agents, which inhibit cell metabolism, have been reported to suppress the stimulating capacity of lymphocytes in a mixed leukocyte reaction. To understand what aspect of the stimulatory mechanism is affected, the effects of these agents were studied on two immune functions of the lymphocytes: (1) their ability to stimulate allogeneic cells and (2) their ability to respond to a stimulus, such as Con A.

As described in 'Materials and Methods', section 2.2.3, mouse splenic leukocytes were treated with ouabain, ultraviolet rays, 5-fluorouracil and colchicine and the effects of these agents were then studied in experiments measuring stimulating ability and Con A response of the treated cells. For assessing Con A response, both pre-S and S-phase events of activation were measured. The parameters studied were increase in K^+ influx, blast transformation (increase in size to 3μ or more) and incorporation of 3H -thymidine. Ability to stimulate was recognized by culturing the treated cells with untreated responders and by examining proliferation in the MLR; incorporation of 3H -thymidine was measured.

3.1.2.1 Ouabain

In human (Christen *et al.*, 1975) and rhesus systems (Kaplan and Owens, 1982) it has been shown that when stimulators in an MLR are pre-treated with ouabain, dual effects

are produced, depending on the dose. Enhancement is seen with a low dose and inhibition with a high one. To see if this was true in the murine system as well, splenocytes were pre-treated with this glycoside and used as stimulators in MLRs. They were examined also for their ability to respond to Con A.

Stimulating capacity

Stimulators in MLR cultures were pretreated with ouabain as described in section 2.2.3. In the course of studies with various doses of ouabain ranging from $10^{-5}M$ to $5 \times 10^{-3}M$, opposite effects of this drug were observed (Figure 3). At $2 \times 10^{-4}M$, there was an enhancement of proliferation compared to the control. At doses lower than that there was no obvious effect and at higher doses there was an inhibition, directly proportional to the concentration of the drug. With $2 \times 10^{-4}M$ pretreatment, there was an enhancement of 1.51 ± 0.22 fold in 3 out of 13 experiments at the peak values on days 3-4 of MLR. This was statistically significant ($p = 0.001$ in seven cases and 0.02 in one). In the rest of the 5 experiments the values were approximately the same as the control. At doses 1 to $5 \times 10^{-3}M$ there was an inhibition of over 50% in all 8 experiments tried. This inhibition persisted even when the number of stimulating cells was increased 4 times (data not shown).

Figure 3. MLR with stimulators pretreated with ouabain.

Curve A represents the MLR with control stimulators; B that with stimulators pretreated with $2 \times 10^{-3}M$ ouabain and curve C with stimulators pretreated with $1 \times 10^{-3}M$. Incorporation of 3H -thymidine (cpm) during 5 days of culture has been shown. Results shown are from one of eight similar experiments.

Bars represent standard deviation.

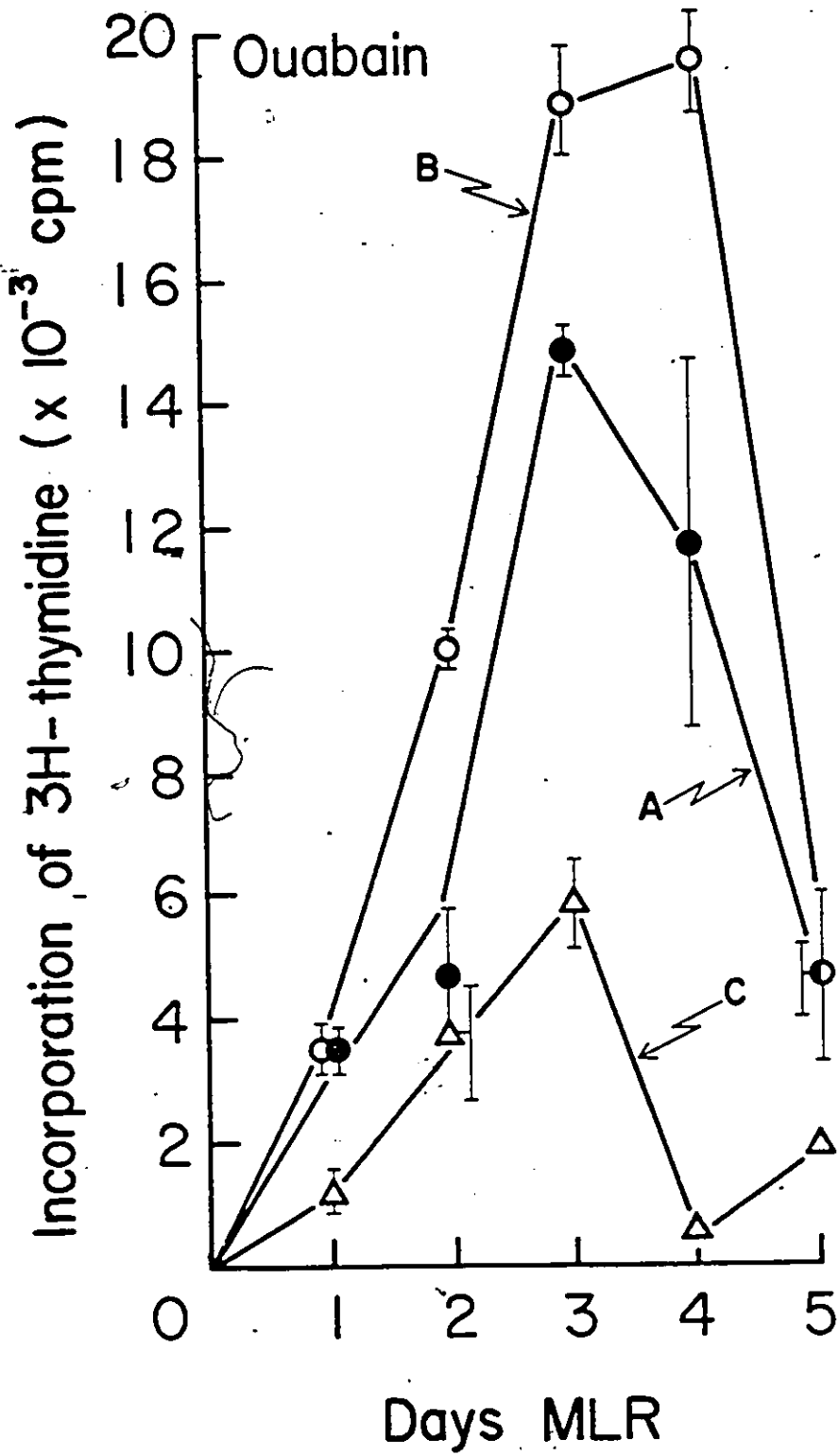
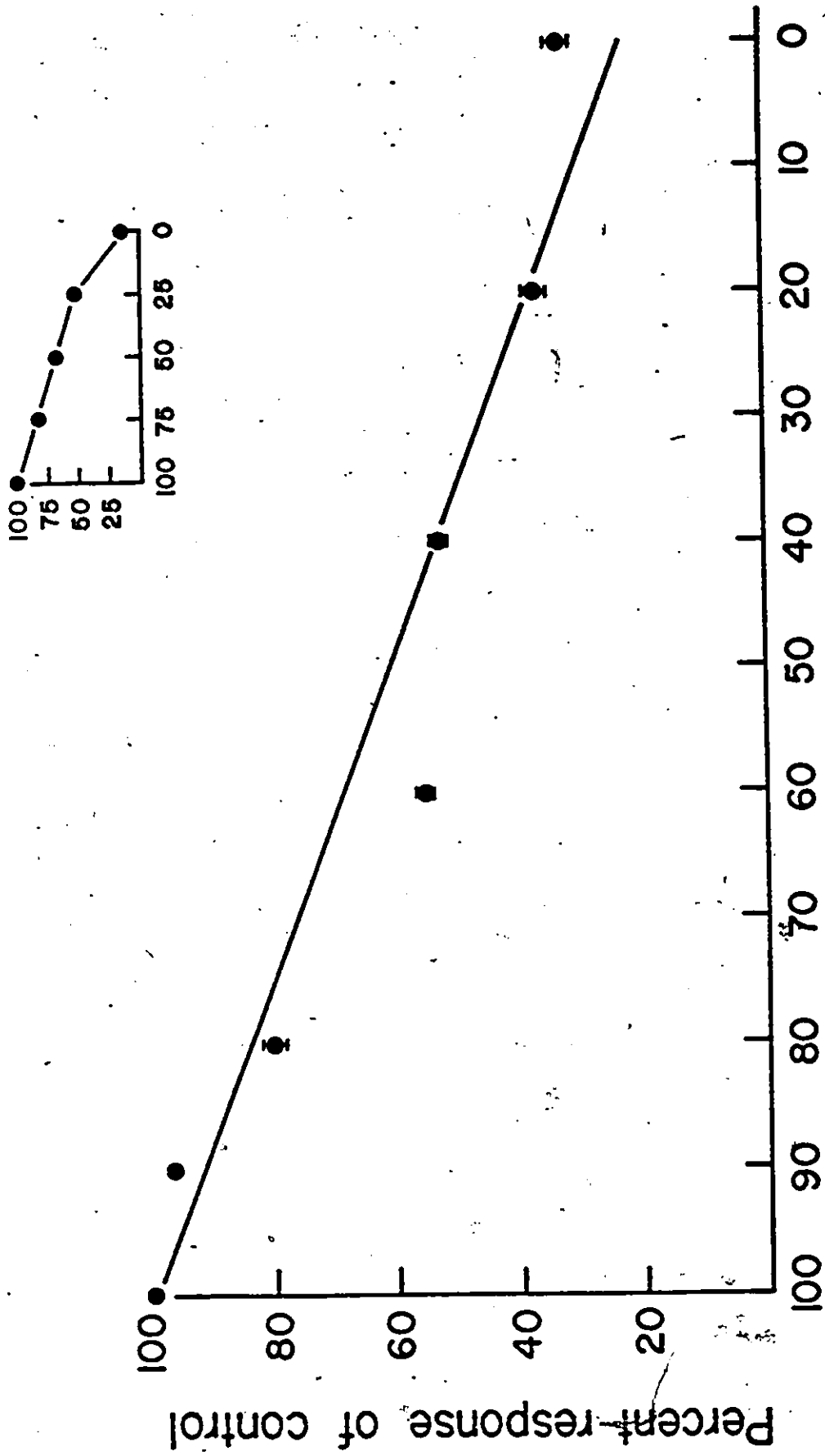


Figure 4. MIR with stimulators consisting of untreated control cells mixed with $1 \times 10^{-3}\%$ ouabain pretreated cells.

C57Bl/6 splenocytes pretreated with $1 \times 10^{-3}\%$ ouabain and mitomycin C were mixed with control mitomycin C treated cells in various proportions, and cocultured with Balb/c splenocytes. Peak MIR values (Day 3) obtained in the different cultures are expressed as percentages of that in the control (100% untreated cells). The composition of the different cultures are expressed in terms of the percentages of untreated control cells they contained.

Bars represent standard deviations.

The inset shows a graph of ^3H -thymidine incorporation in MIR cultures of Balb/c responders and C57Bl/6 stimulators where mitomycin C-treated leukocytes were diluted with glutaraldehyde fixed cells of the same population in different proportions.



Percent of control cells in the stimulators

Effect on the T-helper and T-suppressor balance

To find out if ouabain affected the relative proportions of T-helper and T-suppressor cells in the splenocyte population, ouabain pre-treated splenocytes were mixed with control cells. 1×10^{-3} M ouabain-pretreated C57Bl/6 splenocytes were further treated with mitomycin C and then mixed with control mitomycin C-treated cells in various ratios and incubated with responder Balb/c cells. There was no apparent suppressive effect of the ouabain-treated cells on the controls (Figure 4). A linear relationship between the level of proliferation and the percentage of control cells in the stimulators is seen. The inset shows a similar curve when untreated stimulator cells were mixed with glutaraldehyde-fixed cells in the same proportions.

Response to Con A

Ouabain-pretreated (2×10^{-4} M and 1×10^{-3} M) splenocytes of Balb/c and C57Bl/6 were examined for their Con A response (Table 4). The first indicator of activation was assessed by measuring the influx of potassium at 8-12 hours after the addition of Con A. Pretreatment with the lower dose showed activation of the same or higher magnitude as the control. However treatment with the higher dose abolished the increased uptake of the cations in response to Con A. The number of blasts formed after treatment with the lower dose was the same or higher compared to the control. No blast formation was observed in the case of the higher dose.

Table 4

Con A response of splenocytes pre-treated with ouabain^a.

Treatment	K ⁺ influx f moles/cell/hr ^b	Blast formation Percent blasts ^c	³ H-thymidine incorporation (cpm) ^b
Control	3.35 ± 0.1	7	1001 ± 104
Control + Con A	5.59 ± 0.3	44	13750 ± 694
Control (m) + Con A	5.29 ± 0.5	38	6641 ± 838
2 x 10 ⁻⁴ M	3.22 ± 0.2	NM ^d	998 ± 234
2 x 10 ⁻⁴ M + Con A	5.63 ± 0.3	48	19041 ± 1060
2 x 10 ⁻⁴ M (m) + Con A	5.02 ± 0.28	41	7272 ± 163
1 x 10 ⁻³ M	2.44 ± 0.5	NM	152 ± 21
1 x 10 ⁻³ M + Con A	1.78 ± 0.1	4	2913 ± 665
1 x 10 ⁻³ M (m) + Con A	1.89 ± 0.21	0	415 ± 91

^aSplenocytes from C57B1/6 mice were pre-treated with ouabain for 48 hours and then further treated with mitomycin C (m) at 25 µg/ml. After washing, the cells were cultured with Con A. K⁺ influx was measured at 8 h, cell size at 24 h and ³H-thymidine incorporation at 48 h. Six experiments were done; the range of values did not differ from the mean by more than 10%. Values from one experiment are given.

^bValues for K⁺ influx and ³H-thymidine incorporation are means of triplicates ± SD.

^cFor estimating cell size, over 300 cells were measured for each treatment; blasts were defined as those cells whose diameter was greater than 8 µm.

^dNM = not measured.

3.1.2.2 Ultraviolet rays

Castellanos *et al* (1982) have shown that ultraviolet (UV) affects the initiation of activation at the membrane level. It has also been reported to inhibit the stimulating capacity of leukocytes in MLR (Lindahl-Kiessling and Safwenberg, 1971, Okada *et al*, 1980). Experiments were set up to see how the effect of UV would relate to the hypothesis that the stimulators are required to be activated.

Stimulating capacity

The stimulating capacity of UV-treated cells was examined. In Fig 5, Curve C shows that with 360 ergs/mm² there was inhibition of MLR response. Values on Day 3 of MLR were down to 172 cpm compared to 8201±850 in the control. With 120 ergs/mm² exposure on the other hand, (Curve B in Figure 5) the stimulating ability was not only not inhibited but enhanced as compared to the control in two out of 4 experiments by 53 - 60%.

Response to Con A

The uptake of K⁺ was measured by the transport of its congener ⁸⁶Rb⁺. Table 5 shows the effects of two of the various doses tried: 120 ergs/mm² permitted the activation of K⁺ influx in response to Con A (over 80% of the control value) and 360 ergs/mm² blocked this step. The low dose had no effect on the number of blasts formed but the high-dose totally inhibited this process.

Figure 5. MLR with stimulators pretreated with UV.

Mitomycin C treated control C57Bl/6 splenocytes were reacted with Balb/c responders, resulting in MLR (Curve A), peaking on days 2 -3. When the stimulators were pretreated with 120 ergs/mm², curve B was produced and pretreatment with 360 ergs/mm² gave curve C. MLR is expressed by ³H-thymidine incorporation during 5 days of culture.

One of four experiments done is represented here.

Bars represent standard deviation.

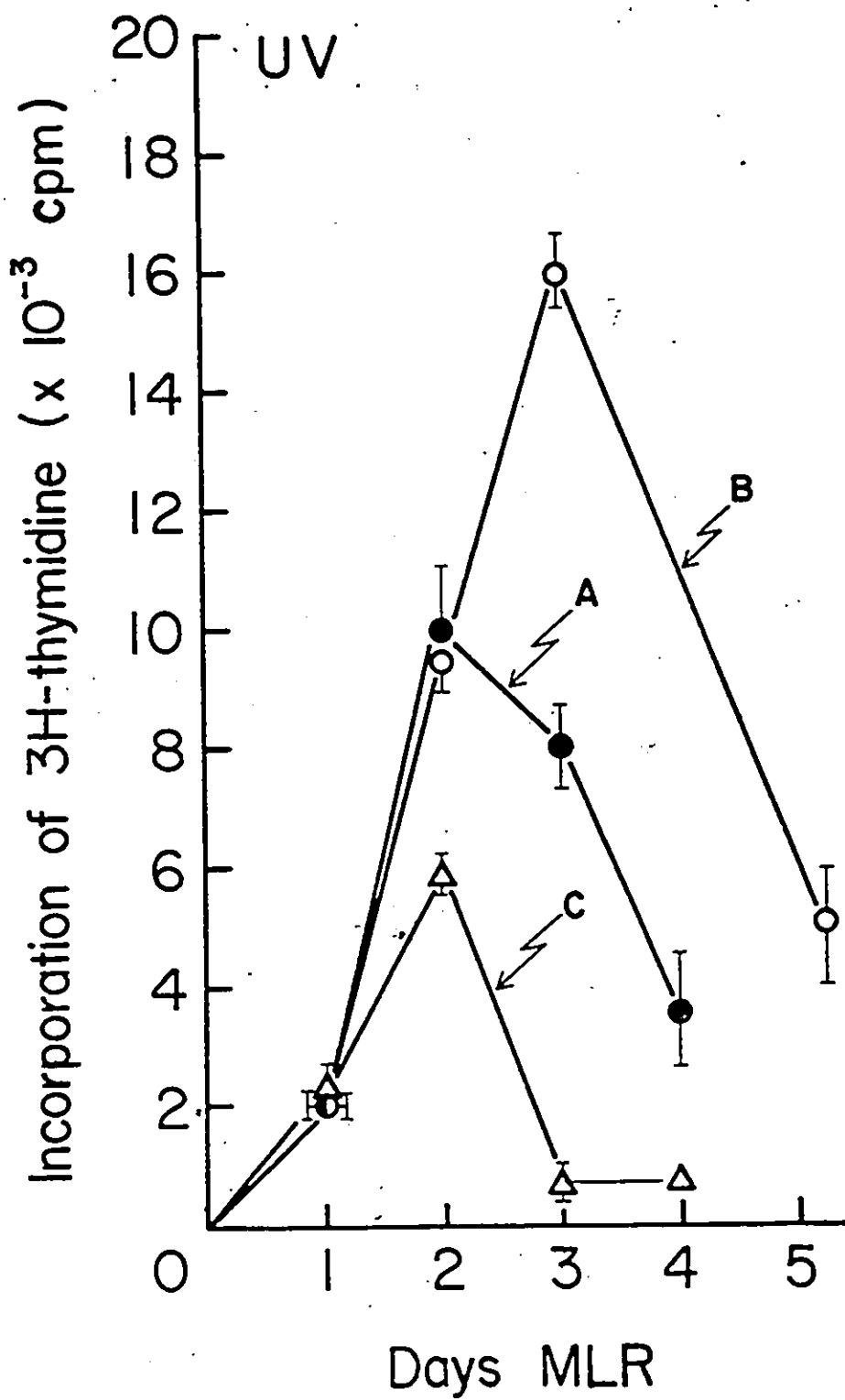


Table 5

Con A response of splenocytes pre-treated with ultra-violet radiation^a.

Treatment	K ⁺ influx f moles/cell/hr ^b	Blast formation Percent blasts ^c	³ H-thymidine incorporation (cpm) ^b
Control	2.48 ± 0.2	8	3282 ± 351
Control + Con A	4.26 ± 0.2	54	111521 ± 2800
Control (cm) + Con A	4.13 ± 0.5	48	26256 ± 285
120 ergs/mm ²	2.58 ± 0.1	NM	2958 ± 985
120 ergs/mm ² + Con A	3.74 ± 0.3	49	61101 ± 212
120 ergs/mm ² (m) + Con A	3.57 ± 1.38	42	22974 ± 1161
360 ergs/mm ²	1.27 ± 0.3	NM	831 ± 38
360 ergs/mm ² + Con A	1.13 ± 0.2	0	1309 ± 18
360 ergs/mm ² (m) + Con A	0.93 ± 0.3	0	1165 ± 28

^aSplenocytes from C57Bl/6 mice were irradiated with 120 and 360 ergs/mm² UV and then further treated with mitomycin C (m). After washing, the cells were cultured with Con A. K⁺ influx was measured at 8 h, cell size at 24 h and ³H-thymidine incorporation at 48 h. Five experiments were done; the range of values did not differ from the mean by more than 10%. Values from one experiment are given.

^bValues for K⁺ influx and ³H-thymidine incorporation are means of triplicates ± SD.

^cFor estimating cell size over 300 cells were measured for each treatment; blasts were defined as those cells whose diameter was greater than 8 μm.

3.1.2.3 5-Fluorouracil

Like mitomycin C, 5-fluorouracil (5-FU), which is a pyrimidine analogue, inhibits DNA replication without affecting the earlier events of cell activation (Boumah *et al.*, 1984). Splenocytes from C57Bl/6 were incubated with various doses of 5-FU for 6 hours. After washing the cells were cocultured with allogeneic cells or Con A.

Stimulating capacity

Figure 6 shows that pre-treatment of stimulators with 6.6×10^{-5} M 5-FU inhibited MIR response by 44%. There was no change from the control when the dose was 3.3×10^{-5} M.

Response to Con A

Measurements of Con A response of 5-FU (3.3 and 6.6×10^{-5} M) -pretreated splenocytes are listed in Table 6. ^3H -thymidine incorporations were inhibited with both the doses. Increase in K^+ influx was seen not only with the low dose but also the higher dose which inhibited the stimulating capacity. Stimulation indices of 1.71, 1.73 and 1.59 were obtained in control and cultures treated with 3.3×10^{-5} M and 6.6×10^{-5} M 5-FU respectively. Assessment of blast formation, however, showed a correlation between the ability to form blasts and to stimulate allogeneic cells. There was a difference between the two doses, with the lower dose the number of blasts was 80% of the control and it was 12% of the control with the higher dose (Table 6).

Figure 6. MLR with stimulators pretreated with 5-fluorouracil.

Curve A represents the MLR of control stimulators of C57Bl/6 with Balb/c. Data for curves B and C are from MLR where the stimulators were pretreated with 3.3 and $6.6 \times 10^{-5}M$ respectively. Incorporation of 3H -thymidine represented proliferation in MLR.

One of the three experiments done is depicted, each experiment being done in triplicate.

Bars represent standard deviation.

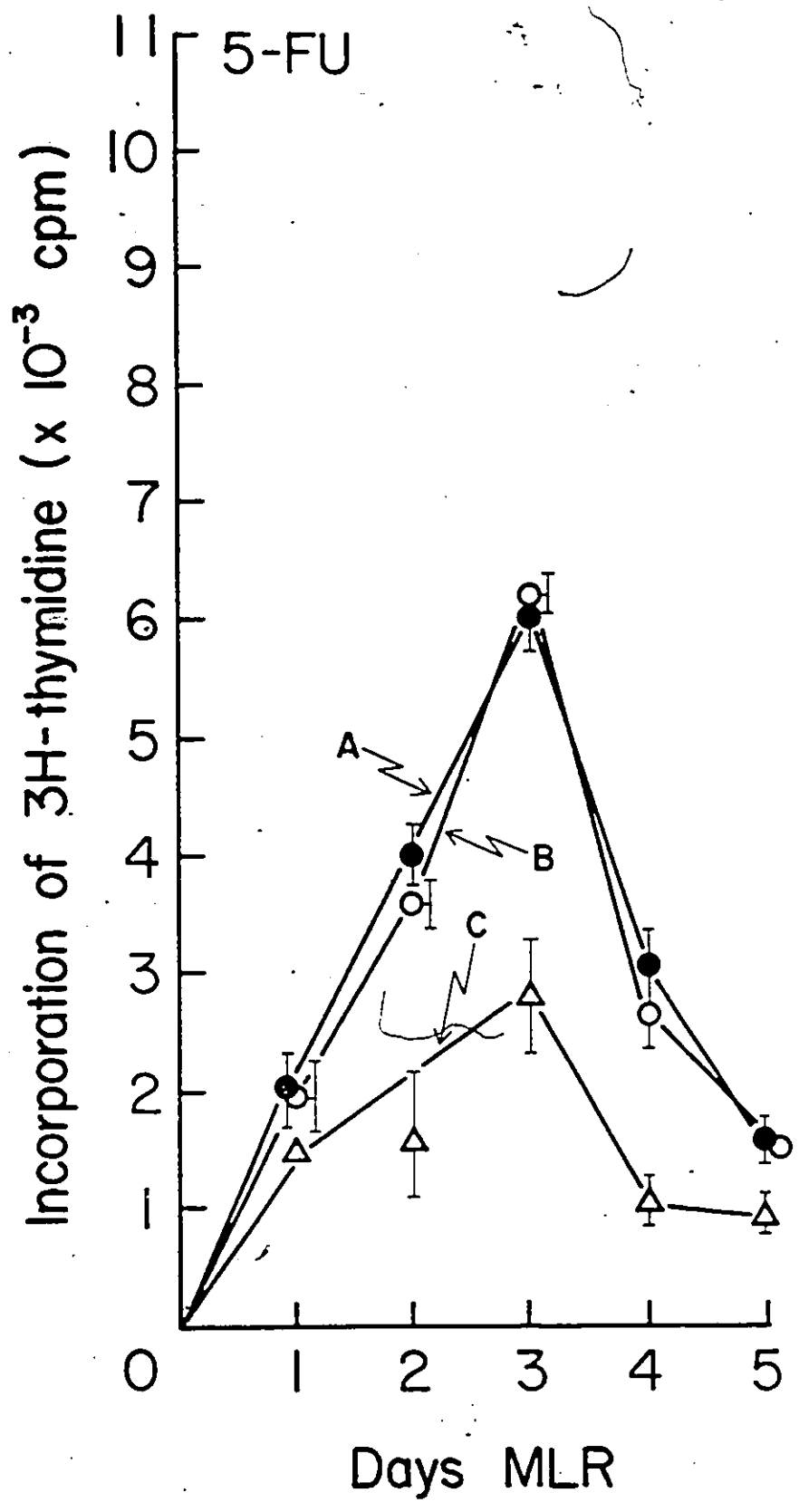


Table 6

Con A response of splenocytes pre-treated with 5-fluorouracil^a.

Treatment	K ⁺ influx f moles/cell/hr ^b	Blast formation Percent blasts ^c	³ H-thymidine incorporation (cpm) ^b
Control	4.40 ± 0.3	8	3123 ± 490
Control + Con A	7.51 ± 0.3	50	151621 ± 3349
3.3 x 10 ⁻⁹ M	4.65 ± 0.7	NM ^d	2750 ± 152
3.3 x 10 ⁻⁵ M + Con A	8.06 ± 0.2	40	22972 ± 636
6.6 x 10 ⁻⁵ M	4.29 ± 0.4	NM	895 ± 238
6.6 x 10 ⁻⁵ M + Con A	6.84 ± 0.3	9	618 ± 149

^aSplenocytes from C57BL/6 mice were pre-treated with 3.3 and 6.6 x 10⁻⁵ M 5-fluorouracil. After washing, the cells were cultured with Con A. K⁺ influx was measured at 8 h, cell size at 24 h and ³H-thymidine incorporation at 48 h. Three experiments were done; the range of values did not differ from the mean by more than 10%. Values from one experiment are given.

^bValues for K⁺ influx and ³H-thymidine incorporation are means of triplicates ± SD.

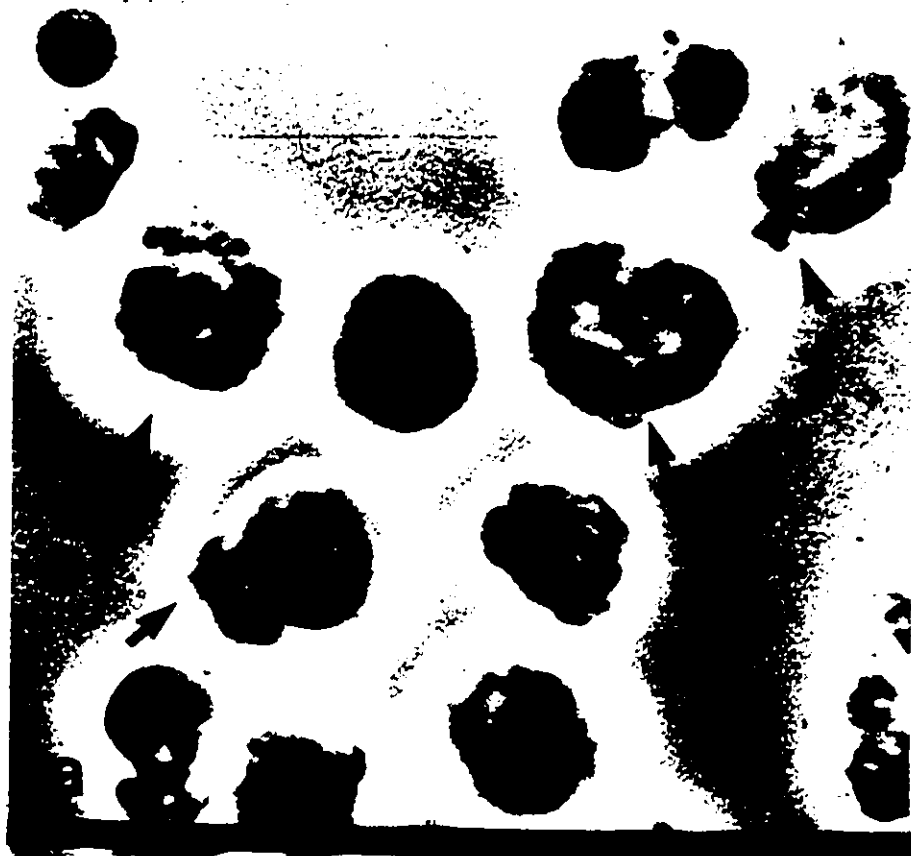
^cFor estimating cell size over 300 cells were measured for each treatment; blasts were defined as those cells whose diameter was greater than 8 μm.

^dNM = not measured.

Figure 7. Light micrographs showing Con A response of lymphocytes pretreated with 5-fluorouracil.

Panel a shows that in response to Con A lymphocytes pretreated with 3.3×10^{-5} M 5-FU formed small blasts (type 2 cells) and hardly any fully formed blasts (type 3) were seen. Panel b depicts only the type 1 cells (size of resting cells); few blasts were formed in a Con A culture where the lymphocytes were pretreated with the high dose, 66.6×10^{-5} M 5-FU.

Bar is 5 μ .



b

Although the number of blasts was comparable in the control and the culture containing cells pretreated with the low dose, only small blasts (type 2) (see section 2.4.2) were observed in the latter, hardly any fully formed blast was seen (Figure 7).

3.1.2.4 Colchicine

Colchicine has been reported to inhibit (Rudd and Kaplan, 1981; Ramsey and Pincus, 1976) or enhance (Stenzel et al., 1978 and Suthantbiran et al., 1980), lymphocyte activation depending on the stimulus and the culture conditions. Experiments were set up to find out if the action of this drug would follow the general pattern noted with the various agents studied, namely the treatment which suppresses the ability to respond to Con A also inhibits the stimulatory capacity of the lymphocytes in an MIF.

Stimulating capacity

C57Bl/6 splenocytes pretreated with colchicine and mitomycin C were cultured with Balb/c or CBA responders. Both enhancement or inhibition were noted, depending on the treatment. Comparatively longer periods of incubation with this drug or treatment with a high dose, 1×10^{-4} M (Figure 8, Curve C) inhibited the stimulatory capacity. 1×10^{-5} M or lower doses on the other hand had either no effect (three experiments) or caused an enhancement of 36 to 56 % (two experiments) (Curve B in Figure 8) when the cells were pretreated for 2 to 6 hours.

Figure 8. MLR with stimulators pretreated with colchicine.

Curve A represents proliferative response of MLC, where the stimulating population was untreated control. Curves B and C represent the responses where the stimulators were pretreated with 1×10^{-5} M and 1×10^{-6} M colchicine respectively.

One of the three experiments done is shown here, each experiment was performed in triplicate.

Bars are standard deviation.

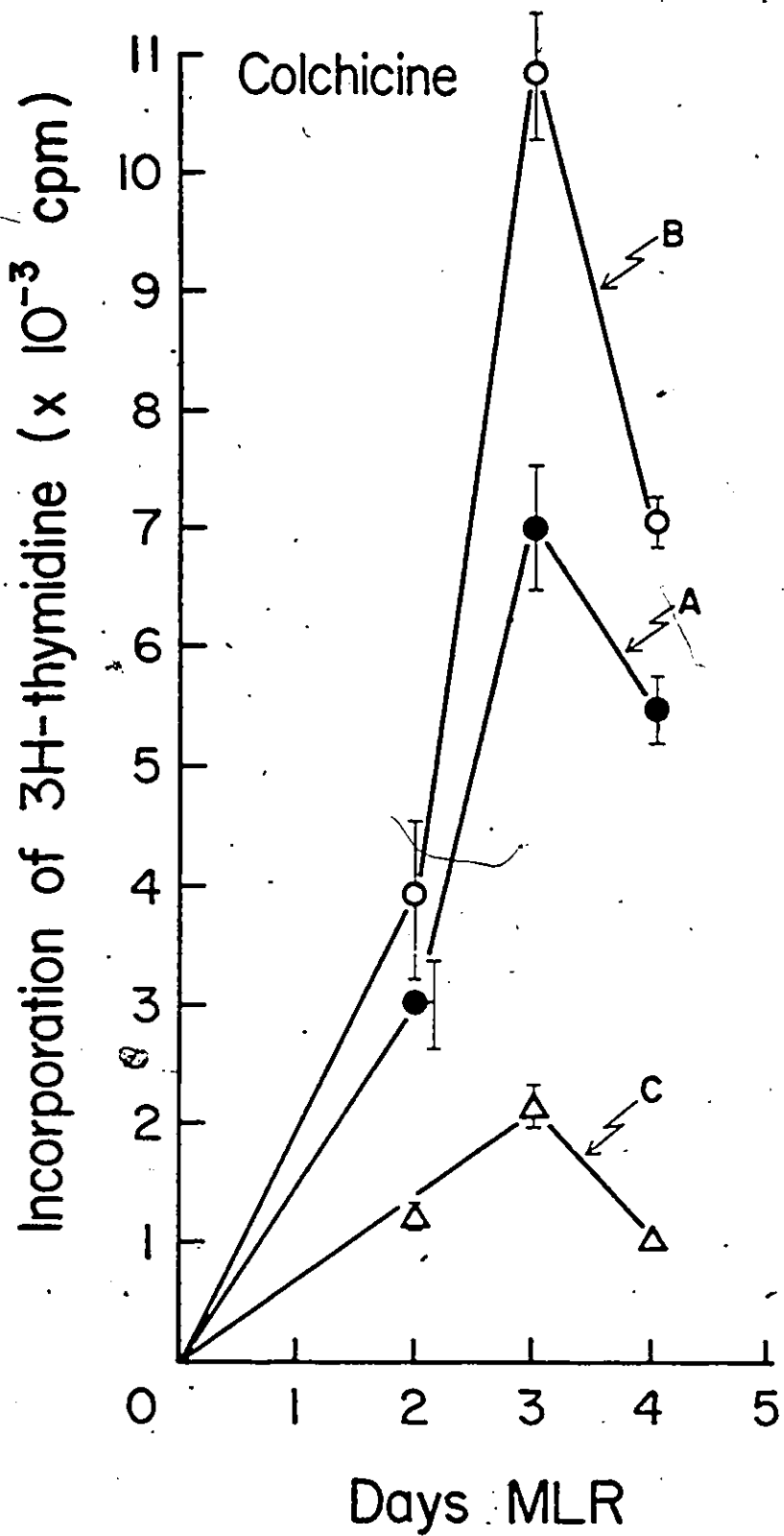


Table 7

Con A response of splenocytes pre-treated with colchicine^a.

Treatment	Blast formation Percent blasts	³ H-thymidine incorporation (cpm)
Control	7	7224 ± 800
Control + Con A	47	136727 ± 3123
Control (m) + Con A	41	31824 ± 848
1 x 10 ⁻⁵ M + Con A	44	141737 ± 1872
1 x 10 ⁻⁵ M (m) + Con A	48	5205 ± 359
1 x 10 ⁻⁴ M + Con A	36	151162 ± 2908
1 x 10 ⁻⁴ M (m) + Con A	6	4271 ± 256

^aSplenocytes from C57B1/6 mice were pre-treated with 1 x 10⁻⁴ M colchicine and then further treated with mitomycin C (m). After washing, the cells were cultured with Con A. Cell size was measured at 24 h and ³H-thymidine incorporation at 48 h. Three experiments were done; the range of values did not differ from the mean by more than 10%. Values from one experiment are given.

^bValues for ³H-thymidine incorporation are means of triplicates ± SD.

^cFor estimating cell size over 300 cells were measured for each treatment; blasts were defined as those cells whose diameter was greater than 8 μm.

Response to Con. A

Table 7 shows that neither of the two doses of colchicine affected blast formation. However, when the colchicine-treated cells were further treated with mitomycin C, once more a correlation was seen between the stimulating capacity of the lymphocytes and their ability to undergo the early stages of activation. After treating the cells with the low dose, 1×10^{-5} M colchicine plus mitomycin C, the number of blasts produced was the same as in the mitomycin C-treated control cells. However, after treatment with 1×10^{-4} M colchicine plus mitomycin C, the number was reduced to 26% of the control; a similar treatment reduced the stimulating capacity of leukocytes in MLR.

Table 8 summarizes the effects of various agents on the functions of leukocytes with respect to their capacity to stimulate an MLR and their ability to respond to Con A and shows that a population of leukocytes could induce MLR optimally only when it could demonstrate the changes characteristic of early (pre-S phase) activation, such as increase in K^+ influx and in cell size. Thus without exception there exists a correlation between these two immune functions, namely, stimulating ability in MLR and Con A responsiveness, suggesting a common mechanism between them (Foy et al., 1982A).

Table 8

Effects of various agents on the early stages of Con A activation of mitomycin C treated cells and on the capacity of these cells to stimulate an MLR.

Treatment ^a	Con A response (Percent of control) ^b	Blast formation	Ability to stimulate in MLR (Percent of control) ^c
	K ⁺ influx		³ H-Thymidine incorporation
Ouabain			
2 x 10 ⁻⁴ M	100-117 ^d	100-120	103-140
1 x 10 ⁻³ M	29-38	0	22-40
UV			
120 ergs/mm ²	76-83	87-100	92-160
360 ergs/mm ²	27-31	0	30-32
5-FU			
3.3 x 10 ⁻⁵ M	106-108	77-96	86-100
6.6 x 10 ⁻⁵ M	89-94	6-18	50-53
Colchicine			
1 x 10 ⁻⁵ M	NM ^e	76-110	100-156
1 x 10 ⁻⁴ M	NM	3-12	28-31

Table 8 (contd.)

^aMouse splenocytes treated with various agents were further treated with mitomycin C and then either cultured with Con A or cocultured with allogeneic responders. Con A response was assessed by measuring ⁸⁶Rb uptake at 8 h after Con A addition and cell size at 24 h. A cell was considered to be a blast when its diameter was 8 μ m or more.

^bAll values are expressed as percent of control; control is the culture treated with only mitomycin C. Percent value was calculated as shown in Table 3.

^cMLR value obtained on Day 3-4 in cultures containing stimulators treated with only mitomycin C is assumed to be 100%.

^dAt least 6 experiments were done for each treatment; the range of values obtained in the different experiments is shown.

^eNM = not measured.

3.1.3 Effect of GVH reaction on stimulating ability in MLR

Having seen above (Table 8) that compromising responsiveness to Con A *in vitro* inhibits MLR stimulating ability of leukocytes, it seemed desirable to find out if splenocytes immunosuppressed *in vivo* would be able to stimulate allogeneic cells. Splenocytes from mice undergoing late graft-versus-host reaction show impaired T cell function (Seddik *et al.*, 1980). Immunosuppressed splenocytes from mice undergoing GVH reaction (20-30 days post GVH) were examined. (See 'Materials and Methods', section 2.2.5, for the induction of GVH reaction and Table 2 in the same section which shows that the splenocytes from these mice were immunosuppressed.)

Table 9 shows that there was an increase of 1.70 to 1.78 fold in K^+ influx when splenocytes from normal CBA x AFI were cultured with Con A, but there was no activation in the uptake of this cation when the Con A-treated splenocytes were from mice undergoing GVH reaction. As predicted from the observations recorded in Section 3.1.2, the splenocytes from the immunosuppressed mouse showed inhibition of stimulating capacity in MLR; they produced only 47% of the proliferative response ($7,487 \pm 243$ cpm) of their normal counterpart ($15,912 \pm 1268$ cpm) on day 3 (peak response) of MLR (Figure 9 and Table 9).

Figure 9. Stimulating capacity of splenocytes from mice undergoing GVH reaction.

Curve A represents MLR between mitomycin C treated splenocytes of CBA x AF1 and Balb/c responders, peaking on days 3 and 4. When splenocytes from GVH mice (induced by injecting CBA x AF1 with parental A strain) were used, a reduced proliferative response was obtained (Curve B) which peaked on days 2 - 3.

One of the three experiments done is shown, each experiment was done in triplicate.

Bars represent standard deviation.

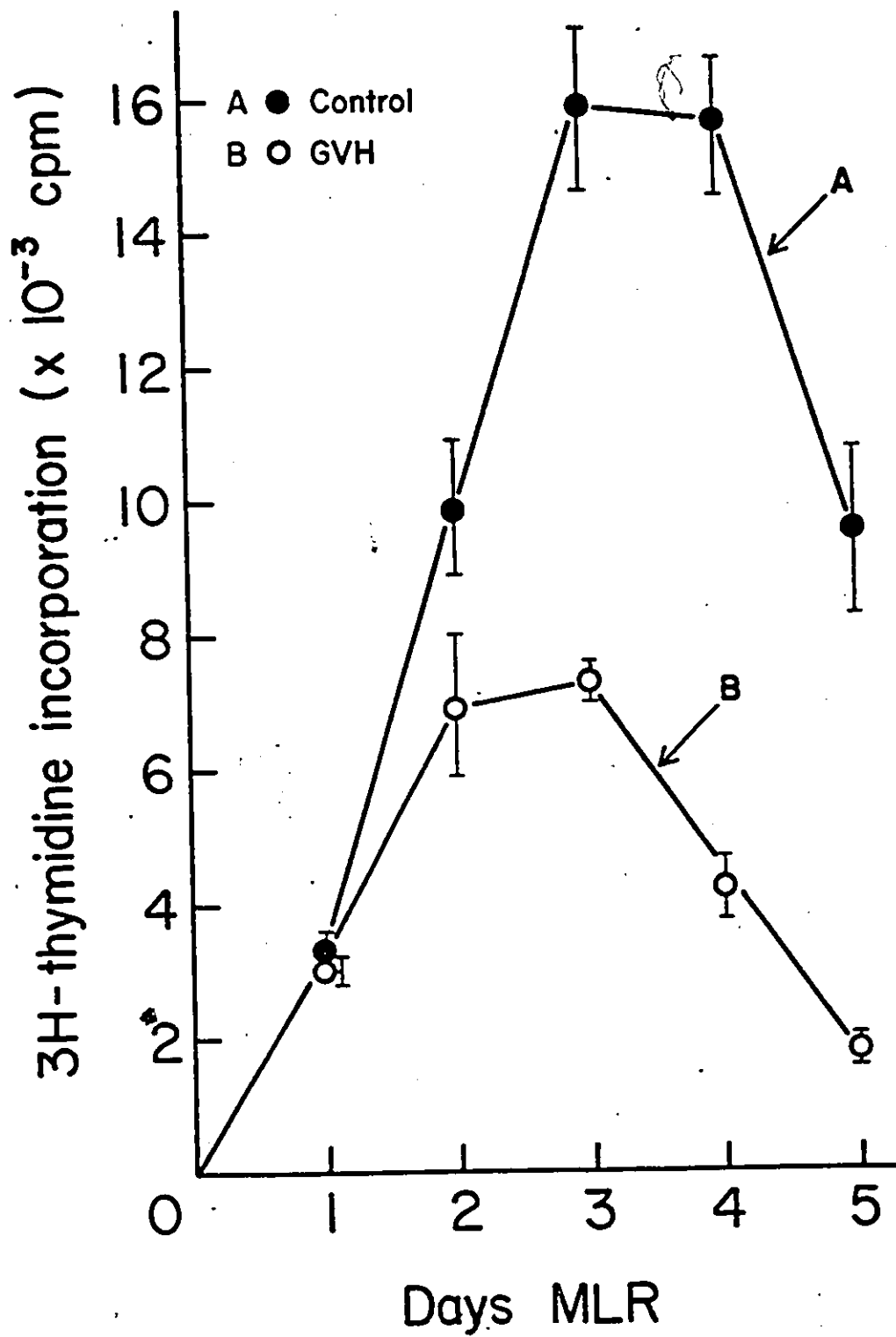


Table 9

Effect of GVH-induced immunosuppression on Con A activated K^t transport and on the capacity to stimulate an MLR^a.

Culture	Response to Con A K ^t influx (Percent control) ^b	Ability to stimulate in MLR ³ H-Thymidine incorporation (Percent control).
Normal	100	100
Normal + Con A	170 ^c , 178 ^d	NA
GVH	100 ^e	47, 39
GVH + Con A	105, 110	NA

^aSplenocytes from normal CBA x AF₁ and GVH mice (CBA x AF₁ with parental A cells) were cultured either with Con A or with allogeneic responders (Balb/c). Con A response was assessed by measuring activation of K^t influx at 8 - 10 h. Stimulating ability in MLR was assessed by measuring peak proliferation which occurred on Day 3-4, ³H-thymidine incorporation was measured.

^bValues are percentages of control; for calculations see Table 1.

^c, ^dData from 2 experiments are included.

^eIn some cases, the GVH control value was elevated over normal control value.

^fNA = not applicable.

3.2 ACTIVATION OF T CELLS IN THE STIMULATORS IN MLR

Results recorded above show that optimal MLR occurs when stimulators can be activated. Since the primary candidate in the stimulator to respond to alloantigens of the responders is the T cell, experiments were planned to determine if the stimulator-T cell is in fact activated during MLR. Assays measuring increase in size and RNA synthesis were used. In cultures containing C57Bl/6 (Thy 1.2) stimulators and AKR responders (Thy 1.1), the stimulator-T cells were identified by FITC-conjugated monoclonal anti-Thy 1.2.

3.2.1 Increase in size

Using FITC coupled anti-Thy 1.2, (see section 2.5.1 for the procedure) stimulator-T cells from cultures containing mitomycin C-treated C57Bl/6 splenocytes and AKR responding cells were identified (Figure 10) (cells in panels b and d and those pointed by arrows in panels a and c). After 24 hours of culture, the cells (panels c and d) showed an increase in size (over 8 μ m) compared to that at 0 hour (panels a and b) where the mean diameter was $6.25 \pm 0.8 \mu$ m. The proportion of activated stimulator-T cells at 0 and 24 hour MLR is depicted in Table 10. At 0 hour 8% of the Thy 1.2 cells were over 8 μ m. After 24 hours of contact with the AKR cells, 40% of Thy 1.2 bearing cells were blasts, which represented one-third of the total blasts in the culture. Under these conditions, then, about one in three of the blasts in MLR were from the stimulating population.

Figure 10. Increase in size of the stimulator-T cells during MLR.

Figures 3b and d are fluorescent micrographs of the fields shown by phase-contrast microscopy in Figures 3a and 3c respectively. Micrographs of cells from MLC containing mitomycin C-treated C57Bl/6 (Thy 1.2) splenocytes and AKR (Thy 1.1) splenic cells show increase in cell size from 0 hour in panels a and b to 24 hours in panels c and d. Arrows in panels a and c indicate the cells that correspond to the cells that are fluorescent with FITC labelled anti-Thy 1.2 in micrographs b and d respectively. Cells larger than 8 μ m in diameter were considered to be activated (pointed with 2 arrows in panel c). The mean diameter of cells in the resting phase (panels a and b) was $5.25 \pm 0.8 \mu$ m.

Bar is 10 μ m.

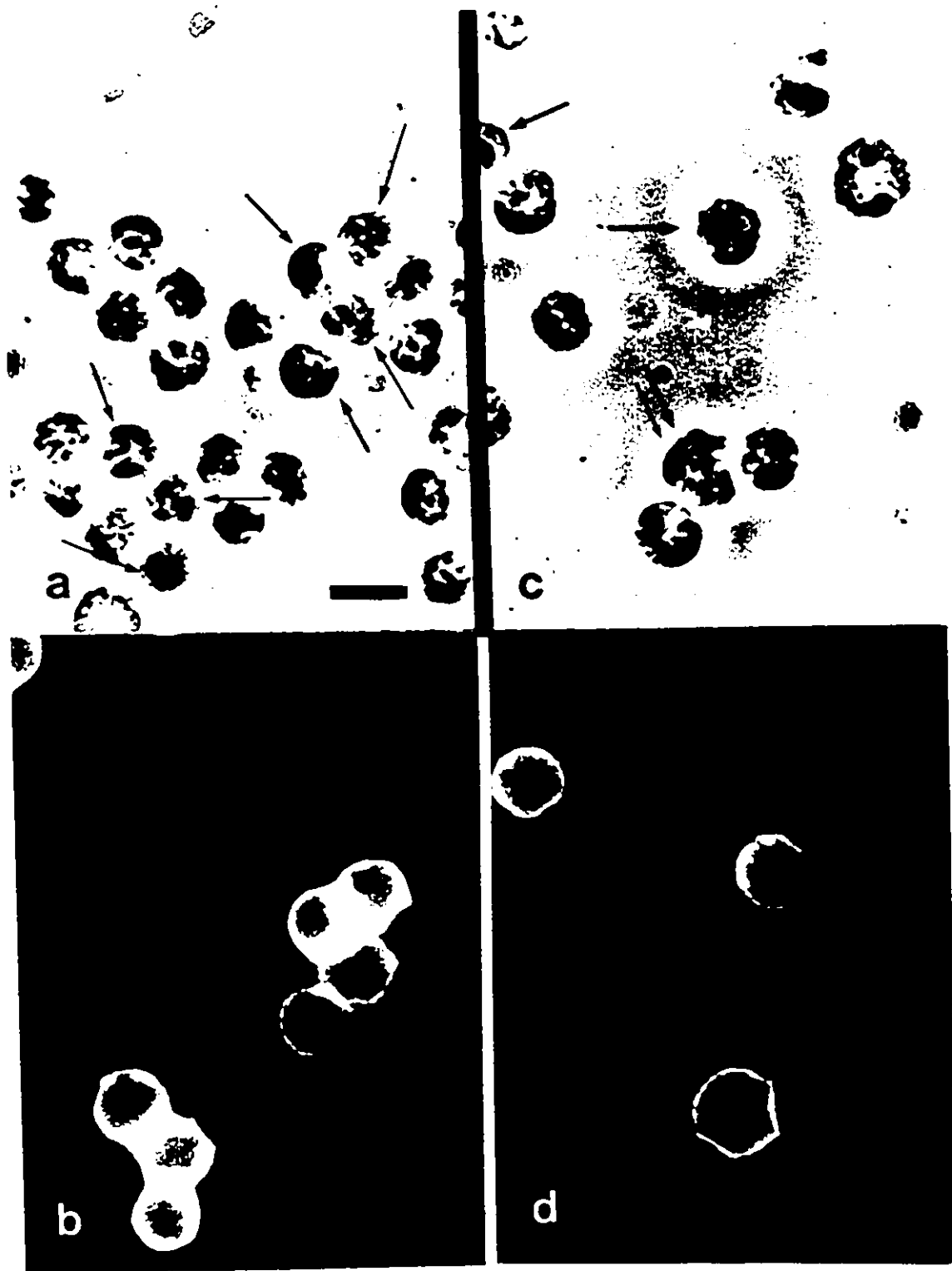


Table 10

Proportion of stimulator-T cells showing increase in size in a 24 hour MLR.^a

Treatment	Thy 1.2 blasts ^b	Total Thy 1.2 cells	Total blasts	Total cells counted
0 hour				
a ^c	8	74	18	263
b ^d	3	57	11	250
24 hour				
a	30	84	94	322
b	18	36	58	200

^aCells from a culture containing C57R1/6 (Thy 1.2) and AKR (Thy 1.1), at a ratio of 2:1, were stained with FITC-labelled anti-Thy 1.2, fixed and scored at 0 and 24 hours after initiation of culture.

^bThe cells were examined for size; cells larger than 8 μm were considered to be blasts.

^{c, d}Results from two experiments are depicted in lines a & b. The absolute numbers are given.

3.2.2 Increase in RNA synthesis

Cultures containing mitomycin C-treated C57Bl/6 splenocytes and AKR responding cells were studied. The stimulator T-cells were double labelled with fluorescein conjugated anti-Thy 1.2 monoclonal antibody and ^3H -uridine as described in 'Materials and Methods' (section 2.5.2). Figure 11 illustrates that stimulator-T cells in a 24 hour MLR show increased incorporation of ^3H -uridine. The cells in phase contrast micrograph (panel b) indicated with arrows correspond to the fluorescent Thy 1.2 bearing cells in panel c. The cells showing more than 10 silver grains (pointed with double arrows) were considered to be activated. The resting cells depicted in panel a showed a mean number of 7 ± 3 silver grains. In MLC cultures pulsed from 0 to 4 hours, 12% of the fluorescent stimulator T cells showed activation (more than ten silver grains) (Table 11). In a culture where allogeneic cells had been cocultured for 24 hours, more than three times as many cells showed increased incorporation of ^3H -uridine. The activated stimulator-T cells comprised 25% of the total activated cells in the culture. Cell counts from three experiments were combined for estimating the overall values. Thus it has been demonstrated unequivocally that a considerable fraction of the stimulating cells undergo activation during MLR (Roy et al. 1982B).

Figure 11. Increase in RNA synthesis in stimulator-T cells during MLR.

Micrographs show cells in a culture containing C57Bl/6 (Thy 1.2) stimulators and AKR (Thy 1.1) responders at 0 hour (panel a) and 24 hours (panels b and c). The cells were pulsed with ^3H -uridine and examined for RNA synthesis by autoradiography. T cells in the stimulating population were identified with FITC-conjugated anti-Thy 1.2. Arrows in the phase-contrast micrograph, panel b, point to the cells that correspond to the cells in the fluorescent micrograph, panel c. In panel c, in the larger Thy 1.2 bearing cell, indicated by two arrows, the density of silver grains was 2.4 times compared to that in resting cells (panel a); the calculations were based on the surface area.

Bar is 5 μm .

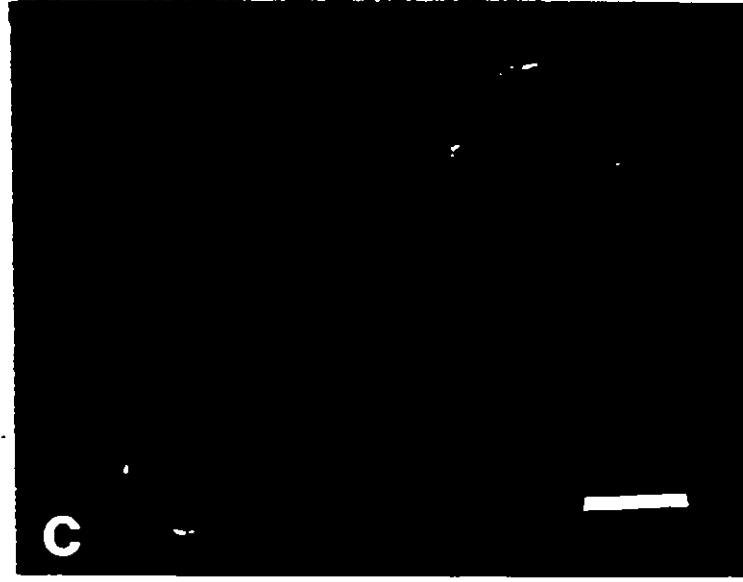
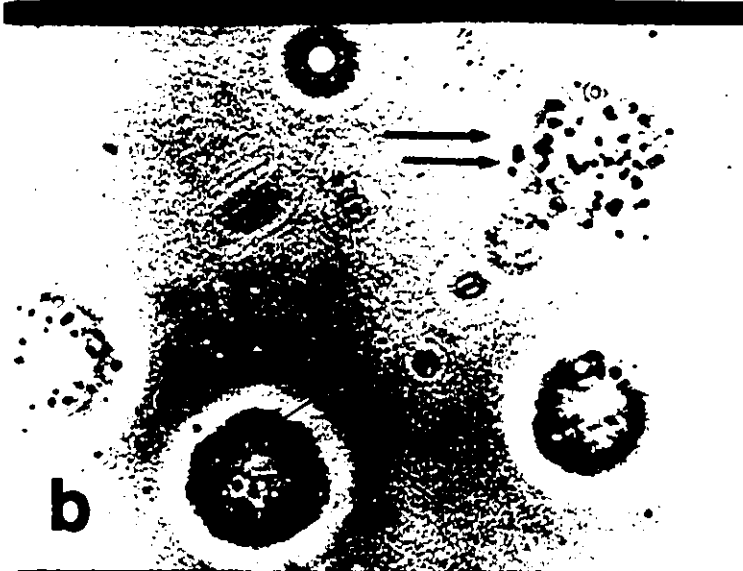


Table 11

Proportion of Stimulator-T Cells Showing Increased RNA Synthesis in a 24 hour MLR^a

Culture	No. of silver grains ^b		No. of Cells Counted	Percent of Activated Cells
	0-10	over 10		
4 hours (a) ^c	100	16	116	14
(b) ^d	84	20	104	16
(c) ^e	84	9	93	10
24 hours (a)	43	57	100	57
(b)	77	43	120	36
(c)	77	39	105	37

^a Cultures containing Balb/c (Thy 1.2) stimulators and AKR (Thy 1.1) responders were examined. Cells were double labelled with FITC conjugated anti-Thy 1.2 and ³H-uridine at 4 and 24 hour after initiation of culture.

^b The fluorescent (stimulator-T) cells were examined for the number of silver grains contained in them; those with more than 10 were considered activated.

^{c,d,e} Results from 3 experiments are depicted in lines a,b&c. The absolute numbers are given.

3.2.2.1 Effect of UV on the activation of stimulator-T cells

Balb/c leukocytes irradiated with low and high doses of UV (120 and 360 ergs/mm²) were used as stimulators in an MLR with AKR responders. After 24 hours of incubation, cells from 1 ml of these cultures and those from the corresponding control culture, which contained untreated stimulators, were processed for immunofluorescence using FITC-labelled anti-Thy 1.2 and then the cells were examined for size. Table 12 shows that although the high dose did not reduce the total number of Thy 1.2 bearing cells in the stimulating population, it prevented them from increasing in size when incubated with allogeneic AKR splenocytes. The low dose allowed the formation of blasts, to the same extent as in the control. Correspondingly, incorporation of ³H-thymidine in cultures, on day 3 MLR, was similar in the control and in the culture containing stimulators treated with the low dose. When the high dose was used, the level of incorporation of ³H-thymidine was similar to that of the responders alone. Thus a definite correlation was seen between the occurrence of blasts in the stimulating population and the level of MLR.

Table 12

Effect of UV on the Activation of T-Cells in the Stimulators

Treatment	Fluorescent Cells	Fluorescent Blasts ^b	Percent Blasts	MLR peak ^c
Balb/c(m)+AKR	83	44	53	15229 ± 562
Balb/((120 ergs)(m)+AKR	114	64	56	16304 ± 244
Balb/c(360 ergs)(m)+AKR	96	5	5	309 ± 52

^a Balb/c (Thy 1.2) stimulators were cultured with AKR (Thy 1.1) responders at a ratio of 2:1. 24 hour culture was stained with FITC conjugated anti-Thy 1.2 and the cells were then examined for size.

^b Cells larger than 8 μ m were considered to be blasts.

^c MLR peak was reached on Day 3; it was measured by incorporation of ³H-thymidine and expressed as counts per minute (cpm).

One of two experiments done is shown here.

Values are means of triplicates \pm SD.

3.3 REQUIREMENT OF T CELLS IN THE STIMULATORS

The experiments described in sections 3.1 and 3.2 led to the conclusion that the splenocytes are required to undergo the early stages of activation in order to be able to stimulate allogeneic cells optimally and that the T cells in the stimulating population do actually undergo activation during the course of MLR. To find out if in fact T cells are required for optimal proliferation, the stimulator population was depleted of these cells and then tested in an MLR.

3.3.1 MLR with T-depleted stimulators

Mitomycin C-treated C57Bl/6 splenocytes were divided into three equal portions: (1) untreated, (2) treated with monoclonal anti-Thy1.2 and guinea-pig complement, (3) treated with complement alone. The cells from each group were then resuspended in the same volume of medium so that the density of the non-T cells was the same in all three fractions, each of which was then used as a stimulating population in MLR with Balb c or with CBA. Incorporation of ^3H -thymidine which was followed for five days and recorded in Figure 12, represented MLR proliferative response. The stimulators treated with complement alone behaved similarly to the untreated controls; however absence of T-cells provoked by complement plus anti-Thy 1.2 caused a reduction of 50% or more in proliferation. This was statistically significant ($p = 0.001$ to 0.05) in all 7 experiments tried.

Figure 12. MLR with T-depleted stimulators of C57Bl/6 and Balb/c responders.

MLR with C57Bl/6 stimulators and Balb/c responders where the stimulators were unfractionated (Curve A) or treated with anti-Thy 1.2 and complement (Curve B) or complement alone (Curve C).

One of 7 experiments done is shown here.

Bars represent standard deviation.

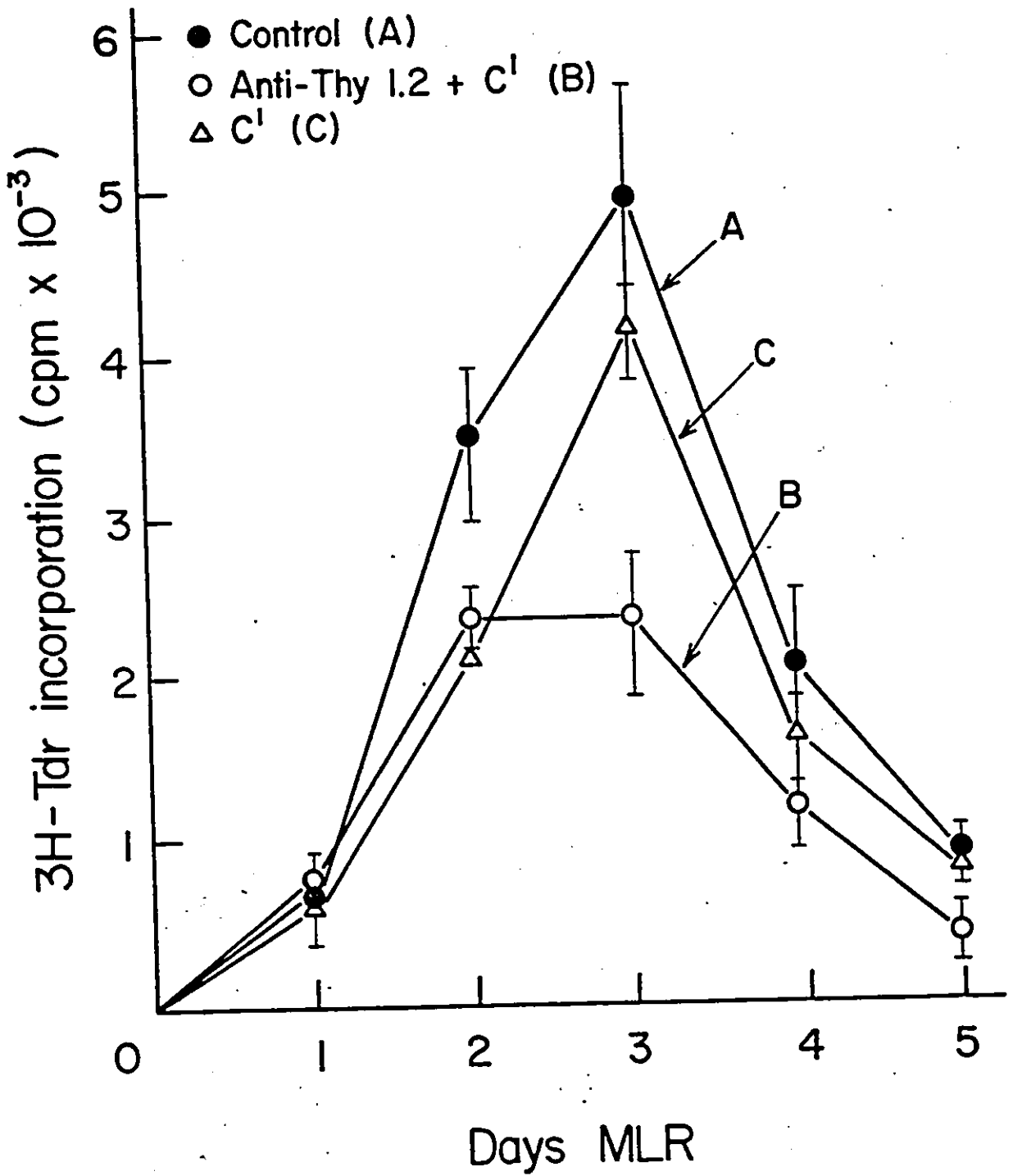
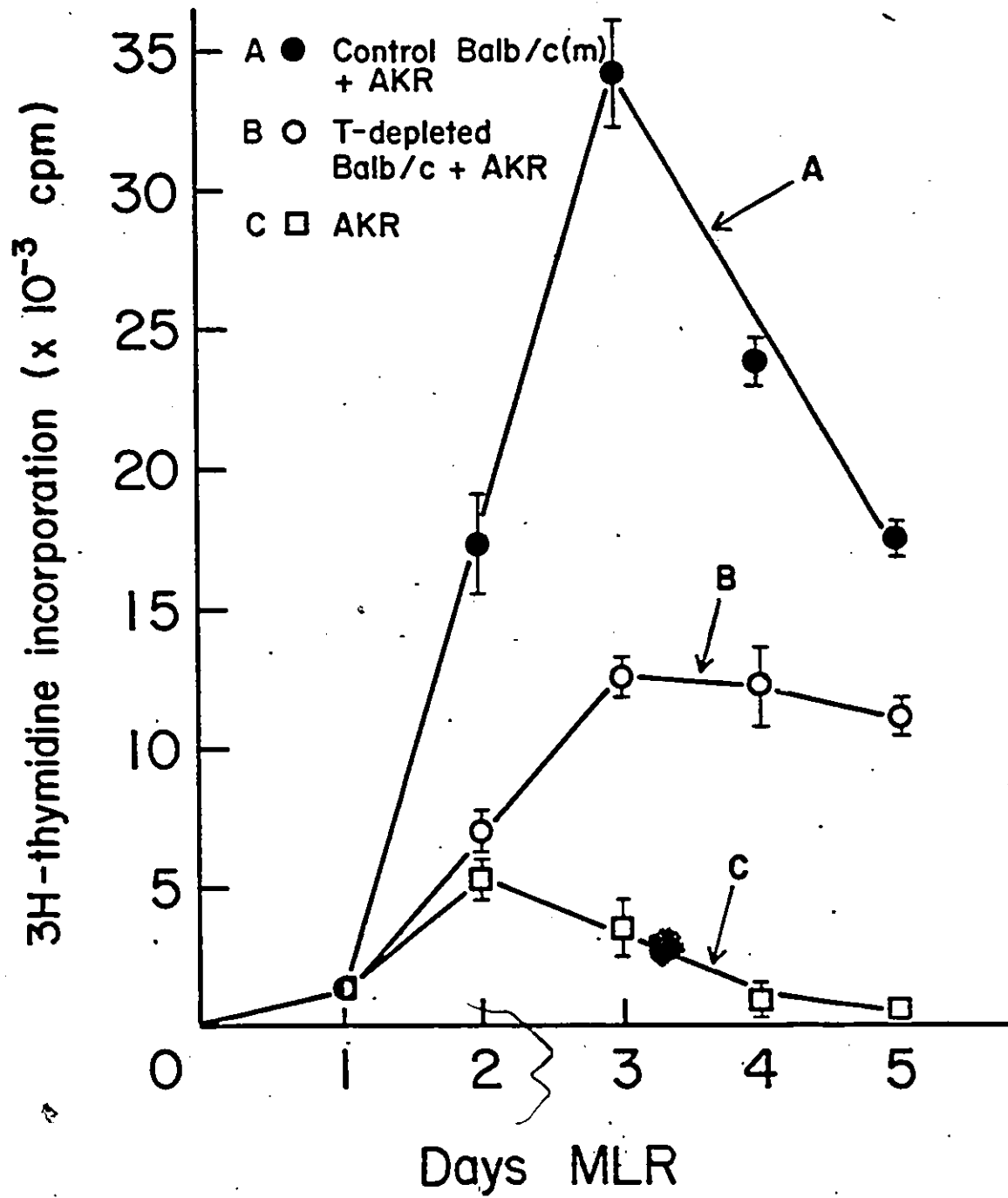


Figure 13. MLR with T-depleted Balb/c (Thy 1.2) stimulators and AKE (Thy 1.1) responders.

Curve A represents MIF with unfractionated splenocytes of Balb/c and Curve B, the reaction where the stimulators were T-deficient. Curve C represents ^3H -thymidine incorporation in the responder alone.

One of the three experiments done is shown here.

Bars are standard deviation.



To rule out the possibility that the reduction of MLR noted above was due to the possible killing of the responder-T cells by a carry-over of anti-Thy 1.2 and complement from the stimulators, an MLC was set up between T-depleted C57Bl/6 (Thy 1.2) stimulator and AKR responder, which is Thy 1.1. Results observed in section 3.3.1 were noted once again (Figure 13). The ^3H -thymidine incorporation after T-depletion was 34% of the control.

3.3.2 Reconstitution of MLR

A population of T-enriched cells was added to T-depleted stimulators to see if the MLR response would be restored.

3.3.2.1 Syngeneic and allogeneic T-enriched cells

T-enriched cells were obtained from the splenocytes of Balb/c (stimulators) and CBA (responders) by panning on Ig⁺ coated plates, as described in section 2.2.2.2. These cells were treated with mitomycin-C and mixed with T-depleted and mitomycin C-treated splenocytes of Balb/c in a 1:1 ratio. The mixed population was then used as a stimulator in an MLR with CBA. Figure 14 shows that the addition of T-enriched cells from Balb/c but not CBA splenocytes restored the MLR proliferation. The peak MLR value on Day 3 was $18,495 \pm 1393$ cpm in MLC with unfractionated control-stimulators; $9,626 \pm 132$ in MLC containing T-depleted stimulators and $18,789 \pm 950$ when T cells from the stimulator were added.

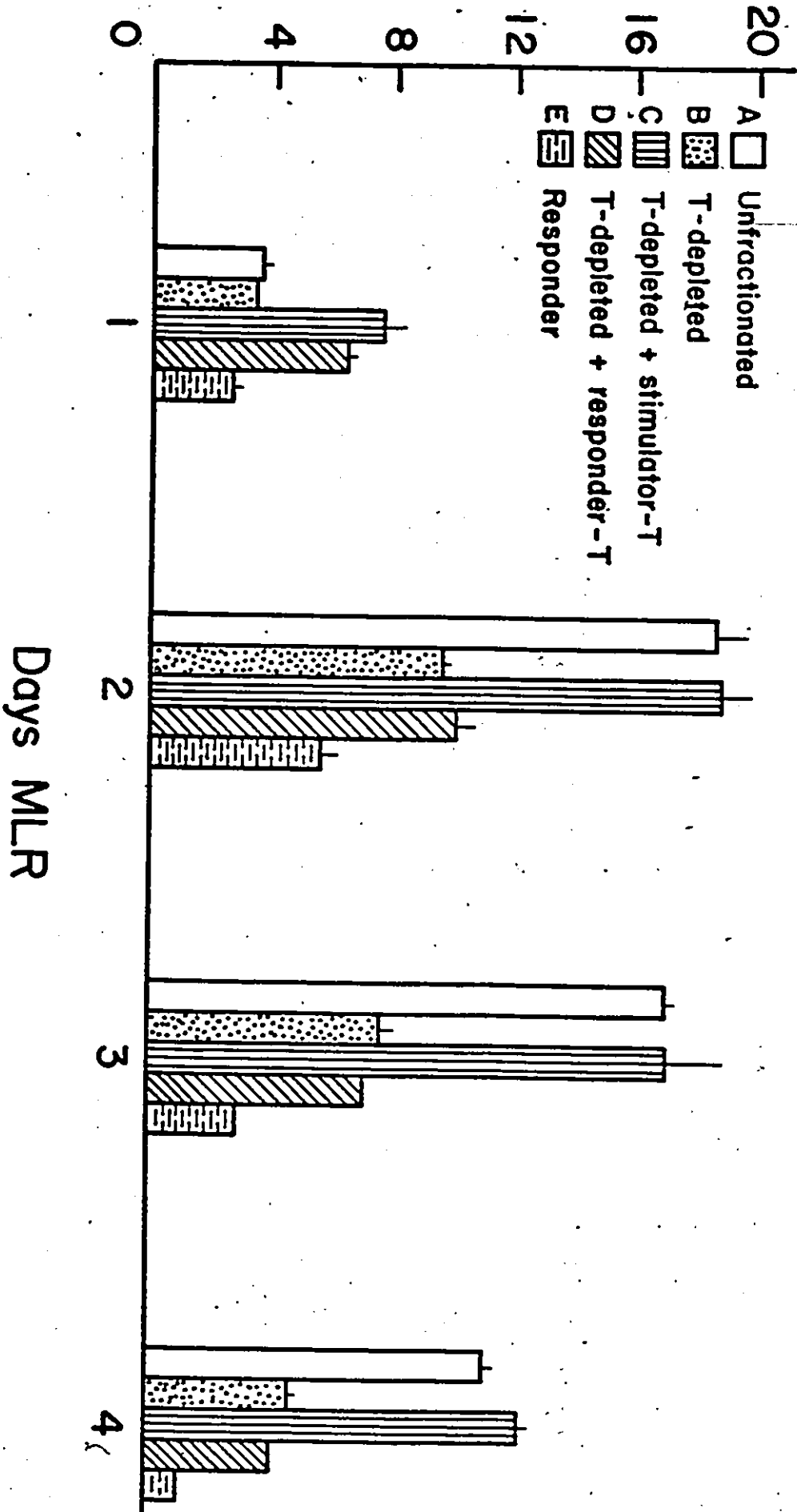
Figure 14. Reconstitution of MLR by syngeneic and allogeneic T-enriched cells.

MLR was followed in cultures containing splenocytes of Balb/c stimulators and CBA responders. Stimulators were (A) unfractionated, (B) T-depleted, (C) mitomycin C treated T-enriched cells of Balb/c mixed with B in a 1:1 ratio, and (D) same as C except that the T-enriched cells were from mitomycin C treated CBA splenocytes. E represents responders alone. MLR proliferation was determined by measuring ^3H -thymidine incorporation (cpm) over a four day period.

One of the three experiments done is shown here.

Values are means of triplicates \pm SD.

3H-thymidine incorporation
(x 10⁻³ cpm)



The value remained at 9966 ± 584 cpm when T-enriched cells from the responder, CBA, were added.

3.3.2.2 Various stimulator to responder ratios

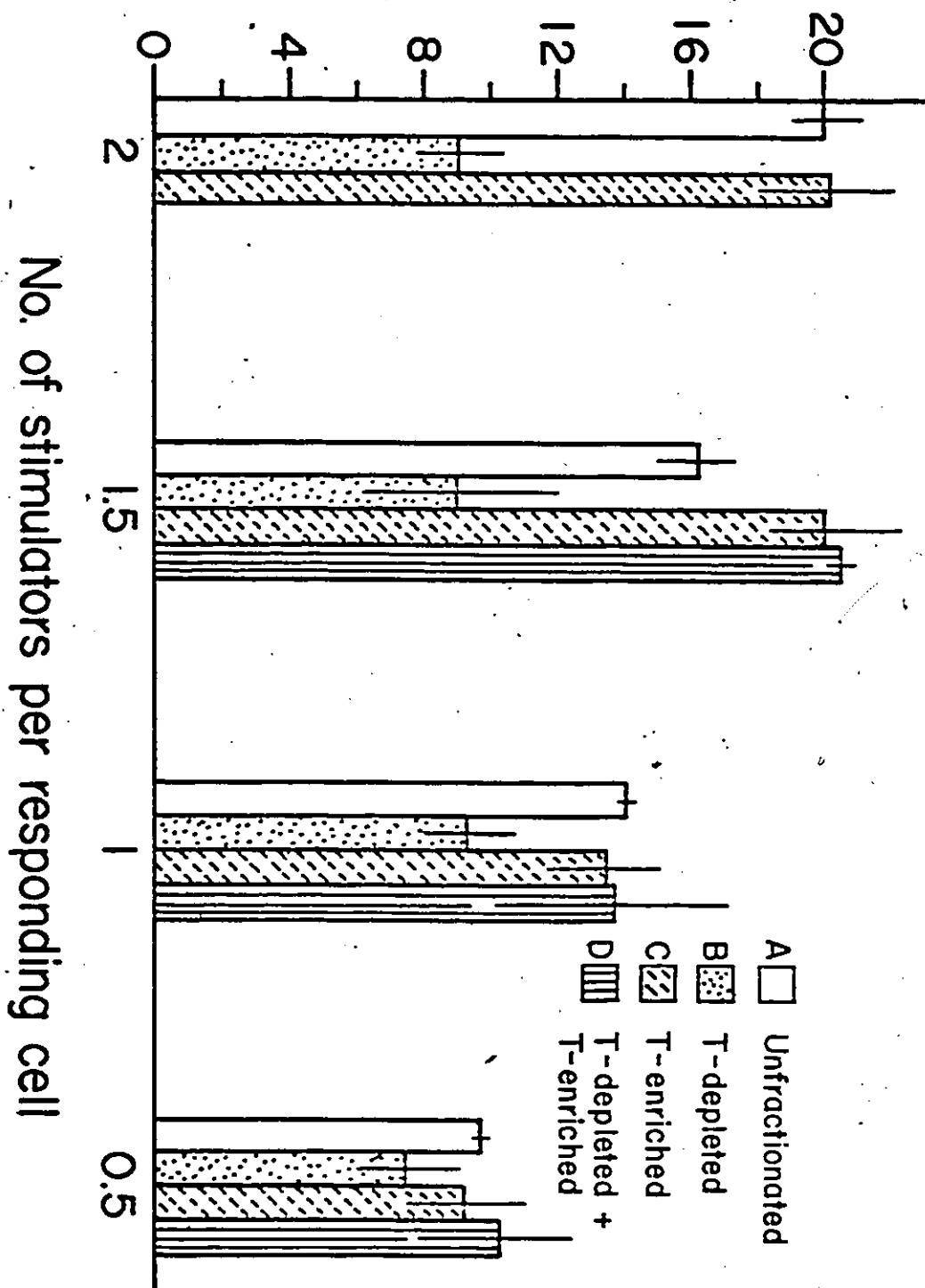
Figure 15 shows the results obtained on Day 3 of MLR from cultures containing stimulators from Balb/c which were: (1) unfractionated, (2) T-depleted, (3) T-enriched and (4) T-depleted splenocytes mixed with T-enriched cells at 1:1. T-depleted splenocytes caused about 50% of the proliferation induced by the unfractionated population when stimulator to responder ratio was 2:1 or 1.5:1. The differences were smaller with ratios of 1:1 and 0.5:1. The proliferation caused by T-depleted cells appeared to be at the same level (around 10,000 cpm) for all the ratios whereas the values with unfractionated or T-enriched fractions were directly proportional to the number of cells in the stimulators: approximately 20,000 cpm at stimulator to responder ratio of 2:1 and 1.5:1; 14,000 at 1:1 and 10,000 at 0.5:1. T-enriched populations by themselves gave the same value as the control with all ratios of stimulator to responder. When the T-depleted and T-enriched cells were mixed, the proliferative response was restored to the value of the control MLRs.

Figure 15. Effect of T-enriched splenocytes of the stimulating population on the stimulating ability of T-depleted splenocytes, with various stimulator to responder ratios.

B6/C was used as the stimulator and CBA as responder. The ordinate represents values of ^3H -thymidine incorporations (cps) in the various cultures on day 3 MLR, and the abscissa the different ratios of stimulators to responders. The various bars represent the different cell populations used as stimulators: unfractionated (A), T-depleted (B), T-enriched (C) and (D) T-depleted and T-enriched fractions mixed in 1:1 ratio. Results from four experiments are combined. Values of peak proliferation (Day 3) are given.

Bars represent standard deviation.

^3H -thymidine incorporation
($\times 10^{-3}$ cpm)



3.4 ROLE OF THE ACTIVATED STIMULATOR-T CELLS

The experiments described above have shown that T cells in the stimulators are activated during MLR and that their presence is required for optimal proliferation. It was then logical to determine what contribution they made to the process of MLR.

3.4.1 Induction of MLR by UV-irradiated Con A blasts

Since it was shown in the preceding sections that activation of T cells in the stimulating population is necessary for inducing optimal proliferation, an experiment was set up to find out if activated T cells were sufficient in inducing MLR and also to find out if metabolic activity in the stimulators was required even when the stimulating population already contained activated cells. 24 hour Con A blasts were washed, irradiated with 360 ergs/mm² of UV, the dose which was found earlier (Table 5) to inhibit the stimulating capacity of resting cells in MLR. These cells were then used as stimulators in MLR. Table 13 shows that when the stimulators were UV-irradiated Con A blasts, there was a reduction of over 50% in MLR response, compared to that produced by Con A blasts treated with mitomycin C alone. However, in the case of the resting cells, UV-treatment caused a reduction of about 30% in MLR response. Thus the stimulating ability of activated cells is more resistant than that of the quiescent cells after a pretreatment which greatly inhibits the latter.

Table 13

Stimulatory Potential of UV-Irradiated Con A Blasts Compared to that of UV-Irradiated Freshly Isolated Splenocytes

Culture ^a	Peak MLR value (Days 3-4) ^b	
	Ba1b/c(m)+C57B1/6 ^c	C57B1/6(m)+Ba1b/c ^d
Responder + Control (m)	6500 ± 1251	6054 ± 1018
Responder + Control (UV) (m)	1486 ± 290 (23%) ^e	971 ± 189 (16%)
Responder + Con A blast (m)	14083 ± 1715	5509 ± 446
Responder + Con A blast (UV) (m)	6684 ± 234 (47%)	2203 ± 226 (40%)
Control (m) ^f	105 ± 19	428 ± 57
Control (UV)	78 ± 28	NM ^g
Con A blast (m)	475 ± 87	478 ± 31
Con A blast (UV)	290 ± 39	NM

^aFreshly isolated splenocytes (control) and 24 hr Con A blasts were washed and irradiated with 360 erg/mm²UV. After mitomycin C-treatment (m), they were cultured with allogeneic responders.

^bPeak MLR Values recorded on Days 3-4 are expressed as cpm.

^{c,d}Results from two experiments are shown.

^eFigures in brackets are percentages of the values in the corresponding cultures containing stimulators not exposed to UV.

^fControl values are shown for cultures whose fresh splenocytes or 24 h Con A blasts (washed) were treated with mitomycin C (m) or UV.

^gNot measured.

3.4.2 Regulation of IL 2 by activated stimulator-T cells

Since primed T cells (Baum and Pilarski, 1978) or IL 2, a product of activated T cells, (Paetkau *et al.*, 1980) can restore MLR in cultures where the stimulatory potential has been compromised, it appeared reasonable to hypothesize that the activated T-cells in the stimulating population regulate the level of IL 2 in the MLC. The following experiments were designed to test this hypothesis.

3.4.2.1 Absence of T-cells in the stimulators

Balb/c stimulators and CBA responders were cultured together at ratios of 2:1 and 1:1. IL 2 or growth factor activity was measured in 72 hour supernatants of MLCs containing stimulators which were (1) unfractionated, (2) T-depleted, (3) mitomycin C-treated T-enriched cells from the stimulators mixed with (2) and (4) mitomycin C-treated T-enriched cells from the responders mixed with (2). A direct correlation was seen between the presence of T-cells in the stimulators and the level of IL 2 in the MLC with both the assays of IL 2 measurement, (see section 2.6.2), the one measuring growth of the IL 2-dependent cell line MTL and the other one assaying the enhancement of proliferation of CBA thymocytes in the presence of suboptimal doses of PBA (Table 14).

Table 14

Presence of T Cells in the Stimulators Associated with IL 2 Activity and MLR

Treatment ^a	IL 2 Activity ^c		
	MLR peak ^b	MTL	Thymocyte
Balb/c(m) + CBA	18495 ± 1393	44853 ± 1241	44337 ± 2721
T-deficient Balb/c(m) + CBA	9262 ± 132	8220 ± 177	4173 ± 170
T-deficient Balb/c(m) + CBA + Balb/c-T	18789 ± 950	47658 ± 10598	43038 ± 9075
T-deficient Balb/c(m) + CBA + CBA-T	9966 ± 584	5824 ± 2882	3689 ± 131

^a Culture consisted of Balb/c and CBA at stimulator to responder ratio of 2:1. The stimulators were

- (1) unfractionated splenocytes,
- (2) T-depleted,
- (3) T-enriched cells from the stimulators (Balb/c-T) mixed with (2), and
- (4) T-enriched cells from the responders (CBA-T) mixed with (2).

^b MLR proliferative value on day 3 is given; it is expressed by ³H-thymidine incorporation (cpm).

^c IL 2 activity was measured in 72 hour supernatants. Both MTL and thymocyte assays were used. Proliferation of MTL cells and thymocytes was measured by ³H-thymidine incorporation (cpm).

Depletion of T cells caused the IL 2 level to be reduced to about 1/5 of that seen in the control; this could be replenished by T cells of the stimulating population but not by the responder T cells. The corresponding reductions in the MLR responses were not so severe; about 50% reductions were seen. This would suggest that factors other than IL 2 participate in the MLR response. Very similar levels of IL 2 were obtained with the two ratios, 2:1 and 1:1, of stimulator to responder; results of the former are shown in Table 14. This is in contrast to the results shown in Figure 15, where a higher MLR value was obtained with a stimulator to responder ratio of 2:1, compared to that of 1:1.

3.4.2.2 Absence of activation in the stimulator T-cells

In MLCs containing UV-irradiated (120 ergs and 360 ergs/ cm^2) Balb/c stimulators and AKR responders, it was found (Table 15) that with 120 ergs the number of activated stimulator cells was about the same (56%) as in the untreated control (53%). Correspondingly, similar results were obtained for the IL 2 level, as measured by the assay using the growth assessment of the IL 2 dependent cell line MTL. With a higher dose of 360 ergs/ cm^2 , which inhibited MLR proliferation, only 5% of the stimulator T-cells were over 8 weeks although the total number of T-cells was comparable to the control (see section 3.2.3); no IL 2 activity was detected.

Table 15

Activation of T Cells in the Stimulators is Associated with IL-2 Activity and MLR.

Culture ^a	Stimulator-T blast ^b	IL 2 activity ^c	MLR peak ^d
Balb/c(m) + AKR	53	6905 ± 496	23586 ± 232
Balb/c(120 ergs)(m) + AKR	56	7227 ± 385	23742 ± 2109
Balb/c(360 ergs)(m) + AKR	5	86 ± 14	6214 ± 232
AKR	N/A	92 ± 9	5691 ± 974

a. MLCs were set up between Balb/c (Thy 1.2) and AKR (Thy 1.1). The stimulators were splenocytes of Balb/c which were (1) untreated, (2) exposed to 120 ergs/mm² and (3) 360 ergs/mm² of UV.

b. In 24 hr MLC, the T-cells in the stimulating population, stained with FITC-labelled anti-Thy 1.2 were examined for size. Cells larger than 8 μ m were considered to be blasts. Percentages of total Thy 1.2 bearing cells are shown.

c. 24 hour supernatants were tested for IL 2 activity, using the IL 2-dependent cell line MTL. Proliferation of the MTL cells are expressed by ³H-thymidine incorporation (cpm).

d. MLR proliferation measured on day 3 by ³H-thymidine incorporation is expressed as cpm.

e. Not applicable.

IL 2 Restores MLR Values in Cultures Containing T-Deficient Stimulators

Treatments	MLR peak
Experiment 1	
Balb/c(m) + CBA ^a	16965 ± 275
T-deficient Balb/c(m) + CBA ^b	6014 ± 29
T-deficient Balb/c(m) + CBA + IL 2 ^c	12954 ± 1280
CBA ^d	657 ± 83
CBA + IL 2 ^d	2860 ± 153
Experiment 2	
C57B1/6(m) + Balb/c ^a	10470 ± 968
T-deficient C57B1/6(m) + Balb/c ^b	3167 ± 18
T-deficient C57B1/6(m) + Balb/c + IL 2 ^a	7531 ± 910
Balb/c ^d	885 ± 121
Balb/c + IL 2 ^d	2024 ± 318

^a Culture contained responders and control stimulators.

^b Culture contained responder and T-depleted stimulators.

^c Culture contained responder, T-depleted stimulator and IL 2 at 1/400 dilution.

^d Controls showing the effects of IL 2 on responder alone.

^e Incorporation of 3H-thymidine measured on day 3 of MLR expressed as cpm.

3.4.2.3 Restoration of MLR by IL 2

IL 2 added to a final concentration of 1/800 to 1/400 restored the proliferative response in cultures containing T-deficient stimulators (Table 16). On depleting the stimulators of T cells, the level of MLR peak was reduced to 30 - 36% of the control. On adding IL 2, the value was restored to 72 - 76 % of the control. This was true in two sets of cultures: Balb/c stimulators with CBA responders and C57Bl/6 stimulators and Balb/c responders.

3.4.3 Identification of the source of IL 2

Table 17 shows that mitomycin C-treated cells were capable of producing IL 2 (treatments a and b). When both allogeneic populations were treated with mitomycin C (treatment b) the supernatant collected on day 3 of MLR showed 75% of the IL 2 activity detected in the control MLC (treatment a) as measured by the assay using the cell line MTL.

In a culture containing T-deficient stimulators and untreated responders (treatment c), the supernatant contained a factor which supported the growth of MTL cells. When the stimulators were depleted of T-cells and the responders were prevented from synthesizing DNA due to mitomycin C treatment there was no detectable IL 2 in the culture (treatment d) suggesting participation of proliferating cells of the responders in IL 2 production in treatment c. Similar results were obtained using the thymocyte bioassay. Thus IL 2 or a

similar growth factor could be produced by both the mitomycin C-treated cells and the responding population.

Table 17

Identification of the Source of IL 2

Treatment	IL 2 activity ^e	MLR peak ^f
Balb/c(m) + CBA ^a	46,200 ± 1976	17243 ± 46
Balb/c(m) + CBA(m) ^b	34,475 ± 2100	1454 ± 61
T-deficient Balb/c(m) + CBA ^c	12,675 ± 1906	8045 ± 481
T-deficient Balb/c(m) + CBA(m) ^d	340 ± 80	476 ± 114

^a MLCs were set up between Balb/c stimulators and CBA responders.

^b Both unfractionated stimulators and responders were treated with mitomycin C(m).

^c T-depleted stimulators reacted with CBA responders.

^d T-depleted stimulators and responders treated with mitomycin C(m).

^e IL 2 activity was measured in supernatants of 72 hour cultures using the MLT assay; proliferation was measured by ³H-thymidine incorporation (cpm).

^f Proliferative response of MLR was measured by ³H-thymidine incorporation on day 3 (cpm).

Chapter IV

DISCUSSION

The purpose of this thesis was to gain a better understanding of the stimulatory mechanism in an MLR, in particular to characterize the functions of the stimulatory population, which must be comprised of metabolically active cells. The stimulators provide the first signal in the form of transplantation antigens, which can trigger the responders into activation. A second signal, which Lafferty and Woolnough (1977) suggested is provided by the metabolically active cells of the stimulators then induces proliferation in the signal 1-activated responding lymphocytes. I hypothesized that to generate a second signal for the induction of detectable proliferation in MLR, the stimulating population should contain activated T cells. My experimental results support this hypothesis (Foy et al, 1982A and B).

Using a variety of agents that affect the stimulating ability of leukocytes in an MLR, it was found that splenocytes could stimulate MLR ~~effectively~~ only when they could respond in parallel experiments to Con A as well. This suggested that an effective stimulating population should not only be metabolically active but must contain activated (pre-S phase) cells. The T cells in the stimulating popula-

tion were indeed found to demonstrate the characteristics of early activation despite their inability to proliferate due to treatment with mitomycin C. I have demonstrated this directly. That the T cells in the stimulating population are required for optimal proliferation was shown in experiments with T-depleted stimulators and reconstitution of these reactions by addition of T-enriched cells from the stimulators. The stimulator-T cells were found to regulate IL 2 content in the MLC. A growth factor resembling IL 2 in some of its biological activities at least, may be produced when T cells are absent from the stimulating population. Therefore, although activated T cells in the stimulating population are required to be present for optimal proliferation, there are other pathways by which signal 2 production and MLR may be induced.

4.1 OPTIMAL MLR REQUIRES ACTIVATED STIMULATOR-T CELLS

4.1.1 Mitomycin C-treated cells can be activated

To test the feasibility of my hypothesis that the stimulating cells should be activated, I initiated an experiment to find out whether treatment with mitomycin C, the protocol used in preparing the stimulators, would allow the cells to be activated; mitomycin C inhibits DNA synthesis (Szybalski and Iyer, 1964). The results in Figure 2 and Table 3 show that lymphocytes pretreated with mitomycin C, when cultured with Con A, were fully capable of demonstrating the features

characteristic of the early phases of cell activation, although DNA replication was effectively inhibited. Mitomycin C, therefore, is similar to hydroxyurea or cytosine arabinoside; these agents also inhibit DNA synthesis without affecting the earlier phases of the cell cycle (Setterfield et al, 1983). My findings with the mitomycin C-treated cells, therefore, lend support to my hypothesis in that the T cells in the stimulating population are capable of undergoing activation.

4.1.2 Stimulators should undergo activation

Having seen that stimulators, leukocytes blocked at G1/S, can undergo the earlier phases of activation, I wanted to see if the entrance of stimulators into the G1 phase was necessary. It was found that a population of leukocytes could stimulate MLR optimally only when it could demonstrate the features characteristic of the early phases of Con A response (increase in K^+ influx and in cell size) (Table 8). This correlation was observed from experiments using a variety of agents which have been reported to affect the stimulatory potential of leukocytes or their ability to respond to a stimulus. The agents used were ouabain, UV, 5-FU and colchicine. When mouse splenocytes pretreated *in vitro* (consequently immunosuppressed) with different doses of a variety of agents were cultured with Con A, inhibition occurred at various stages of lymphocyte activation (Figure 16).

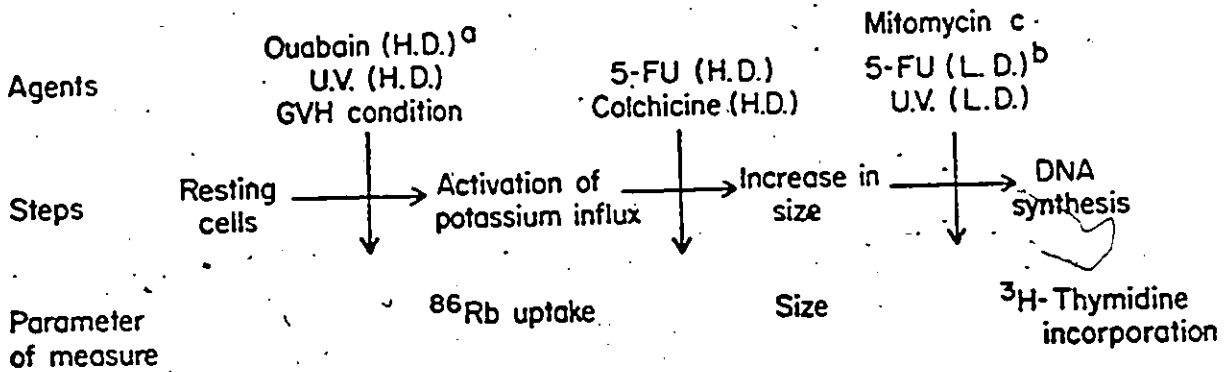


Figure 16. Site of action of agents blocking Con A induced activation of lymphocytes. All these agents, irrespective of where they act, were applied to resting cells and then removed prior to addition of the mitogen.

^aHD high dose

^bLD low dose

Only the treatments which allowed the cells to be activated to a point where small blasts (type 2) were formed, permitted the splenocytes to induce MLR optimally. The correlation between Con A responsiveness and stimulating ability was found to exist also in experiments using splenocytes which were immunosuppressed in vivo (in GVH-mice).

4.1.2.1 Ouabain

Ouabain produced two opposite effects in MLR and Con A response. When the stimulators were pretreated with $1 \times 10^{-3} M$ ouabain, they failed to induce proliferation in the responding cells in an MLR and these treated cells did not show any response to Con A (Figure 3, Table 4). When the stimulators were pretreated with a lower dose, $2 \times 10^{-4} M$ ouabain, their stimulating capacity and their responsiveness to Con A was comparable to that of the control untreated splenocytes. In more than half the instances, however, the treatment with the low dose caused an enhancement of the proliferative response both in MLR and in Con A cultures. The enhancement of the MLR response, although highly variable in terms of reproducibility, was similar to the phenomenon of "superstimulation" reported by Kaplan and his colleagues. They found that a 48 hour pretreatment of human lymphocytes with $2 \times 10^{-6} M$ ouabain caused an irreversible loss of their subsequent capacity to stimulate in the MLR. At a concentration of $1 \times 10^{-7} M$, the treated stimulator cells were far more ef-

fective than untreated controls, causing a 2-fold enhancement of incorporation of ^3H -thymidine in MLR (Christen et al, 1975; Dornand and Kaplan, 1976). Similar results were obtained in the rhesus system using $2 \times 10^{-6}\text{M}$ and $5 \times 10^{-6}\text{M}$ ouabain; inhibition occurred with the high dose and 3-fold enhancement with the lower dose (Koller, cited in Kaplan and Owens, 1982).

Kaplan and Owens (1980; 1982) have put forward a hypothesis to explain the mechanism of superstimulation. They suggest that the pretreatment with the low dose causes a "switching down" of the sodium pump which may prompt a state of differentiation that might be necessary to cause superstimulation. Low doses of ouabain have been shown to cause differentiation in the Friend cell (Bernstein et al, 1976). Recently, Dornand et al (1984) have reported that treatment of leukocytes with ouabain induces the production of IL 7. This, of course, will produce an augmenting effect on immune response. These findings are consistent with Kaplan and Owens' hypothesis of ouabain-induced differentiation. Another explanation for "superstimulation" may lie in an altered ratio of helper to suppressor cells, due to selective inhibition of suppressors by $2 \times 10^{-6}\text{M}$ ouabain.

Unlike the finding of Wagner (1973) where he found increased immunogenicity by increasing the dose of stimulating cells, whose stimulatory potential was suppressed with metabolic inhibitors, I failed to observe such an increase in

the stimulating capacity by raising the number of stimulator cells pretreated with the inhibitory dose, $1 \times 10^{-3} M$ ouabain.

Thus the ouabain experiments indicate that the stimulatory potential of immunogenicity is dependent not on the antigenic dose but on the functional ability of the leukocytes, possibly on their ability to undergo activation.

4.1.2.2 Ultraviolet radiation

As with ouabain, irradiation of stimulators with UV caused a dual effect. When lymphocytes were irradiated with 120 ergs/cm^2 , either there was no change from the untreated control or an enhancing effect occurred in the stimulating capacity in MIR. A higher dose, 360 ergs/cm^2 , caused a loss in the stimulating ability of the splenocytes. Again, as with ouabain pretreatment, irradiation of the stimulators with the lower dose produced an enhancing effect on the stimulating population in about 50% of the experiments (Figure 5). It is difficult to explain why the enhancement occurred; due to its poor reproducibility, further experiments could not be planned. It is possible that UV at low doses induce the kind of differentiation one ascribes to ouabain-induced MLR effects. Correspondingly, the lower dose had no effect on Con A response; increase in K^+ influx and cell size were in the range of the untreated controls (Table 5). No increase in K^+ influx was noted when cells irradiated with the high dose were cultured with Con A.

Although no other laboratory has reported results similar to the ones with the low dose of UV, my findings on the effect of high dose of UV irradiation on the stimulating capacity in MLR are consistent with the earlier reports. Using comparatively high doses of UV, inhibitory effect of UV on the stimulating capacity has been noted by several workers (Lindahl-Kiessling and Safwenberg, 1971; Lafferty *et al.*, 1974; Schendel and Bach, 1975; Okada *et al.*, 1979; Paetkau *et al.*, 1980). UV does not affect the normal levels of Class I MHC antigens as detected serologically (Lindahl-Kiessling and Safwenberg, 1971; Lafferty *et al.*, 1974), nor does it affect their antigenic activity in the MLR, although their stimulatory capacity, that function of the stimulators which requires metabolic activity, is lost. UV irradiated cells can induce CTL production in the MLR provided the culture is fortified with γ -irradiated-third party stimulators (Lafferty *et al.*, 1974) or in the presence of I-region-incompatible population (Schendel and Bach, 1975) or by exogenous addition of IL-2 (Okada *et al.*, 1979; Paetkau *et al.*, 1980; Wagner *et al.*, 1980; Larsson *et al.*, 1982). Kelso and Boyle (1982A), however, have shown from quantitative studies that not only is there loss of immunogenicity, there is impairment of T cell recognition of the K/D antigens as well, although UV-treated cells retained the K/D and I-region determinants, as recognized by antibodies. They measured the capacity of cell monolayers to absorb CTL-precursors.

My results on the Con A response of lymphocytes irradiated with 360 ergs/mm² are in accord with the findings of Castellanos *et al* (1982), although there is a difference between human and murine systems with respect to dose sensitivity. They found that UV, in human lymphocytes, inhibited an early event at the membrane level; with 84 ergs/mm² there was neither any increase in K⁺ influx, nor any ³H-thymidine incorporation. Perhaps like ouabain, the redox system is affected by a higher dose of UV compared to the human system.

4.1.2.3 5-Fluorouracil

Like mitomycin C, 5-fluorouracil has been shown to inhibit DNA replication without affecting the pre-S phases of cell activation process (Bouman *et al* 1984). It was found that pretreatment with $3.3 \times 10^{-5} M$ of 5-FU, which prevented ³H-thymidine incorporation in response to Con A but allowed activation of K⁺ influx to the same degree as the untreated cells (Table 6), stimulated allogeneic cells to the same extent as the control mitomycin C-treated cells. Pretreatment with $6.6 \times 10^{-5} M$ 5-FU, the dose which blocked DNA synthesis severely (Bouman *et al*, 1984), blocked the stimulating capacity (Figure 6) but contrary to expectation, activation of K⁺ influx was not prevented. There was, however, an inhibition of increase in size. Therefore, it appears that optimally effective stimulators should be activated to a stage in the

cell cycle which proceeds beyond the stage where Con A-induced increase in cation fluxes were measured (10 - 12 hours post stimulation). Formation of small (type 2) blasts (see Materials and Methods), appeared to be adequate (Figure 7). Increase in RNA synthesis precedes the appearance of blasts (Setterfield *et al.*, 1983). Transcripts of new RNA appear at 12 - 14 hours post stimulation (Mitchell *et al.*, 1978). Thus it may very well be that immunogenicity is related to the ability of the stimulators to synthesize new messenger RNA. Lindahl-Kiessling and Safwenberg (1972) suggested that the ability to synthesize RNA may be a necessary feature of the stimulating population.

4.1.2.4 Colchicine

Since there are conflicting reports regarding the effects of colchicine on early events of cell activation, it was considered interesting to use this drug in the present series of experiments. Inhibition of RNA synthesis by colchicine has been reported by Hausser *et al.*, 1976; Greene *et al.*, 1976; Rudd *et al.*, 1979). In contrast, no inhibition has been found by Sherline and Mundy (1977), Resch *et al.* (1977) and Cutbert and Shay, (1983). The results reported in this thesis show that there was no inhibition in Con A responsiveness when the cells were pretreated with 1×10^{-5} M to 1×10^{-4} M colchicine for 4 to 6 hours. The number of blasts formed and the incorporation of ^3H -thymidine was the same as

in the untreated control (Table 7). However, when splenocytes pretreated with the high dose (1×10^{-6} M) were further treated with mitomycin C, the cells failed to exhibit stimulating capacity in MLR and inhibition in Con A response occurred. On treating the cells with 1×10^{-5} M, either there was no effect on the stimulating capacity or else an enhancement (156% of the control) occurred in the MLR (Figure 3). Unfortunately, this enhancing effect was reproducible in only 50% of the experiments, making it difficult to analyze it. Steen et al (1978) and Suthanthiran et al (1980) found enhanced MLR with brief pretreatment of stimulators or responders with 1×10^{-6} to 1×10^{-7} M colchicine and inhibition with longer treatments and higher doses. Panney and Fincus (1976), who found inhibition of the stimulatory capacity, did not find any quantitative difference in the surface antigens. Teas et al (1977) provide some suggestion which might explain the phenomenon of enhanced MLR seen with lower doses or short periods of treatments with colchicine. They found in their work using chick embryo fibroblasts that with lower concentrations of colchicine (1 to 2×10^{-7} M) there was an increase in the number of cells entering S phase, compared to the normal. In our system entry into S phase is prevented by mitomycin C treatment, therefore, one might expect an increased number of cells in G1, which appears to be endowed with good stimulatory capacity, including an ability to produce IL 2 (Stadler et al, 1981).

Consistent with my other experimental results, the experiments with colchicine also showed MLR stimulating capacity only in the cell population which could show early phases of Con A response (Figure 8 and Table 7).

4.1.3 Cells immunosuppressed in vivo are poor stimulators

The use of immunosuppressed splenocytes from mice undergoing GVH reaction gave me an opportunity to test and subsequently substantiate my hypothesis that to stimulate MLR optimally, it is not enough for the leukocytes to be just metabolically active; they should be able to demonstrate an ability to respond to an activating stimulus.

Induction of GVH reaction causes immunosuppression in lymphocytes (Lapp and Møller, 1969; Møller, 1971); they fail to proliferate in response to Con A (Lapp and Kirschner, 1979). In all my experiments, described in Section 3, (Table 3), the cell populations that failed to show the pre-S phase responses to Con A, showed impairment of stimulating capacity in MLR. Splenocytes from mice induced with GVH reaction, which were shown to be immunosuppressed in assays measuring plaque-forming cells to sheep erythrocytes, (Table 2) could cause only 50% of the MLR response produced by their normal counterpart (Figure 9). As predicted from the studies reported above, these cells showed no activation of K^+ influx when cultured with Con A (Table 9). Thus immunosuppressed splenocytes behaved similarly to the leukocytes

whose ability to respond to Con A was blocked (Figure 16) in the pre-S phase. The mice used were in a late GVH period, day 20 to 27 post GVH, where immunosuppression has been shown to be due to impairment of T-cell function (Seddik *et al.*, 1980). Thus participation of immunocompetent T-cell is indicated in the stimulatory mechanism.

We can conclude from the series of experiments described above that a correlation exists between the ability of leukocytes to demonstrate the changes characteristic of early blastogenic response and their ability to stimulate allogeneic cells optimally (Table 3). This correlation between Con A responsiveness and stimulating capacity suggested that there is a common mechanism between the two processes. Synthesis of RNA may be the common feature. Using actinomycin D and anisomycin A, inhibitors of RNA and protein syntheses, Wagner (1973) suggested that the production of these two substances are necessary for the action of the stimulators in MLP. Lindahl-Kiessling and Safwenberg (1972) associated immunogenicity of leukocytes with their ability to synthesize new RNA. Syntheses of proteins and RNA are necessary also for the mechanism by which cells demonstrate blast-formation, a feature characteristic of G1 cells; RNA synthesis must occur in the first 0 to 16 hours of cell activation (Setterfield *et al.*, 1983). This leads to the conclusion that the stimulators inducing optimal MLP, although unable to synthesize DNA, nevertheless undergo the pre-S

phase of activation, perhaps upto a point where new RNA is transcribed (13 hours post-stimulation, Mitchell *et al.*, 1973). Of course, the key step may be another metabolic event occurring at the same time, or slightly later, than the increase in RNA synthesis and dependent on the production of newly synthesized transcripts.

4.1.4 Stimulator-T cells are activated during MLR

That the stimulator-T cells are actually activated during the course of MLR was demonstrated morphologically; they were identified by a fluoresceinated monoclonal antibody and were found to demonstrate increase in size and in RNA synthesis (Figures 10 and 11). That the mitomycin C-treated or X-irradiated stimulator-T cells are activated was suggested by experiments of Gordon *et al.* (1979) when they observed "back stimulation"; anomalous proliferative response occurred in the primed lymphocyte typing test due to growth factor produced by the stimulating cells. Such a factor can be released only by activated cells (Stadler *et al.*, 1981). Moyers and Broege (1983) have shown that CTL precursors were best activated if the stimulating cell population contained alloantigen activated-T cells; this became apparent under conditions of limiting helper factor. Their experimental systems included P1 stimulators and parental responders. The observation that the stimulating population includes T cell blasts (or that back stimulation exists) is of biological

cal importance. They express new surface antigens (Letarte *et al.*, 1980; Bonavida *et al.*, 1982) and may elaborate IL 2 and other lymphokines (Stadler *et al.*, 1981; Prystowski *et al.*, 1982). All this would be expected to increase the stimulatory potential and augment the MLR response, supporting the suggestion that activation of stimulator cells constitute an integral part of the stimulatory mechanisms.

4.2 OPTIMAL MLR REQUIRES T CELLS IN THE STIMULATORS

It has been established in the experiments described in 'Results' that the stimulating population should contain T cells in order to induce optimal proliferation in MLR. By depleting T cells with the appropriate monoclonal antibody and complement it was shown (Figures 12 and 13) that ³H-thymidine incorporation in the MLR was reduced 50% or more. Others have also noted the requirement of stimulator-T cells for optimal response in MLR in cultures containing stimulating cells obtained from nude mice (Coyers and Drege, 1982). They observed in microcultures and *in vivo* that mixtures of stimulating cells from allogeneic athymic and euthymic strains of different H-2 haplotypes activate substantial cytotoxic responses only against H-2 antigens of the euthymic strain. Using guinea-pig cytotoxic T cell lines as responders, Malek and Shevach (1981) found that MLR response could occur only if the stimulating population contained T cells. There are other reports, however, where absence of T

cells did not reduce MIR response (Lutz et al., 1981; Widmer and Bach, 1981). These discrepancies may be explained by heterogeneity of Thy 1.2 antigen as detected by monoclonal antibodies. Del Guercio et al. (1982) have shown that monoclonal antibody F7D5 abolished all T cell functions studied in C57Bl/6 spleen cells whereas it was totally or partially ineffective on DBA/2. Similar differential activity of monoclonal anti-Thy 1 was shown by Prystowski et al. (1982).

We found that the reduction caused by T cell depletion of the stimulators could be restored by a mitomycin C-treated T-enriched population of the stimulators but not the responders (Figure 14) as noted by Moyers and Droege (1982) also. This indicated that the stimulator-T cells are more than just members of the T cell (IL 2 producer) pool. Stimulator T cells are probably effective when present along with other syngeneic cells of the stimulating population. Such a conclusion was reached by Moyers and Droege (1982) who found that lymph node cells, poor in Ia⁺ cells, were consistently inferior to spleen cells as stimulators and that purified T cell population was inferior to unfractionated population. All these observations would suggest that an interaction between activated stimulator-T cells and other syngeneic cells, perhaps the macrophages, generate Lafferty's "allogeneic stimulus", that function of the stimulators which requires metabolic activity.

4.3 ROLE OF THE ACTIVATED STIMULATOR-T CELL

As suggested above, the activated T cells of the stimulators can be envisaged to contribute to the stimulatory mechanism by enhancing the repertoire of alloantigens and/or generating the second signal.

4.3.1 Stimulation by new antigenic determinants?

Activated T cells per se appear to have certain stimulatory capacity even after UV exposure (Table 13). Once the T cells are activated, the requirement of metabolic activity in the stimulators does not appear to be very stringent for the stimulatory mechanism. Formation of T cell blasts is accompanied by the expression of a series of new surface antigens (Bonavida *et al.*, 1982). Some of these, like the transplantation antigens may be resistant to UV (Lafferty *et al.*, 1974) and be able to arouse proliferative response in some of the responding cells (Table 13). Blasts have been shown to be capable of activating syngeneic unstimulated cells, possibly through the production of novel antigens (Lindahl-Kiessling and Säfvenberg, 1971; Kaplan *et al.*, 1976; Owens *et al.*, 1984). Coutinho *et al.* (1981) have shown that there are certain non-H-2 and non-Mls coded antigens that are able to effect MLR. Moreover, activated T cells have been shown to express increased Ia, a 10-fold increase over the quiescent cells (Letarte *et al.*, 1980). In the human system Ia bearing T cells have been shown to have a high stimulatory potential

(Indiveri *et al.*, 1980; Schuurman *et al.*, 1980; Wollman *et al.*, 1980; Forre *et al.*, 1982). However, in UV-irradiated blasts the action of such Ia antigens may be suppressed; Schendel and Bach (1976) have shown that inhibition caused by UV irradiation may be restored by untreated Ia bearing cells of the appropriate haplotype. It is unlikely that the increased stimulating potential of the UV-irradiated Con A blasts is due to the lymphokines they might release. Kelso and Boyle (1982B) found that the production and release of IL 2 was inhibited by UV.

It would thus appear that the stimulatory capacity exhibited by UV-irradiated Con A blasts may lie in the new surface antigens expressed during blast formation, some of which may be UV resistant and to which some responding clones may be sensitive. Glutaraldehyde-fixed LPS blasts and tumour cells have been described to exhibit stimulating potential (Lightbody and Kong, 1976). UV-irradiated cells from a lymphoma cell line are exceptionally potent activators of normal human lymphocytes (Hardy *et al.*, 1970). One may deduce from this that stimulatory capacity may be associated with cycling cells.

4.3.2 Regulation of IL 2 in MLC

The presence of activated T cells in the stimulators has been correlated with the detection of IL 2 in the cultures: (1) the absence of T cells in the stimulating population re-

duced IL 2 activity (Table 14); (2) absence of activation in the stimulating cells suppressed IL 2 availability. UV irradiation of stimulators prevented the formation of T cell blasts in the stimulating population and no IL 2 was detected in the corresponding cultures (Table 15); of course the accessory cell-induced stimulation could be blocked as well (Germain, 1981) and (3) exogenously added IL 2 could compensate for the function of activated stimulator T cells (Table 16). Similar findings have been reported by Moyers and Droege (1982;1983) also. They have shown that under experimental conditions where IL 2 production is inhibited, CTL production can be achieved by adding growth factors (supernatants from Con A cultures of leukocytes) to the cultures. In their experiments the presence of T cells in the stimulating population was found to be necessary for optimal activation in MLR, responses against athyric stimulators could be obtained only in the presence of helper factors. Activation of the T cells in the stimulating population has been shown to be a requirement for optimal responses by Moyers and Droege (1983) also. Experiments in microcultures revealed that homozygous C3H (H-2^k) or CBA (H-2^k) responder cells failed to react against semiallogeneic or haptenated syngeneic stimulator cells, unless helper factor was added to the culture or unless the stimulators were activated by exposing them to cells differing from them in the I-region. Addition of irradiated spleen cells of C3H or A.TL (K^s I^k D^d)

did not restore the reaction. When the stimulating cells could be activated, as by the addition of A.TH ($K^s I^s D^a$), MLR was induced. Neonatal cells which express B-2 antigens but which fail to undergo activation in the presence of Con A, stimulate allogeneic cells poorly; their immunogenicity was found to be 2 - 10 fold below that of adult cells (Kelso and Boyle, 1982B). Backstimulation, therefore, appears to constitute an integral part of the mechanism for producing a second signal, such as IL 2.

Moyers and Orcege measured CTL responses and I have measured the proliferative responses in the MLR; I added purified IL 2 and they added the crude Con A supernatant; still comparable results were obtained, in the two laboratories, confirming that activated T cells in the stimulating population must be present for optimal allogeneic response.

4.4 SOURCE OF A SECOND SIGNAL IN MLR

An analysis of the experiments designed to determine whether IL 2 is produced by the responding or the stimulating cells suggests that both the allogeneic populations are effective (Table 17) and that there must be more than one pathway of producing growth factors in MLC.

The primary source of IL 2 is generally considered to be the responding population. It is commonly accepted that the alloantigen-triggered cytotoxic precursor cells of the responders receive proliferation-inducing signal 2 from the

Lyt 1⁺ T cells of the responding population, which produce a non-specific helper factor during MLC (Wagner *et al.*, 1980). This growth factor, considered to be IL 2 (there is no direct evidence) is produced by activated T cells in G1 phase (Stadler *et al.*, 1981).

A second source of IL 2 appears to be the stimulators. It has been shown that mitomycin C-treated cells, (therefore, the stimulators (Treatment b in Table 17)), can also produce a growth factor, possibly IL 2, which can support the growth of the IL 2-dependant cell line MTL. This is in accord with the findings of Stadler *et al.* (1981) that IL 2 is produced in G1 cells and the finding that stimulator-T cells are in G1 (Figures 10 and 11). Release of potentiating factors by irradiated or mitomycin C-treated leukocytes have been reported by several workers (Kasakura 1971; Hirschberg *et al.*, 1974). Reddehase *et al.* (1982) have shown that a factor required for CTL production is derived from the activated stimulators. The identity of the factors noted by these laboratories has not been established. Palacios (1982) has shown with IL 2 producing clones that treatment with mitomycin C or X-ray did not affect their capacity to synthesize IL 2 when they were cultured with PHA; treatment with 3000 rads or more inhibited the production.

My experiments point to yet another source of growth factor where the factor generated has IL-2-like biological activity, in that it can support the growth of the cell line

CTL, but unlike IL 2 appears to be produced by cells capable of synthesizing DNA. When there is no T cell in the stimulating population the IL 2-like growth factor in the culture supernatant must be derived from the responding T cells (Treatment c in Table 17). However, if in such a culture, the responders are blocked at G1/S phase with mitomycin C treatment (Treatment d), no growth factor is detected, indicating that in Treatment c the factor is generated by responding T cells which are able to enter the S phase. It could be that DNA synthesis is required because only one round of cell activation does not provide with a sufficient number of responding cells in G1 to produce an adequate amount of IL 2.

However, mere presence of a large number of responder cells may not be sufficient either. It was shown in the experiment reported in the 'Results' (Table 14) that reduction in IL 2 caused by depletion of T cells from the stimulator could be restored only by adding stimulator-T cells and not responding-T cells.

One has to be cautious regarding the specificity of the biological assays for IL 2. It has been found that there are several lymphokines which are biochemically distinct from IL 2 but may perform similar immunoenhancing activities (Farrar et al., 1982). A growth factor, which Ching and Miller (1982) have found not to be IL 2, is required for the growth of some CTLs. Werber et al. (1983) reported that colony

growth of T cells in a soft agar was promoted by a T-cell derived factor which was different from IL 2; upon hydrophobic chromatography, it showed two peaks of activity, neither of which coincided with that of IL 2. Thorpe et al (1983) have reported the detection of a growth factor which supports proliferation of Con A-stimulated murine T cell blasts, a bioassay for IL 2, but which can be produced by unstimulated thymocytes in vitro. Kasakura (1971) had also noted the release of growth factors by unstimulated leukocytes. IL 2 has been shown to be produced only by activated T cells (Caplan et al, 1981). I think that only biochemical characterization and not a bioassay can establish if a growth factor is actually IL 2.

It is tempting to speculate that the growth factor detected in cultures with T-depleted stimulators (Table 17, treatment c) is not IL 2 and that it is not produced by Lyt 1⁺ lymphocytes. Paetkau et al (1981) have shown Lyt 1⁺ to be the producer of IL 2. I think that this growth factor with IL 2-like immunoenhancing properties is produced by Lyt 2⁺ cells of the responders. Okada and Benney (1980) have shown that a growth factor is produced by Lyt 1⁺ in response to I-region difference and by Lyt 2⁺ when there is a K/D difference. Czizrom et al (1983) showed that Lyt 1⁺ lymphocytes are required to be stimulated by Ia-bearing stimulators (Class II antigens) whereas Lyt 2⁺ would respond to any cell demonstrating allogeneic differences. I think that the acti-

vation of IL 2 producing Lyt 1⁺ cells requires the presence of T cells in the stimulating population, which exert their effect in association with syngeneic Ia bearing cells and that Lyt 2⁺ cells produce an IL 2-like growth factor when they proliferate in response to Class I antigens. One of the functions of the activated stimulator-T cells may lie in enhancing the stimulatory capacity of Ia bearing cells, that is, in the induction of Class II-induced responses. All this conjecture is, of course, open to question. It may be that this growth factor is not produced by Lyt 2⁺ cells but by Lyt 1⁺ cells when they are uninhibited by agents such as mitomycin C. There are other alternative possibilities as well.

4.5 AMPLIFICATION OF RESPONSE IN ALLOGENEIC REACTIONS

The work reported in this thesis answers, partly, my question regarding the function of stimulating population and suggests an answer to the general question as to why the allogeneic response exceeds by 100 - 1000 times the magnitude of immune response expected according to the clonal selection hypothesis (Wilson and Fox, 1971).

To answer the question regarding the function of the stimulators it can be said that the work reported in this thesis has shown that activation of the stimulator-T cells raises the stimulating potential; they may display new surface antigens (Latarte et al. 1980; Echnavida et al. 1982).

which may activate previously unresponsive clones in the responders and in the syngeneic cells (Kaplan *et al.*, 1976; Owens *et al.*, 1984). These newly activated cells may produce growth factors and the stimulator T cells themselves may produce lymphokines (Table 17). In either case, the result would be a cascade of events, involving cell-cell and cell-mediator interactions, eventually leading to optimal proliferation.

The lymphokines released in MLR may act directly as the second signal for the alloantigen-triggered responders or they may act by exerting a secondary effect by activating syngeneic B cells (Glasebrook *et al.*, 1982) and macrophages (Oppenheim *et al.*, 1981). B cell blasts are far superior stimulators than the corresponding quiescent cells (Chestnut *et al.*, 1982). Macrophages are biologically more efficient in producing cytokines when they are activated (Oppenheim *et al.*, 1981).

The cumulative effects of the products of activated T cells from both responders and stimulators, may partly be responsible for the large number of alloreactive cells: considering the stimulus of the major histocompatibility antigens, the number of cells responding in allogeneic reactions exceed by far the number estimated by the clonal selection hypothesis (Burnet, 1969; Wilson and Fox, 1971; Matzinger and Bevan, 1977).

The proportion of histocompatibility antigen-reactive cells in an individual which respond to the MHC alloantigens on the cells of another member of the same species is unusually large. Estimates derived from studies with GVH reaction (Szenberg et al., 1962) and MLR are in agreement and place this number at 2-4% of the cells of the circulating lymphocyte pool (Wilson and Nowell, 1971). This finding is in conflict with Burnet's (1969) clonal selection hypothesis and various suggestions have been made to account for this (Wilson and Fox, 1971). One of the possibilities is that the high frequency of alloantigen-reactive cells represents the activity of a large number of subpopulations of antigen-reactive cells stimulated by an equally large number of antigens. There is no direct supportive evidence for this although Matzinger and Bevan (1977) suggest that the products of MHC influence the antigenicity of other cell surface components. Another possible explanation may be found in the effects of lymphokines which may non-specifically activate a large number of clones. The third possibility stems from a combination of factors: (1) new antigens develop during MLR on both responding and stimulating activated cells which stimulate new clones (syn- and allo-geneic) responsive to them (Table 13; Kaplan et al., 1976; Owens, et al., 1984) and (2) and an array of growth-promoting lymphokines are released from both allogeneic populations (Table 17), culminating in a complex interplay among them.

The current concept assigns the stimulatory role to Ia bearing cells (Sunshine et al, 1982; Steinman et al, 1983). My results emphasizing the importance of activated T cells in the stimulating population do not necessarily contradict this. The activated T cells may directly act as stimulators and/or function synergistically with the syngeneic Ia bearing cells. It is suggested that the activation of T cells in the stimulating population constitutes an amplifying force for the allogeneic response and that it may be considered to form an integral part of the stimulatory mechanism. Immune recognition of foreign antigens is highly specific but the "allogeneic stimulus", which probably arises from activation of the stimulators, amplifies the response of a few responding cells so that the allogeneic response can be detected.

Chapter V
CONCLUSIONS

5.1 CONCLUSIONS AND INFERENCES

The conclusive point that has emerged from the work done for this thesis is that for optimal proliferation in MLR, the stimulating population is required to contain activated T cells, which themselves produce IL 2 and which regulate the generation of growth factors by responding cells.

It is inferred from preliminary evidence that there is ~~more~~ than one pathway for inducing proliferation in MLR; there may be two or more mechanisms of producing the second signal which must synergise with one another; IL 2 is not the only second signal.

The conclusion is based primarily on the following experimental results. I have shown that the stimulating population must include T cells, which undergo pre-S phase activation during MLR in order to induce optimal proliferation. T-depleted stimulators can induce MLR but to a reduced level. I have described some of the functions of the stimulator-T cells and suggested that, overall, they raise the stimulating capacity so that the immune response induced in the responders by alloantigens of the stimulators is amplified to a detectable level.

A correlation has been found between the capacity of leukocytes to stimulate an MLR response and the ability to undergo activation (pre-S phase), suggesting that the stimulating cells themselves must be alloantigen-activated for inducing optimal response in the responding population. It appears likely that the ability to synthesize new RNA is associated with immunogenicity of the stimulators. That the T cells in the stimulating population do actually undergo activation (pre-S phase) was shown morphologically. Using a fluorescein-labelled monoclonal antibody the stimulator-T cells in a 24 hour MLR were identified and it was shown that they incorporated ³H-uridine to a greater extent than the resting population. To my knowledge, this is the first direct demonstration that the stimulator T cells in an MLR undergo activation. The current literature says that the stimulator cells must be metabolically active but I say that to be metabolically active is not enough, they should be immunoresponsive: one-way MLR must involve two-way activation.

The T cells are not commonly thought to have a stimulatory function in an MLR. However, in my experiments, the presence of T cells in the stimulating population was shown to be necessary for the induction of optimal proliferation in MLR. Depletion of T cells by treatment with anti-Thy 1 serum and complement reduced significantly the proliferative response in the MLR which could be restored by the addition of T cells from the stimulators but not the responders. It

seems obvious that the T cells in the stimulators exert a function other than the release of IL 2, otherwise the extra responder T cells should be effective. This function most likely includes other cells syngeneic to the stimulator T cells.

The experiments performed to characterize the functions of the activated stimulator T cells show that they can induce MLR even when irradiated by an inhibitory dose of UV and that the T cells in the stimulating population can regulate the level of growth factors in the culture supernatant. The mechanisms underlying these functions have not been resolved.

Generally, IL 2 produced by T_H helper cells in the responders is considered to be the second signal in an MLR, without any regard to the presence of T cells in the stimulators. In the present series of experiments, it has been shown that the stimulators themselves are capable of producing IL 2 and that the T cells in the stimulating population participate in the generation of growth factors in the responders. It has been further suggested that a growth factor or a second signal may be produced by a pathway where participation of stimulator T cells is not necessary and which may involve DNA synthesizing cells in the responders. Thus there should be more than one pathway for generating the necessary growth factors.

Thus MLR may be observed when a population of lymphocytes is exposed to alloantigens along with an adequate amount of growth factors, which may be produced by more than one mechanism. Lafferty and his co-workers suggested that metabolic activity of the stimulators is required for that function of the stimulators which generates a second signal and I have found that the generation of the second signal involves the action of stimulator-T cells which are activated; the process of activation would require metabolic activity.

5.2 THE MODEL

Several laboratories and the experiments described in this thesis show that the stimulating cells should be metabolically active to induce MLR. My experimental results lead to a proposal that metabolic activity in the stimulating cells is required for amplifying the response generated by alloantigens (Class I MHC antigens); the immunoenhancing forces would include the ability of the stimulators to be immunoresponsive and to undergo activation in response to alloantigens of the responders. I hypothesize that the stimulator T cells which I have shown to undergo activation during MLR, interact with syngeneic Ia bearing macrophages whose Class II antigens arouse the Lyt 1⁺ lymphocytes in the responders. When the stimulators are depleted of T cells, the stimulating Ia bearing cells function in some other way or in a limited way.

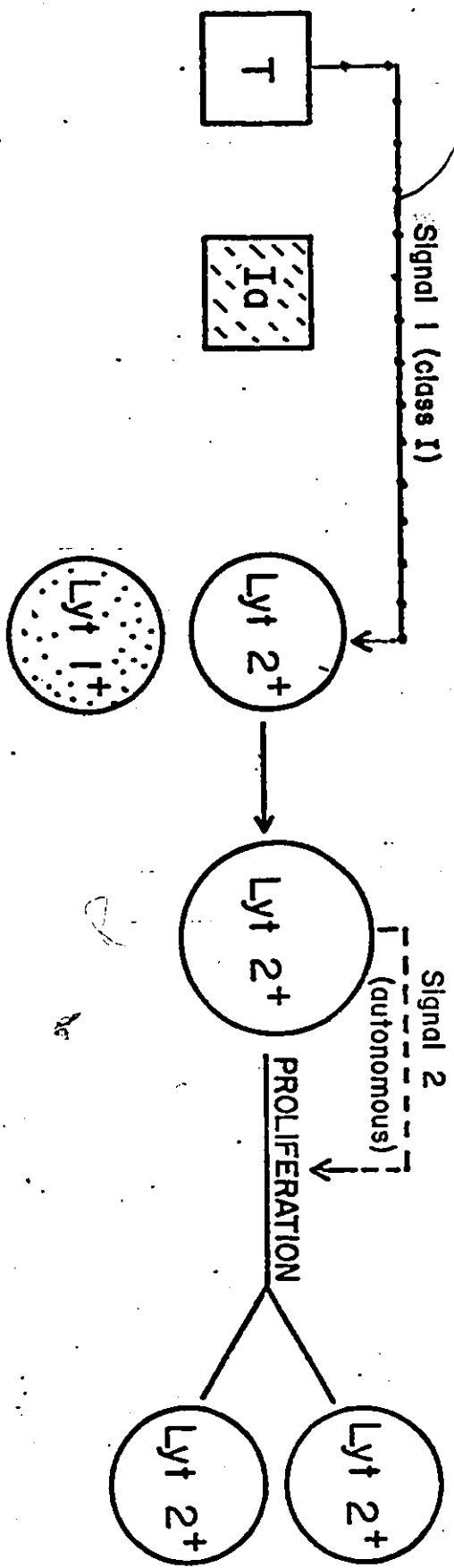
Figure 17. Scheme showing the proposed model for the mechanism of MLR.

Responses induced by metabolically inactivated stimulators are shown in (A) and that by the metabolically active stimulators in (B).

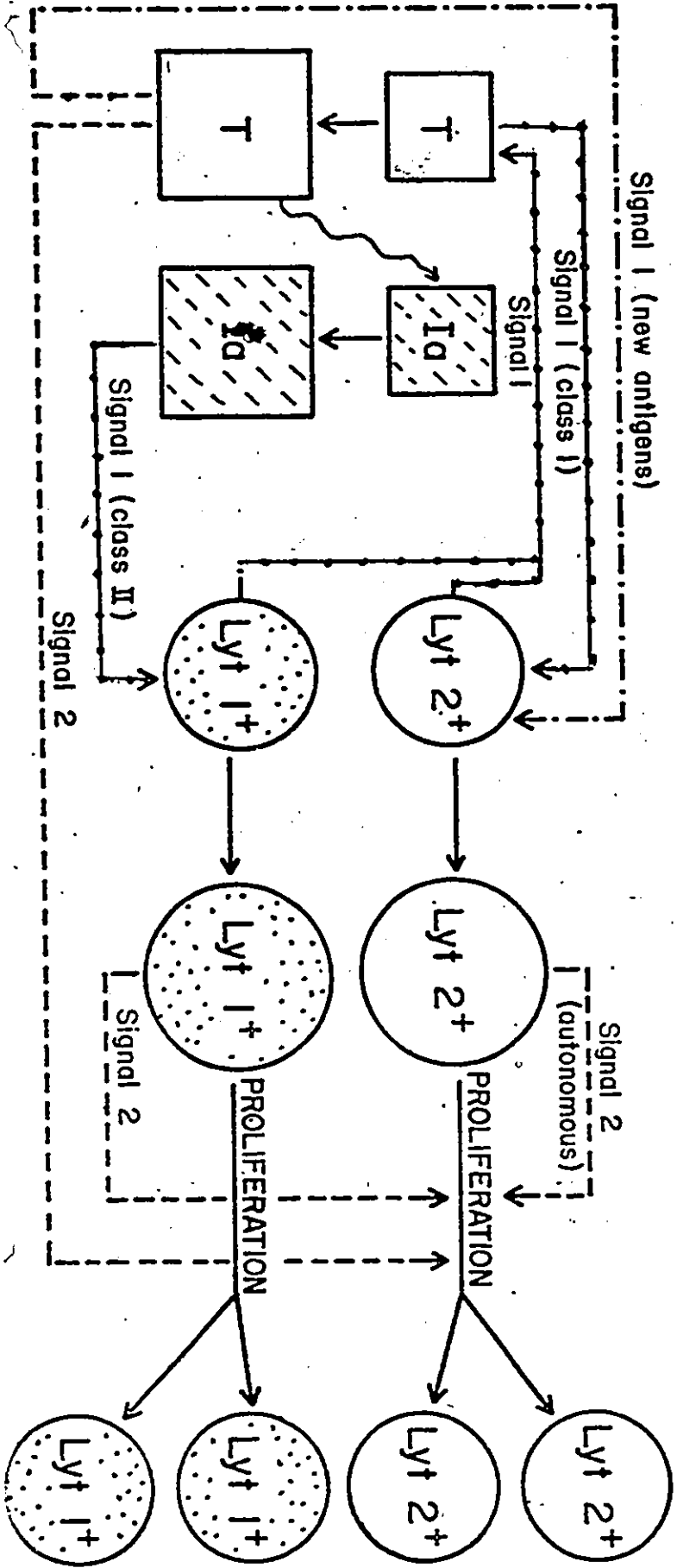
Interaction of stimulators (□) with responders (O) and the sites of origin and of action of signal 1 (→) and signal 2 (---) are depicted. Activated cells are represented by enlarged size and all the amplifying forces are in dashed lines.

Abbreviations used are: T, T cells; Ia, Ia bearing cells; Lyt 1⁺, Lyt 1⁺ bearing cells; Lyt 2⁺, Lyt 2⁺ bearing cells.

A. Metabolically inactive stimulators



B. Metabolically active stimulators



It is possible that signal 1 or Class I antigens of the stimulators induce immune response in the Lyt 2⁺ population of the responders by a mechanism which is independent of metabolic activity of the stimulators or of the presence of stimulator T cells. At least some Lyt 2⁺ cells can release growth factors and proliferate autonomously (Widmer and Bach, 1981; Glasebrook *et al.*, 1983). The amplifying forces can then enhance the responses of the Lyt 2⁺ cells and bring that to a detectable level.

The alternative model would be that the metabolic activity is required not only for the induction of Class II-induced responses but for that of the Class I-induced responses as well, as suggested by Lafferty *et al.*, (1980). Kelso and Boyle (1982A) found that the recognition of K/D antigens required metabolic activity in the cells capable of absorbing the precursors of cytotoxic cells.

5.3 FUTURE DIRECTIONS.

During the course of the work described in this thesis a few questions have come up: these and the model proposed above may be experimentally resolved or tested. Some of the suggestions for future work have been listed below:

(1) Explaining the possible association between small blasts and stimulating capacity. Perhaps the new RNA synthesized during cell activation is correlated with the emergence of new surface antigens or with the production and re-

lease of lymphokines. Examining the effects of RNA inhibitors on these features may lead to some answers.

(2) Elucidating the mechanism by which UV-irradiated Con A blasts induce MLR, whether by virtue of the antigens peculiar to the blasts or by releasing pre-formed IL 2.

(3) Identifying the IL 2-like growth factor detected in cultures with T-depleted stimulators. One has to first establish the requirement of proliferating cells; that additional mitomycin C-treated cells will not generate more growth factor. Its biochemical characterization will reveal whether or not it is IL 2.

(4) To resolve if the action of alloantigens or signal 1 requires metabolic activity. If the sole requirement of the metabolic function is for generating IL 2 or a similar factor, then signal 1 or alloantigens from metabolically inactivated cells should be able to stimulate responders at least up to the pre-S phase, a stage where IL 2 acts.

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