National Library of Canada

Acquisitions and Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada

Direction des acquisitions et des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

SUPPRESSION OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELL RESPONSE TO MITOGEN BY TETANUS TOXOID. A STUDY OF THE POSSIBLE MECHANISMS

Thesis Submitted To
The School Of Graduate Studies And Research
The University Of Ottawa

In Partial Fulfillment Of The Requirements For The Degree Of
Master of Science
Department of Microbiology And Immunology
School of Medicine

By

Helen Stefanovic

© Helen Stefanovic, Ottawa, Canada, 1992

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-85846-X

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

Previous studies have shown that certain antigens can down-regulate immune responses of human peripheral blood mononuclear cells (PBMC) both *in vivo* and *in vitro*. This study demonstrates that tetanus toxoid (TT), in a dose-dependent fashion, suppresses the induction of a blastogenic response of PBMC by phytohemagglutinin (PHA), monoclonal anti-CD3, and anti-CD4 antibody. Pokeweed mitogen (PWM)-induction of IgG and IgM is suppressed as well. The suppression is partially reversed by indomethacin and IL-2, but not by IL-1 or tumor necrosis factor (TNF-α). PBMC pre-incubated with TT could suppress the PHA blastogenic response of fresh autologous cells during co-incubation. The removal of $CD4^+$ cells prior to induction of suppression greatly diminished the suppression of PHA blastogenic response, whereas the elimination of $CD8^+$ cells had no effect. Therefore it is concluded that TT induces non-specific suppressor cells that can strongly dampen mitogenic responses, and that $CD4^+$ cells play an important role in this suppression. $CD4^+$ cells, together with monocytes, may suppress mitogenic responses involving a prostaglandin-dependent pathway.

ACKNOWLEDGEMENTS

I would like to gratefully acknowledge the help and guidance given to me by my supervisor, Dr. Lionel Filion, and whose patience was a comfort especially in the later stages of writing.

At this time I thank Dr. Maxwell Richter for his help and support at the Civic Hospital, and Dr. Carlos Izaguirre for his guidance in immunofluorescence. I would also like to acknowledge the influence of Dr. Ralph Germinario, a professor from my undergraduate days who memorably spoke of "serendipity" in research.

I heartily thank Jamie Waring who has been a constant companion and sounding board.

I also wish to thank all my friends and colleagues, both past and present for their friendship and encouragement.

DEDICATION

I would like to dedicate this thesis to my parents who have given their love and support throughout my every endeavour.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

aa	Amino acid
Ab	Antibody
AC	Accessory cell
AMLR	Autologous mixed lymphocyte reaction
APC	Antigen-presenting cell
BCDF	B cell differentiation factor
BCGF	B cell growth factor
BHV	Bovine herpes virus
C	Complement
Ca^{++}	Calcium ion
CD	Cluster of differentiation (antigen cluster designation)
CON	Control
CON A	Concanavalin-A
CTL	Cytotoxic T lymphocyte
DAG	Diacylglycerol
DNP	Dinitrophenol
DTH	Delayed-type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FCS	Fetal calf serum
Fc	Crystallisable fragment of Ig

FITC	Fluorescein isothiocyanate
G	Gravity
g	Gram
gp	Glycoprotein
HBsAg	Hepatitis surface antigen
HETE	Hydroxyeicosatetraenoic acid
HLA-DR	Human class II Histocompatibility antigen (subtype: DR)
HPLC	High pressure liquid chromatography
hr(s)	Hour(s)
HSV	Herpes simplex virus
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IFN	Interferon (alpha, beta, gamma)
IL-	Interleukin (1,2,4,5,6,)
IL-2R	Interleukin-2 receptor
LT	Lymphotoxin
LTB_4	Leukotriene B_4
mAb	Monoclonal antibody
MΦ	Macrophage
MHC	Major histocompatibility complex
mg	milligram

ml	millilitre
MLR	Mixed leucocyte reaction
ng	nanogram
NK	Natural killer (cell)
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PG	Prostaglandin
PHA	Phytohemagglutinin
PIP_2	Phosphoinositol diphosphate
PKC	Protein kinase C
PPD	Purified protein derivative
PMN	Polymorphonuclear cell
PT	Pertussis toxin
PWM	Pokeweed mitogen
RIF	Rosette inhibitory factor
RNA	Ribonucleic acid
RPMI-1640	Rosewell Park Memorial Institute nutrient medium 1640
SIF	Serum inhibitory or immunosuppressive factor
SRBC	Sheep red blood cell
TA	Toxoplasma antigen
TCGF	T cell growth factor
TCR	T cell receptor for antigen

^{3}H-TdR	Tritiated thymidine
Ti	T cell antigen receptor idiotype
TNF	Tumor necrosis factor (alpha, beta)
r-TNF	Recombinant TNF
TT	Tetanus toxoid
T_C	T cytotoxic cell
T_D	T delayed-type hypersensitivity cell
T_H	T helper cell
T_S	T suppressor cell
U	Unit
μg	microgram
μl	microlitre
μM	micro molar
UV-B	Ultraviolet radiation, B rays

I. INTRODUCTION

The immune system has evolved to protect the host from infection and climinate pathogens. The external environment provides humans and all living creatures with a constant challenge from a myriad of microbial infectious agents. Infection can result from virus, bacteria, fungus or parasite. If allowed to penetrate, colonize and multiply successfully, these organisms would eventually kill their host. Immunity against a pathogen can be divided into innate (non-specific) and adaptive (specific) components. Non-specific immunity is the first line of defense against most organisms and prevents most infectious agents from establishing a niche and flourishing. Innate immunity is comprised of physical barriers (skin) and biochemical defences (mucus, lysozyme, complement proteins, and acute phase proteins). In addition, cells such as phagocytes and natural killer (NK) cells also play a central role in this type of immunity (Roitt, 1989).

The adaptive immune response is comprised of cells and factors which enable the system to react quickly and specifically to a challenge by an invading organism. The cells of the adaptive immune system arise from pluripotent stem cells which in turn give rise to 2 main lineages of immune cells, the lymphoid and the myeloid lineages. The differentiation of the lymphoid line results in the production of the lymphocytes, which confer specificity to the immune response, whereas the myeloid

line gives rise to a variety of cells which include phagocytic cells and granulocytes. The antigen-presenting cells such as monocytes, macrophages and dendritic cells arise from this branch of development. This latter group of cells also functions in the innate immune system and bridges both the innate and adaptive systems (Cantor *et al.*, 1986).

There are 2 major populations of lymphocytes, T and B cells, which are morphologically similar but have different functions and development patterns. The term T cell was coined because these cells were found to differentiate and mature in the thymus, whereas B cells were recognized as cells that differentiated in the Bursa of Fabricius in birds. In mammalian species, B cells differentiate initially in the fetal liver, and in the spleen and bone marrow in adult mammals (Paul, 1984; Richter, 1982).

Lymphocytes are produced in the primary lymphoid organs (thymus and bone marrow). Some of these cells migrate to the secondary lymphoid tissues (spleen, and lymph nodes). Lymphoid cells represent 20% of the total leukocyte population. B cells represent 5-15% of the circulating lymphoid pool and are classically defined by the presence of surface immunoglobulins or "antibodies". The antibodies have a specificity for some structure on the surface of a pathogen. When released from the mature B cell into circulation, they can bind to the structure that has elicited them.

Foreign material which is capable of eliciting antibodies has classically been termed antigen, for "antibody generating". The structure that is the binding point for the antibody is termed an epitope or antigenic determinant. These terms have been employed interchangeably. Cross-reactivity of antibody to determinants, which possess enough conformational homology for an approximate "fit" but not a perfect match, does occur, but the binding affinity is usually stronger for the original structure by which the antibody was elicited. Only T and B lymphoid components of the immune system are capable of recognizing foreign molecules by their molecular features and mounting an antigen-specific response that leads to the elimination of the antigen. B cells participate in "humoral immunity" (antibody-mediated) by differentiating into antibody-secreting cells. Antibodies are especially effective against extracellular phases of bacterial, viral and parasitic infections. Cytotoxic T cells (T_C) participate in what is termed the effector arm of cellular immunity, via recognition and cytolysis of infected cells, tumors, and foreign tissue. In addition to their cytolytic function, T cells participate in regulating the immune response by either promoting or inhibiting various immune functions. In this capacity, T cells have classically been generally further divided into T helper/inducer (T_H) and T suppressor (T_S) cells.

The understanding of B and T cell function is partially focused on the physical structure of antigen that induces these cells to proliferate and differentiate.

Immunogenicity of a foreign substance indicates the ability to elicit an immune response and is the summation of a variety of influences that reflect the previous history of the host as well as its genetic attributes. This involves the interactions of the available B cell repertoire, activity of T helper cells, T suppressor cells, and MHC complex. Antigenicity implies the ability of a structure, *eg.* protein, to be recognized by a product of the immune response, *ie.* antibodies or T cells (Benjamin *et al.*, 1984).

Conformational determinants are recognized by antibodies and are dependent on the native spatial conformation of a given structure. Native proteins are usually formed into tertiary conformations and are based on the 3-dimensional arrangement of the amino acids. A degradation of the native protein can result in the breakdown of linkages (disulfide bonds) between key amino acids that hold the conformation together. This would form a secondary conformation which may have retained enough bonds between the amino acids to include a fold or loop in the original amino acid sequence. If these last forces are broken, the result would be a primary conformation which is comprised of the straight linear arrangement of amino acids. Sequential determinants are based on the primary and secondary amino acid sequence of a protein and are not generally recognized by the antibody that would recognize the tertiary sequence. The binding of immunoglobulin molecules to antigen is particularly sensitive to the tertiary structure of the antigens on a microorganism.

What constitutes a determinant for a B cell is quite different from that for a T cell. In contrast to conformational recognition, the epitopes recognized by T cells are different, or rather the T cell receptor for antigen (TCR) does not recognize free antigen but recognizes small peptide fragments derived from the proteins of the microorganisms that are associated with the products of the MHC. Fragmentation of the viral, bacterial or parasitic polypeptides is presumed to be carried out by the action of cellular proteases. The degraded fragments are associated with the cellular "self" MHC molecule and are expressed on the cell surface (Briacle and Briacle, 1991).

A peptide-binding cleft occupied by 7-10 amino acid residues is typically found associated with MHC I molecules (Van Bleek and Nathenson, 1990). A peptide fragment recognized by the T cell receptor for antigen (TCR) is 6-11 amino acids long (Royer and Reinherz, 1987; Brown *et al.*, 1988).

Differences in the epitopes recognized by subpopulations of T cells may exist. For example, T helper cells may recognize one peptide sequence while T suppressor cells recognize another (Sercarz and Krzych, 1991). The strict associations of the CD4 molecule with MHC II, and of the CD8 molecule with MHC I, support the distinction of epitopes if it is assumed that the same protein fragment is not presented by both classes of MHC molecule. A further difference in epitope recognition has

been seen among T suppressor and T cytotoxic cells which can be ascribed to the T cell receptor and not to antigenic processing (Brondz *et al.*, 1987).

A. Clonal Selection and Expansion

The population of T and B cells expresses specific receptors for a great variety of determinants that it may encounter. This has been referred to as the T or B cell "repertoire". Cells bearing receptors that bind strongly to a given determinant are stimulated to multiply. This results in the formation of a clone of thousands of daughter cells specific for that determinant. T cells are activated when they bind antigen in conjunction with accessory cells. Antigen may bind to the limited number of cells in circulation that recognize it and induce them to proliferate, so that the population can increase to a sufficient number of cells to enable the host to mount an adequate immune response. In this way antigen drives the expansion of specific clones of antigen-binding cells. Clonal selection occurs both for B lymphocytes through their surface immunoglobulin and for T lymphocytes through their T cell receptor for antigen (TCR). Recognition results in the clonal expansion of cells which terminally differentiate and mature into effector cells or give rise to memory cells.

Most of the accessible surface of any globular protein may be antigenic, however the differences in structure between the antigen and self- protein (MHC), as well as the host's immunological regulatory mechanisms, are important factors in

influencing the outcome of the overall immune response. An understanding of host regulatory mechanisms is therefore crucial in the understanding of pathogenesis, immune responses to antigen, and the clinical manipulation of those responses to our advantage.

B. Cell Function and Networks

The T cell population is heterogeneous and functions by many interactions between several cell types which include those cells designated as antigen-presenting (accessory), B cell, T helper or T suppressor. In cellular immunity, effector cells, such as cytotoxic T lymphocytes (CTL or T_C) can lyse specific target cells. T lymphocytes can also act on other cell types such as macrophages or NK cells and induce them to eliminate a foreign body. This type of cellular response is referred to as delayed-type hypersensitivity (DTH). The interactions among cells within the response are often mediated by the release of lymphokines and cytokines. Other interactions of lymphocytes involve T helper cells (T_H) which up-regulate immune responses by delivering stimulatory signals to B cells. This causes the B cells to differentiate and secrete antibody. T_H can also deliver signals to other cells, including T_C and phagocytic cells.

A third major population of T cells are the T suppressor cells (T_S). T_S were initially discovered when vaccinated rabbits were not able to generate antibodies

immediately following the peak of antibody synthesis after primary immunization. T_S are responsible for inhibiting immune responses in a feedback mechanism, and ensure that unnecessary immune responses do not persist following clearance of antigen from the host. The exact nature of antigen-specific T_S is not known since suppressor cells have not been successfully cloned *in vitro*, however Dorf and Benaceraf (1984) have described a suppressor network of cells in mouse studies; T_S1 serves as an inducer cell, and T_S2 serves as an effector cell. They have also described a 3-cell system with T_S1 as inducer, T_S2 as a transducer cell which amplifies the interaction between the inducer and the effector cell, which is denoted by T_S3.

Each of these cell types is thought to elaborate their own suppressor factors, which removes the need for actual contact between the T cells. In man, the T_H cell has been identified by the binding of monoclonal marker for CD4, and T_S have been identified by CD8.

C. Characteristics of T Cells: Cell Surface Molecules

With the aid of monoclonal antibody (mAb) technology, T cells are now defined by the surface expression of CD3 molecules. CD3 is complexed with the TCR. Those cells bearing $\alpha\beta$ heterodimers (TCR) can be further subdivided into those populations expressing either CD4 or CD8 antigens. Historically these markers

have been associated with T_H ($CD4^+$) or T_S and T_C ($CD8^+$) phenotypes. The $CD4^+$ population has been further segregated according to the presence of CD45R, CDW29 and the $CD8^+$ population by CD11 and CD18. The association of help and suppression with these markers is not absolute, as many have overlapping functions. A suppressor circuit identified in man has been shown to involve $CD4^+$ inducer cells of the CD45R phenotype, and $CD8^+$ effector cells. Many other determinants have been defined by monoclonal antibodies but are quite numerous and may be receptors for cytokines, adhesion molecules, or may not have been assigned any function yet.

D. $CD4^+/CD8^+$ T Cells and MHC Association

CD4 and CD8 molecules are normally expressed on mutually exclusive populations of peripheral cells. T cells expressing CD4 almost invariably recognize foreign antigens as peptides presented by self MHC class II molecules, while $CD8^+$ cells recognize foreign antigens expressed as peptides presented by self MHC class I molecules (Meuer *et al.*, 1982; Gay *et al.*, 1987). Most "helper" cells express CD4 but not all T cells expressing CD4 are capable of helping B cells to produce antibody. The association of CD8 molecules found on cytotoxic lymphocytes and MHC class I antigen which is present on all nucleated cells in the body, provides the basis for recognition of foreign cellular material or infected cells.

E. Requirements for the Initiation of T-cell Activation

1. *Biochemical Events*

TCR must be able to bind with Ag/MHC II (recognition) and must be able to convert the binding event to signal transduction across the plasma membrane in which intracellular secondary messengers are generated. Occupancy of the TCR by antigen or mAb must result in intracellular biochemical events to initiate a response. These events occur during the first few minutes or seconds following stimulation of T cells by antigen, lectins or mAb to TCR. The changes include increases in cytoplasmic free calcium [Ca^{++}], pH changes, protein phosphorylations, and changes in cyclic nucleotides.

An increase in [Ca^{++}] and activation of PKC (protein kinase C) are not restricted to the activation of T cells; this change is also observed in platelets, neutrophils, hepatocytes, and B cells. The hydrolysis of a membrane phospholipid, phosphoinositol diphosphate (PIP_2) is involved. In all these systems, receptor stimulation results in activation of an intracellular enzyme, phospholipase C which cleaves the phosphodiester linkage of PIP_2 resulting in 1,4,5,-IP_3 and 1,2-diacylglycerol (DAG). These molecules function as intracellular messengers to induce an increase in [Ca^{++}] and PKC (Imboden *et al.*, 1985). Second messenger generation persists as long as occupancy of the receptor persists. A sustained increase in [Ca^{++}] seems to be necessary for the initiation of IL-2 gene transcription. Other activation

pathways also proceed by the same mechanism (CD2 or T11) (Pantaleo *et al.*, 1987; Bagnasco *et al.*, 1989). Activation through the CD3 molecule may also be linked to the transduction of a tyrosine kinase (Rudd, 1990).

The consequence of T cell stimulation is the release of lymphokines. Although the release of lymphokines may be the direct result of antigenic stimulation, the effects of such lymphokines are not antigen-specific. Therefore, lymphokines act independently of antigen and are pleiotropic.

2. *The Role of IL-2*

Once T cells are stimulated to secrete IL-2, the soluble factor can interact with the same cell in an autocrine fashion or on other cells in a paracrine manner. The activated T cells can act on cells that express IL-2R but cannot produce their own IL-2. This has been seen in murine clones T_H1 and T_H2 (Swain *et al.*, 1988).

Expression of IL-2R can be induced on the surface of T cells in the absence of proliferative response to anti-TCR mAb alone. The production of IL-2 by previously unstimulated resting T-lymphocytes is more stringently regulated than IL-2R expression and requires additional stimuli to cross-linked anti-TCR mAb (Sabath and Prystowsky, 1990). The additional stimuli are provided by APC in the form of IL-1 and IL-6 (Holsti and Raulet, 1989).

A major part of regulation of effector function is carried out by T cell subsets secreting different patterns of cytokines. The regulation of T cell function involves the control of different T cell subsets. An example of this is the opposite secretory patterns observed with the mouse T_H1 and T_H2 clones. Initial cell separation experiments revealed two distinct "helper" populations, where T_H2 helped B cells in cognate responses and T_H1 had a non-specific role. Today, functions have been assigned to these sub-populations based on their secretory products, T_H1 produce IL-2, γ-IFN, Lymphotoxin (LT) but not IL-4 or IL-5. IL-3 is produced by either population and T_H2 are major producers of IL-4 and IL-5 but not IL-2 or γ-IFN (Mossmann *et al.*, 1986; Swain *et al.*, 1988). Human equivalents to T_H1 and T_H2 have been found, and are denoted by the surface expression of CD45R and CDW29 markers (Rudd *et al.*, 1987).

F. Basis for Immunoregulation for Accessory Cells

Cells bearing MHC class II molecules degrade, associate and express antigen in conjunction with MHC class II. Monocytes, B-cells and dendritic cells can present antigen to T cells and are termed accessory (AC) or antigen-presenting cells (APC). Each of these cell populations presents antigen to varying degrees (Paul, 1984).

Monocytes or tissue macrophages (MΦ) are phagocytic cells that play a pivotal role in host protection by virtue of their role in both innate and acquired immunity.

As they are exposed to both antigenic stimulus and the products of surrounding cells, monocytes progressively acquire new functional capabilities, change their surface phenotype and secrete a series of products which in turn may regulate the activities of other leukocytes, like leukotrienes, prostaglandins (PG), HETE's (hydroxyeicosatetraenoic acids), interleukins and reactive oxidative intermediates (Goodwin and Ceuppens, 1983; Rola-Pleszynski, 1985; Goldings, 1986).

Macrophages have a unique place in the tissue response to external stimuli. They can interact with many extracelluar molecules, proteins, and polysaccharides, and can internalize and subject them to intracellular metabolic changes. They are highly secretory cells; the secreted products include proteases, complement proteins, growth and regulatory factors such as IL-1, IL-6, TNF and arachidonate derivatives like PGE_2 and leukotrienes. They also have receptors for lymphokines, the regulatory proteins released by lymphocytes. Upon activation by lymphocytes, monocytes acquire new properties, such as up-regulation of MHC class II molecules in response to γ-IFN (Adams and Hamilton, 1987; Koerner and Hamilton, 1987).

1. *Co-stimulatory Function of APC's*

The APC initiates the immune response through its capacity to generate both specific and non-specific signals recognized by the T cells. The specific signal is the complex of peptide fragments of a protein antigen bound to self-MHC molecules on

the cell surface of APCs, to be recognized by a clonally restricted TCR on T cells. The binding of class II molecules to class II receptors on T and B cells is thought to be the bridge which stabilizes interactions.

Expression of MHC-antigen complexes non-specifically promotes cell adhesion, which allows the APC to provide costimulatory activity for T lymphocyte stimulation. The regulation of macrophage secretory events is complex and involves multistep pathways. Arachidonic acid may be mobilized from cellular phospholipids in response to surface stimuli via Ca^{++} dependent phospholipase A or by a pathway involving phospholipase C and diacylglycerol lipases (Prpic *et al.*, 1987). The metabolic process can be up-regulated via pathways that require an influx of Ca^{++}. The regulation of monocyte cell surface receptors/molecules is also a complex process and is dependent on the interaction with the MΦ and extracellular factors such as γ-IFN derived from T cells (Adams and Hamilton, 1987). In addition, bacterial lipopolysaccharides prime macrophages for enhanced release of arachidonic acid metabolites, and stimulation of the monocyte B-glucan receptor during phagocytosis can result in the generation of leukotrienes (Czop and Austen, 1985; Aderem et al., 1986).

The functions of antigen presentation to lymphocytes, secretion of arachidonate metabolites and reactive oxygen intermediates, and ability to kill microorganisms may

not be mutually exclusive; the relative importance of each may vary depending on the nature of the stimulus and the kinetics of the resulting response.

2. *The role of Macrophage-derived mediators: IL-1, IL-6, TNFα*

Soluble mediators released by these cells are non-specific in contrast to immunoglobulins which are antigen-specific. Many monokines are simultaneously produced by activated monocytes. They are related molecules with related biological specificities.

IL-1 is an important mediator of inflammation. *In vitro*, IL-1 is pleiotropic. IL-1 induces the release of PGE_2 from macrophages and dendritic cells. IL-1 has different effects on the 2 types of T cell clones distinguished by their differential production of IL-2 and IL-4. The IL-4 producers have IL-1 receptors and could respond to IL-1 in the presence of IL-4. The IL-2 producers seem to lack IL-1 receptors and do not respond (Swain *et al.*, 1988). Similarly, differential requirements for IL-1 (co-stimulatory signal) were demonstrated for T_H1 and T_H2 clones by using MΦ or B cells as the APC (Weaver *et al.*, 1988). The positive effects of IL-1 on T_H cells may be countered *in vivo* by negative effects of PGE_2 which is induced by IL-1 and is a powerful inhibitor of T cell replication. T cells can induce monocytes to produce IL-1 by cell-cell contact (Weaver and Unanue, 1990)

in an MHC class II-restricted manner. Expression of IL-1 receptors is down-regulated by IL-1 following internalization.

IL-6 (originally called BCDF or BSF-2) is produced by T cells upon mitogen stimulation and acts on the late stages of B cell differentiation that lead to secretory immunoglobulin production (Kishimoto, 1985). Other functions of IL-6 include potent growth stimulation of myeloma cells, differentiation of neuronal cells, synergy for proliferation of myeloid leukemic blast cells and IL-3-dependent hematopoietic blast-cell colony formation. It has also been identified in T cell activation and cytolytic differentiation and in fever induction (Wong and Clark, 1988). Recently, monocytes have been shown to produce IL-6 and it has been identified as a necessary factor (Ceuppens *et al.*, 1988; Lorre *et al.*, 1990) and a cofactor to IL-1 in the activation of T cells where IL-6 was required for IL-2 production (Holsti and Raulet, 1989).

Tumor necrosis factor (TNF) is another cytokine which has diverse cellular function. It was initially named for its tumoricidal activity. It is now known that two forms of TNF exist, TNFα (cachectin) which is generally produced by monocytes (Fiorentino *et al.*, 1991) and TNFβ (lymphotoxin) which is produced by T lymphocytes. Human T_H1 and T_H2 cells have both been shown to produce TNF-α (Wierenga *et al.*, 1991) but this finding remains controversial. TNFα has a direct

effect on human T cells, enhancing their capacity to proliferate in response to different stimuli in antigen- and mitogen-induced systems (Yokota *et al.*, 1988). TNF also augments MHC I and MHC II antigens and augments expression of IL-2 receptors, thus it may have an immunologically synergistic role with IL-2 (Hamblin, 1988). Prostaglandins and glucocorticoids can up-regulate the expression of functional receptors (Akahoshi *et al.*, 1988) on PBMC , and can also suppress the production of TNF by macrophages responding to LPS (Waage and Bakke, 1988).

The interferons (IFN-α, β, γ) although not themselves produced by monocytes, have a prime role and influence on the activity and regulation of monokines and lymphokines. The α and β forms of IFN are produced by virally infected cells and can up-regulate the expression of MHC I products. IFNγ, derived from T cells can activate both MHC I and MHC II products which may have an important effect on T lymphocyte function. It is a main response of T cells to mitogenic stimulation and has been termed "macrophage activating factor". Activation of the IFNγ gene by T cells is directly signalled by changes in Ca^{++} and phosphatidyl inositol metabolism resulting from membrane perturbation and independent synthesis of other proteins (Croll *et al.*, 1987). IFN has also induced antigen-presenting activity in B cells when added concurrently with TNF. Monocytes which have been induced by IL-1 or LPS to produce PGE_2 show a decrease in IL-1 production when given IFNγ (Hawrylowicz and Unanue, 1988). Interleukins, interferons, cytokines and monokines can be

triggered by many factors and form a complex network of interaction with key cell types. This can be abrogated at many levels and ultimately lead to the suppression of immune response.

G. Suppression of Cellular Responses by Antigen

The cellular immune response is elicited and regulated by the cooperative interactions of various T cell subpopulations and macrophages. The level of responsiveness of the cellular arm of the immune system may be demonstrated *in vitro* by the polyclonal activation of T cells to blastogenesis by mitogens like PHA or CON A or mixed lymphocyte reactions (MLR) (Richter, 1968; Warren and Benzos, 1987; Sopori *et al.*, 1987; Suzuki and Sakane, 1988). PHA is a specific T-cell mitogen and requires cooperation between monocytes and T cells to induce their proliferative responses (Maizel *et al.*, 1979).

The humoral response refers to the production of antibody towards antigens and requires active communication among B cells, T cells and monocytes. Inactivation of one or more of these cell types may alter humoral responsiveness and lead to immunosuppression.

Viruses can cause immunosuppression by a variety of mechanisms. Immunosuppression can occur as a result of direct or indirect effects of the virus on

leukocyte populations. In the case of direct effects, virus may infect and destroy specific cells involved in immunity. Also viral components may be released which may interact directly with specific cells and affect accessory or effector functions (Petit *et al.*, 1988). Indirect effects can be produced by the release of mediators, hormones, prostaglandins, and complement. Inhibition of mediators can reduce cellular reactivity. The resultant immunosuppression may be confined to specific antigens that induced suppression or a more generalized suppression to a wide variety of antigens or stimuli (Reinherz and Schlossman, 1981).

Immunosuppression has been seen in many clinical states and seems to occur with infection from viral, bacterial and fungal agents. This includes clinical states resulting from *Hepatitis B* virus (HBV) (Kakumu *et al.*, 1980), *Herpes Simplex* virus-type 1 (HSV-1), *Bordetella pertussis* infection, toxoplasmosis resulting from *Toxoplasma gondii* (Luft *et al.*, 1988), *Mycobacterium ulcerans* (Pimsler *et al.*, 1988) and during Influenza (Roberts and Domurat, 1989) and measles infections (causative agent, genus *morbillivirus* of the family Paramyxoviridae) (Rouse and Horohov, 1986; Casali and Nakamura, 1989).

Influenza viral infection is common and a significant cause of morbidity and mortality from secondary bacterial infections. Research *in vivo* and *in vitro* has demonstrated that following influenza infection complex immune events lead to

suppression of non-virus specific responses (Kilbourne *et al.*, 1987). In addition to B cells and T_H cells with which they interact, other sub-populations of lymphocytes are involved. These include CTL, their precursors and their helpers. Also delayed hypersensitivity (DTH) lymphocytes (T_D) recognize influenza antigens and are in turn modulated by their helpers and suppressors.

Several groups have reported depression of mitogen- or antigen-stimulated human lymphocytes, proliferative responses and decreased DTH in patients with influenza infection. This is due to virus-induced alterations in peripheral blood monocyte accessory cell function which is required for such responses (Roberts *et al.*, 1980).

In cattle, during Bovine herpes virus (BHV) infection, virus can interact with monocytes which are good producers of IFN-α and PGE_2. These components are known to be able to depress the proliferation of T and B lymphocytes and have a negative effect on their effector functions. Similarly, BHV-1 can stimulate PMN to produce leukotrienes and reactive O_2^- molecules. Infection can also alter cell surface markers on MΦ or PMN such as FcR, complement and class II antigens. This may interfere with APC function. Specific effects of BHV-1 on T_S and T_H are not well characterized (Babiuk *et al.*, 1989).

Inhibition of antigen-induced and IL-2-induced proliferation of bovine peripheral blood leukocytes has been demonstrated using inactivated Bovine Herpes virus-1 (Hutchings *et al.*, 1990). Natural infection results in immunosuppression which predisposes the cattle to bacterial pneumonia (Filion *et al.*, 1983). The consequences of any modulation of the immune response are very complex since enhanced activity of one specific cell type may have a negative effect on another cell type.

The inability of certain individuals to mount an effective antibody response to HBV is well-documented and reviewed by Escobar (1989). Increase in suppressor T cell (T_s or $CD8^+$) function has been implicated, as many occurrences of depressed $CD4^+/CD8^+$ ratios have been seen in both fulminant and acute infection (Thomas *et al.*, 1982; Barnaba *et al.*, 1983). Soluble suppressor factors from the serum of hepatitis patients have also been identified and termed RIF (Rosette inhibitory factor), and SIF (Serum immunosuppressive factor) (Escobar, 1989).

In vitro examples of immunosuppression have been gathered from agents that have been associated with clinical immunosuppression. The envelope protein, gp120 from HIV-1 suppresses *in vitro* PHA-induced lymphocyte blastogenesis. Mann *et al.* (1987) showed that gp120 added to normal PBMC at the start of culture, along with PHA, was suppressive to the blastogenic response in a dose-dependent fashion. This

suppression was most effective when the viral protein was added at the same time as PHA. This group postulated that the suppressive effects were due to the specific binding of gp120 to the CD4 molecule on T cells which would serve as an "off" signal to the cell or perhaps would interfere with the association of MHC II and CD4.

In the late 1970's another retrovirus, feline leukemia virus was being studied for its immunosuppressive characteristics and a protein, P15e emerged as the suppressive agent. Mathes *et al.*, (1978) showed that this protein was highly suppressive to lymphocyte PHA blastogenic responses *in vitro* and established a correlation in vivo when immunization with this protein resulted in diminished PHA blast responses following immunization (Mathes *et al.*, 1979).

Immunosuppressive properties of a soluble toxin from *Mycobacterium ulcerans* (Pimsler *et al.*, 1988) have been studied since the lack of clinical response in infection is striking. A tiny colony of mycobacterium can cause widespread tissue necrosis well into the underlying fatty tissue of the host. This lack of response has been attributed to immunosuppression. Toxin added at culture initiation suppressed CON A proliferation in a dose-dependent manner. Toxin similarly suppressed the MLR reaction suggesting the possibility of a common mechanism of suppression of CON A and alloantigen-induced proliferation. The mechanism of action involving different populations of lymphocytes were not, however, examined.

Immunosuppression resulting from measles virus has been known since the early 1900's and was initially documented by Von Pirquet (1908) and Wainberg and Mills (1985). Vydelingum *et al.* (1989) have studied the mechanism of virus-mediated immune suppression *in vitro* by using a heat-inactivated form of measles virus. They showed that immunosuppression was not due to a cytopathic effect of virus. Cultures enriched for monocytes (adherent cells) demonstrated the strongest immunosuppressive effect suggesting that monocytes played a role in measles virus-induced immunosuppression.

Immunosuppression is not limited to viral components. The evidence for *Candida albicans* immunosuppression comes from *in vivo* and *in vitro* studies. Chronic candidiasis in humans has demonstrated a decrease in response to common skin-test antigens and a decreased *in vitro* response to mitogens. Furthermore, immunosuppressive serum factors and suppressor cells have been described in human chronic candidiasis. This suppression for mitogens can be induced *in vitro* by addition of soluble extracts from *Candida albicans*. Damle *et al.* (1987) have found that patients with candidiasis possess strong suppressor cell activity of both $CD4^+$ suppressor and $CD4^+$ inducer cells. Cuff *et al.* (1989) have demonstrated the induction of suppressor cells by a soluble extract from Candida. This extract was shown to induce suppressor cells which inhibit both primary and secondary anti-SRBC

responses in a dose-dependent manner. Both suppressor B cells and suppressor T cells have been implicated in their model.

Pertussis toxin (PT), the major toxin produced by *Bordetella pertussis* (bacteria) has been reported to suppress immune responsiveness (Arora *et al.*, 1987). PT is immunosuppressive to the primary and secondary *in vitro* CTL responses of mouse spleen cells. The suppression was dose-dependent, with 1 μg of PT being optimal. PT must be present during the initial stages of CTL generation (0-24 hr). Removal of PT from culture prior to 2 hr incubation was not sufficient to generate suppression. Finally, they concluded that the suppression was being mediated by L3T4$^+$, Ly2$^+$ suppressor T lymphocytes (Arora *et al.*, 1987).

Adherent MNC cells (enriched in monocytes) have been implicated in suppression of lymphocyte proliferation resulting from toxoplasmosis (causative agent, Toxoplasma gondii) or in vitro infection by Toxoplasma antigen (TA) (Luft *et al.*, 1988). Monocytes which were removed from a MNC population which had been treated with TA were suppressive to fresh autologous cultures. When these monocytes were removed from culture after one day, no suppression was seen; culturing for an 8-day period resulted in an optimal amount of suppression. Exposure of MΦ to TA in combination with CD4$^+$, CD8$^+$ or both together revealed that the

presence of $CD4^+$ cells and not $CD8^+$ cells was required for monocytes to become immunosuppressive.

The evidence for the involvement of $CD4^+$ cells in the induction of suppression is steadily accumulating. As early as 1982, it was shown that both $T4^+$ ($CD4^+$) and $T8^+$ ($CD8^+$) populations were necessary for the induction of suppression (Morimoto *et al.*, 1982). Communicative interactions between $CD4^+$ and $CD8^+$ were required for the induction of suppressor-effector function in an *in vitro* primary antidinitrophenol (DNP) antibody response. The effector cell of the suppression remained the $CD8^+$ cell; however, suppression could not be exerted without the initial induction by $CD4^+$ cells. Both the $CD4^+$ inducers and $CD8^+$ effectors were sensitive to low-dose irradiation.

The cellular and molecular basis for immunoregulatory function of $CD4^+$ cells was studied following autologous mixed lymphocyte reaction (AMLR) (Takeuchi *et al.*, 1987). $CD4^+$ cells were separated on the basis of expression of 2H4 antigen (now termed CD45R). The AMLR preferentially stimulated this population. Furthermore, the $CD4^+$ population activated by AMLR exerted suppressor activity on PWM-stimulated IgG synthesis of fresh autologous PBMC.

The $CD4^+$ $CD45R^+$ population induces suppression through the $CD8^+$ population. Monoclonal antibodies binding to 2H4 abrogated the suppression, suggesting that the 2H4 molecule itself may be involved in the suppressor-inducer function. Small numbers of $CD4^+$ $CD45R^+$ cells were required to exert suppression and the suppression was dose-dependent.

The increased ability of AMLR-activated cells to induce suppression may be due to a quantitative change in the number of CD45R polypeptides present on the surface of $CD4^+$ $CD45R^+$ cells. Further dissection of immunoregulatory pathways and characterization of immunoregulatory molecules may provide new insights into immunoregulation. Since T cell activation by self-MHC may lead to the generation of a predominantly suppressive immunoregulatory network, the role of this circuit in self-non-self recognition may be of great importance. Perturbation of the $CD4^+CD45R^+$ subset could lead to serious systemic consequences such as autoreactivity and tissue destruction, in addition to suppression.

In vivo modulation of antigen presentation has been shown to generate T_S rather than T_{DH} in herpes simplex type 1 (HSV-1) infection (Howie *et al.*, 1987). The nature of initial antigen presentation to the specific cells of the immune system determines whether or not a positive immune response or suppression of immunity will be generated. These researchers were able to induce T_S cells for the normal DH

response to HSV-1, by UV-B irradiation of APC. They determined that the suppression is T-cell-mediated and the T_S cell induced is of a T_H1^+, $L3T4^+$, $Ly2^-$ phenotype ($CD4^+$ analog in humans).

Immunosuppression seen with HSV-1 and HSV-2 seems to activate a suppressor cell circuit and demonstrates specific and non-specific components. The question arises as to whether all HSV proteins can raise T_S cells or just particular ones are involved. This has major implications in vaccine production since it is not known how those "suppressor" epitopes differ from those that induce protective immunity. This may have broader implications regarding antigens in general.

Vaccination with hepatitis surface antigen (HBsAg) or tetanus toxoid has been shown to suppress *in vitro* responses of PBMC (Filion *et al.*, 1989). In addition HBsAg-specific responses were shown to be suppressed *in vivo* (Filion *et al.*, 1988). *In vitro* exposure to HBsAg suppressed the PHA blastogenic responses of PBMC, closely mimicking the effect observed through vaccination. The *in vivo* suppression was partially mediated by a $CD4^+$ and/or $CD8^+$ T lymphocyte.

II. OBJECTIVES

Previously, this laboratory has shown that human vaccination with hepatitis surface antigen (HBsAg) resulted in temporary immunosuppression of the PBMC responses to PHA and PWM *in vitro* soon after vaccination (Filion and Saginur, 1988; Filion *et al.*, 1989) and following each booster vaccination. Furthermore, administration of HBsAg *in vitro* to PBMC from both immune (but without circulating Ab) and non-immune donors during mitogenic stimulation was itself immunosuppressive. It was reasoned that the antigen might be triggering a suppressive regulatory pathway that could have clinical significance.

The purpose of this thesis was (1) to determine if tetanus toxoid, a bacterial antigen commonly employed in vaccination, could suppress the PHA and PWM blastogenic responses *in vitro*, and if so (2) to study the mechanism(s) involved in the generation of that suppression.

III. MATERIALS

A. Materials Used in Culture and Isolation of PBMC

CULTURE MEDIUM - RPMI-1640 powder containing L-glutamine was obtained from Gibco Laboratories, Toronto, Ontario, and prepared according to manufacturer's instructions. The medium was prepared in 10 litre batches and the pH was adjusted to 7.2; it was filter sterilized and aliquoted. Prior to use the medium was enriched with 20 % fetal bovine serum (FBS), 25 mM HEPES buffer, 4 mM L-glutamine, gentamicin (8 μg/ml), and amphotericin B (5 μg/ml).

FETAL BOVINE SERUM (FBS) - (heat-inactivated) was obtained from Gibco Laboratories, Toronto, Ontario.

FICOLL - Ficoll Hypaque was obtained from Pharmacia Fine Chemicals AB, Upsala, Sweden.

GARAMICIN - (40 mg/ml) Gentamicin, 2 ml vials were obtained from Schering Inc., Pointe Claire, Quebec.

HEPARIN - 1000 U.S.P. units/ml, 30 ml vials were obtained from Organon Canada Ltd., Toronto, Ontario.

HEPES BUFFER - was obtained from Gibco, Toronto, Ontario.

OKT4 and OKT8 - were used in the selective isolation of $CD4^+$ and $CD8^+$ subpopulations of cells. The monoclonals were obtained from Ortho Pharmaceuticals, Toronto, Ontario.

PBS - (0.01 M) was prepared from 1.7 g Na_2HPO_4 dibasic, anhydrous (Sigma)+ 0.35 g $NaH_2PO_4 \bullet H_2O$ monobasic (Sigma)+ 11.25 g NaCl (Sigma), brought up to 1 litre with distilled, deionized water; pH was adjusted to 7.2.

PHYTOHEMAGGLUTININ - Vials of lyophilized phytohemagglutinin were obtained from Gibco Laboratories, Toronto, Ontario.

POKEWEED MITOGEN - Vials of lyophilized pokeweed mitogen were obtained from Gibco Laboratories, Toronto, Ontario.

RABBIT COMPLEMENT (low tox) - was used in the selective killing of $CD4^+$ and $CD8^+$ subpopulations of cells. Complement was obtained from Cedarlane Labs, Whitby, Ontario.

WATER SOURCE - distilled water was passed through the NANOPURE water filtration system.

B. Reagents Added During Culture as Part of Experimental Protocol

INDOMETHACIN - was obtained from Sigma Chemical Co., St. Louis, Missouri.

INTERLEUKIN-2 & Recombinant TNF - Human Ultrapure IL-2 and recombinant TNF were obtained from Genzyme, Boston, Massachussetts.

TETANUS TOXOID (Adjuvant-free) - was obtained from Institute Armand Frappier, Laval, Quebec.

C. Reagents Used in Enzyme-linked Immunosorbent assay

ABTS - 2,2-azino-di-[3-ethylbenzethiozolsulfonate] - was obtained from Fisher Scientific, Orangeburg, New York.

CITRATE BUFFER - was prepared from 1.11 g citrate (Fisher), 0.117 g Na_2HPO_4 dibasic (Sigma, St. Louis, Missouri) dissolved up to 100 ml H_2O. The pH was adjusted to 4.2 with NaOH.

GOAT ANTI-IgG or ANTI-IgM (Affinity purified) - were obtained affinity-purified from Intermedico, Toronto, Ontario.

GOAT ANTI-HUMAN IgG or IgM-HORSERADISH PEROXIDASE (Affinity-purified) - were obtained from Intermedico, Toronto, Ontario.

IgG or IgM (Affinity-purified) - were obtained from Intermedico, Toronto, Ontario. They were used as standards for the assay.

HYDROGEN PEROXIDE - was obtained from Fisher Scientific, Orangeburg, New York.

POLYETHYLENE GLYCOL - (1%), was obtained from Fisher Scientific, Orangeburg, New York.

SUBSTRATE - was prepared for each ELISA plate in the following ratio: 5 ml citrate buffer, 5 ml H_2O, 5 μl H_2O_2, 2 mg ABTS.

D. Reagents Used in Staining of Lymphocytes

CD4-FITC, CD8-FITC, CD25-FITC - were obtained from Becton Dickinson, Toronto, Ontario.

PARAFORMALDEHYDE - was obtained from Fisher Scientific, Orangeburg, New York, and prepared as a 1% solution.

PHYCOERYTHRIN-STREPTAVIDIN (PE-SA) cytochemical staining kit- catalog no. RPN.1172, was obtained from Amersham, Oakville, Ontario.

SODIUM AZIDE - 100 g bottles were obtained from Fisher Scientific, Orangeburg, New York.

TRYPAN BLUE - was obtained from Gibco, Toronto, Ontario.

E. Reagents Used in Assessing Blastogenesis

TRITIATED THYMIDINE - purchased at 5.0 Ci/mmol from New England Nuclear, Boston, Massachussetts.

F. General Supplies

CONICAL TUBES - 50 ml graduated, tissue culture grade, Falcon, Fisher Scientific, Orangeburg, New York.

CULTURE TUBES - 5 ml (12x75 mm) Falcon 2003 and 15 ml (17x100 mm) Falcon 20 ml round-bottomed polystyrene tubes were obtained from Becton Dickinson, Lincoln Park, N.Y.

CULTURE PLATES - 12-well tissue culture grade plates were obtained from Corning Laboratories, Corning, New York.

ELISA PLATES - 96-well, Immulon II Elisa Plates, were obtained from Fisher Scientific, Ottawa, Ontario.

STERILE PIPETTE TIPS - (250 μl, 1000 μl) were obtained from Biorad, Toronto, Ontario.

EPPENDORPH TUBES (1.5 ml) - were obtained from Fisher Scientific, Orangeburg, New York.

MICROSCOPE SLIDES (25x75 mm) - frosted at one end were obtained from Corning Glassworks, Corning, New York.

MICROTITRE CULTURE PLATES - flat-bottomed, Falcon Microtest III, Fisher Scientific, Ottawa, Ontario.

NALGENE FILTERS (0.22 μm) - were obtained from Nalgene Labware, Rochester, New York.

NEEDLES (18, 22 gauge, 1") -were obtained from Becton Dickinson Labware, Oxnard, California.

PIPETTES - 1, 2, 5, and 10 ml polystyrene sterile disposable serological pipettes were obtained from Fisher Scientific, Orangeburg, New York.

SYRINGES - 1, 10, and 60 ml plastiplate sterile disposable syringes were obtained from Becton Dickinson Labware, Oxnard, California.

G. EQUIPMENT

CELL HARVESTER - cultures were harvested in a Skatron Cell Harvester, Mandel Scientific, Toronto, Ontario.

CO2 INCUBATOR - cultures were established in a NAPCO 6300, CO_2 incubator set at 37°C and 5% CO_2.

CENTRIFUGE - refrigerated centrifuge, model IEC CENTRA-7R by International Equipment Company (obtained through Fisher Scientific, Ottawa, Ontario).

ELISA PLATE READER - the BIO-TEK EL/310 ELISA plate reader, was obtained through Mandel Scientific, Toronto, Ontario.

ELISA PLATE WASHER - the SKATRON II MICROWASH, was obtained through Mandel Scientific, Toronto, Ontario.

FLUORESCENT MICROSCOPE - C. Zeiss, Oberkochen, Germany.

GILSON PIPETTEMAN - (100 μl, 200 μl and 1000 μl) were obtained from Mandel Scientific, Toronto, Ontario.

LIGHT MICROSCOPE - BH-2 Olympus microscope, Japan.

LIQUID SCINTILLATION COUNTER - Beckman LS3801, Toronto, Ontario.

TISSUE CULTURE HOOD - the BioGARD HOOD by the Baker Company Inc., Sanford, Maine.

IV. METHODS

A. BLOOD SUPPLY

Human peripheral blood was obtained from normal healthy laboratory volunteers by venipuncture, or from buffy coat obtained from the Ottawa chapter of the Canadian Red Cross Society (unscreened blood).

B. ISOLATION OF CELLS

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation of blood on Ficoll-Hypaque gradient at 400 G for 30 min. The cells at the interface were collected and washed with PBS at 300 G once and at 200 G twice. The cells were suspended at a concentration of 10^6 cells per ml in culture medium as described in Materials section.

C. OPTIMIZATION OF CULTURE CONDITIONS FOR BLASTOGENIC RESPONSES INDUCED BY PHA AND PWM

The blastogenic assays were performed using various PBMC concentrations ($1x10^5$, $2x10^5$, $3x10^5$,$4x10^5$/well) and PHA concentrations (0.065 - 0.26 μg/ml) to determine an optimal proliferative response. Similarly, the blastogenic response induced by PWM was optimized by using various concentrations of PBMC ($0.5x10^5$,

$1x10^5$, $2x10^5$, $4x10^5$/well), and PWM (.013 - 0.26 μg/ml). The time of culture was also assessed.

D. Stimulation Using Monoclonal Antibody

Stimulation of PBMC with the mAbs anti-CD3, anti-CD4 or anti-CD8 was performed as follows. PBMC were incubated with an optimal dilution of appropriate monoclonal for 1 hr at 37°C and 5% CO_2 (previously determined from dose responses). The cells were washed with RPMI three times and resuspended in culture medium. Triplicate cultures of $2x10^5$ PBMC were established in microtitre plates and incubated at 37°C and 5% CO_2 for 3 days for assessment of blastogenic response using ^{3}H-Thymidine incorporation.

E. Thymidine Incorporation Assay

The cultures were pulsed with 1 μCi of tritiated thymidine (^{3}H-Thymidine) 18-24 hours prior to culture termination and harvested with the Skatron Cell Harvester. The radioactivity was determined in a liquid scintillation counter.

F. ELISA

The IgM and IgG levels in culture supernatant were measured by an ELISA performed in the following manner: affinity-purified goat anti-human IgM or anti-human IgG (1 μg/ml in PBS) was dispensed into Immulon II ELISA plates and

incubated overnight at 4°C. The plates were washed with 0.1% PBS-Tween 20 using the Skatron II Microwash. Dilutions of IgM or IgG standards or culture supernatant were added. These plates were incubated at room temperature for 2 hr and washed. Goat anti-human IgM-horseradish peroxidase (anti-hIgM-HRPO) or goat anti-human IgG-horseradish peroxidase (anti-hIgG-HRPO) was added (1:1600 dilution) and incubated for 1 hr at room temperature. The plates were washed and 100 μl substrate (ABTS and hydrogen peroxide (0.1%)) in citrate buffer were added. The plates were read at 405/490 nm employing the BIO-TEK EL/310 ELISA plate reader.

The standard curves relating IgM and IgG concentration and optical density were plotted. The concentrations of IgM and IgG in the culture supernatant were calculated from the optical density of the 100 μl culture supernatant sample.

G. Suppression of the Mitogenic Response by TT

Suppression of the mitogenic responses was achieved by the addition of TT at varying concentrations (1-5 μg/ml) to culture as listed in Results section. The TT was either present throughout culture or removed by washing at times indicated.

II. Additions To Culture

Human ULTRAPURE IL-2 , recombinant Tumor Necrosis factor (rTNF) or indomethacin was used at various concentrations: IL-2, 1 to 50 units per culture; rTNF, 1 to 100 ng/culture and indomethacin at 0.5 to 4 μM/culture.

I. Enumeration of Cell Surface Markers

1. *Staining of Cells with a Single Fluorochrome*

The number of cells with CD4, CD8 or IL-2 receptor (CD25) was assessed using a fluorescent antibody assay. Briefly, 10^6 PBMC were incubated with 0.01 μg of anti-CD4, anti-CD8 or anti-CD25 for 30 min on ice in culture medium containing 0.1% Na azide. The cells were washed once with cold PBS containing 0.1% Na azide, and 100 μl affinity-purified goat anti-mouse IgG-FITC (1:20 dilution) was added to the cells for an additional 30 min at 4°C. The cells were rewashed with PBS-Na azide, and fixed with 0.05% paraformaldehyde for 10 minutes. The cells were washed in PBS-Na azide and resuspended to 100 μl. A 10 μl smear was made and air-dried in the dark. A drop of fluorescence mounting fluid, NPG (n-propyl-gallate 5% (w/v) in glycerol) was applied prior to viewing with a fluorescent microscope at 40x magnification. Cells were counted in a 4 fields totalling 100 cells.

2. *Staining of Cells with two Fluorochromes Simultaneously*

The number of cells expressing both CD4 or CD8 and IL-2 receptor (CD25) was assessed using a double fluorescence technique. PBMC ($1x10^6$) were exposed to 0.01 μg of anti-CD4-FITC, or anti-CD8-FITC for 30 min on ice in culture medium containing 0.1% Na azide. The cells were washed once with cold 0.1% Na azide-PBS. The Phycoerythrin-streptavidin cytochemical staining kit was used to stain the second marker and kit instructions were followed. Anti-CD25-biotin was incubated with the cells for 1 hr on ice in Na azide culture medium. The streptavidin-PE complex, (1:50) was added and kept on ice for 40 min. The cells were washed in 0.1% PBS-Na azide and fixed with 0.05% paraformaldehyde. The cells were washed and mounted on slides and air-dried. The stabilizer compound was applied (20 minutes), decanted and the slides air-dried. A drop of the mountant was placed under a coverslip prior to viewing on the fluorescent microscope at 40x magnification. Cells were counted in 4 fields toalling 100 cells.

J. Enrichment of $CD4^+$ and $CD8^+$ Cells

Enrichment of $CD4^+$ and $CD8^+$ populations of cells was achieved by incubating 10^6 PBMC with 0.01 μg of anti-CD4 or anti-CD8 monoclonal antibodies, on ice for 1 hour. Rabbit complement was added and the mixture was heated in a 37°C water bath for an additional hour. Cell viability was assessed using the trypan

blue dye exclusion method. The treated cells were washed 3 times in sterile PBS and resuspended in culture medium prior to plating.

K. Assessment of Cell Viability

Viable cell counts were performed on cell culture samples at various points during the experiments using trypan blue dye exclusion. A 50 μl sample of cell suspension was mixed with 20 μl trypan blue and 10 μl was loaded onto a hemocytometer. Cells were viewed under 10x magnification and a viable cell count was taken. Viable cells did not take up the dye; non-viable ones were stained blue.

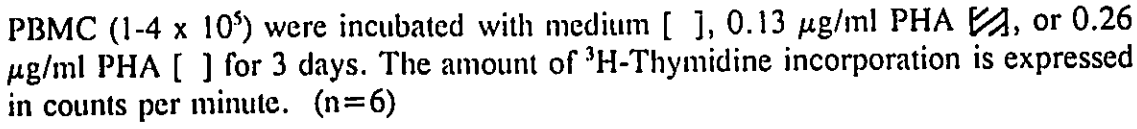
V. RESULTS

A. Culture Conditions for Mitogenic Stimulation

The stimulation by PHA, PWM, and monoclonal anti-CD3 and anti-CD4 stimulation was determined from the experiments reported in Figures 1 to 4. The optimal PHA response was determined by varying the cell concentration and mitogen dose in 3-day culture (Figure 1), as measured by ^{3}H-Thymidine incorporation. A pre-plateau of this response was observed when $2x10^{5}$ cells were cultured with a concentration of 0.13 μg/ml PHA. These conditions were chosen so that the variability in the response due to fluctuations in cell number was limited, while minimizing the amount of cells necessary to obtain a sufficient blastogenic response.

The PWM response is reported in Figure 2 & 3. Figure 2 shows the results of the IgG and IgM response of the cultures with varying cell concentration and PWM dilution during a 7-day incubation period. Stimulation of $4x10^{5}$ cells showed the highest response at all concentrations of PWM used. A maximal IgG and IgM response was observed at a 0.30 μg/ml concentration of PWM (Figure 2). The optimal culture time was assessed using $4x10^{5}$ cells in culture and incubating 3, 5, and 7 days (Figure 3). The concentration of PWM was varied as above to verify optimal combination of time and mitogen concentration. Figure 3 shows that a 7-day

FIGURE 1. PHA DOSE RESPONSE.



PBMC (1-4 x 10^5) were incubated with medium [ ], 0.13 μg/ml PHA [▨], or 0.26 μg/ml PHA [ ] for 3 days. The amount of ^{3}H-Thymidine incorporation is expressed in counts per minute. (n=6)

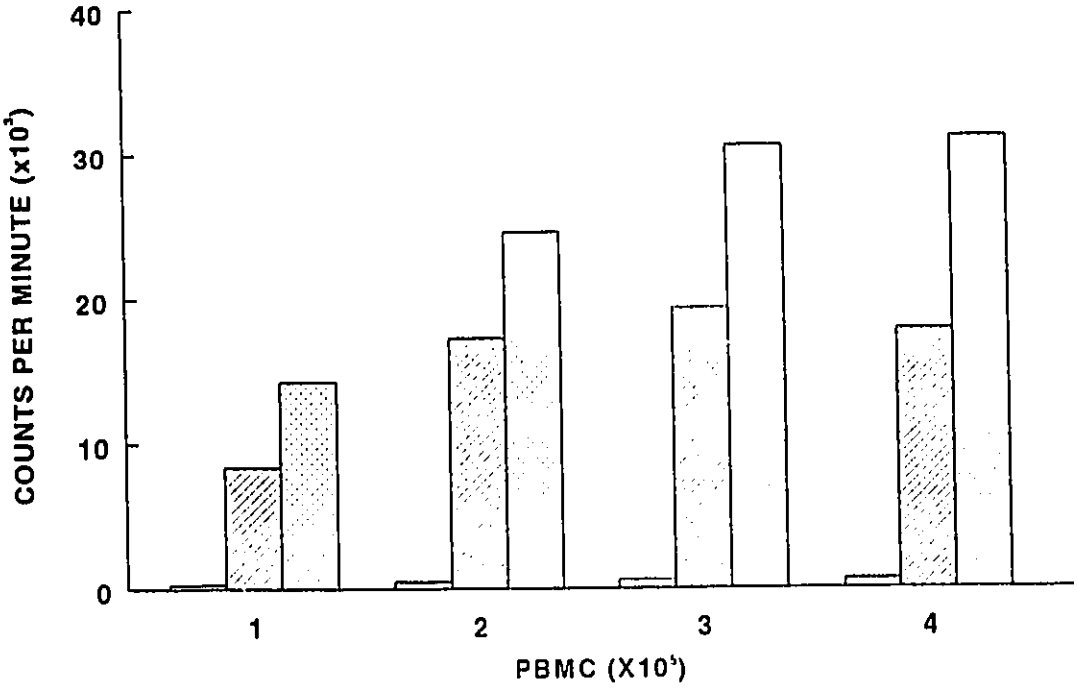

40
30
20
10
0
COUNTS PER MINUTE (x10³)
1
2
3
4
PBMC (X10⁵)

FIGURE 2. PWM-INDUCED IgG AND IgM PRODUCTION. EFFECT OF CELL CONCENTRATION AND PWM DILUTION.

PBMC ($4x10^5$ [ ], $2x10^5$ [::], $1x10^5$ [▨]) were incubated with medium (CON), or various doses of PWM (.15-1.2 μg/ml) for 7 days. A 100 μl aliquot of supernatant was taken and the amount of IgG or IgM was assessed using an ELISA. Immunoglobulin is expressed in ng/ml. (n=4)

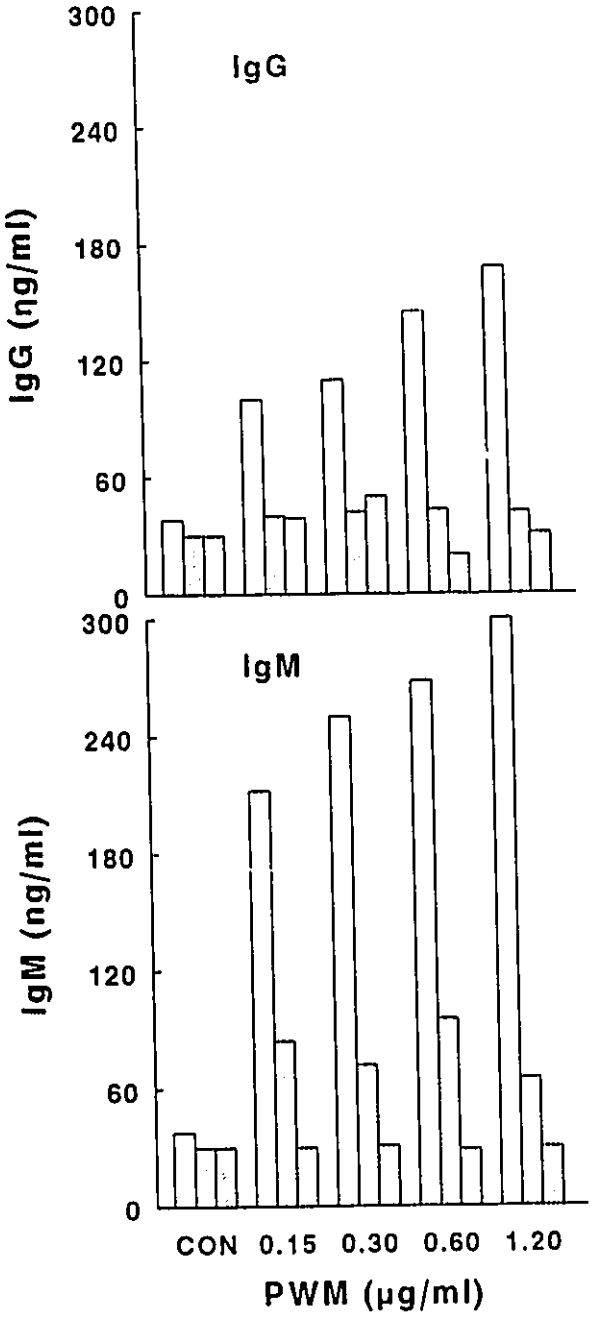
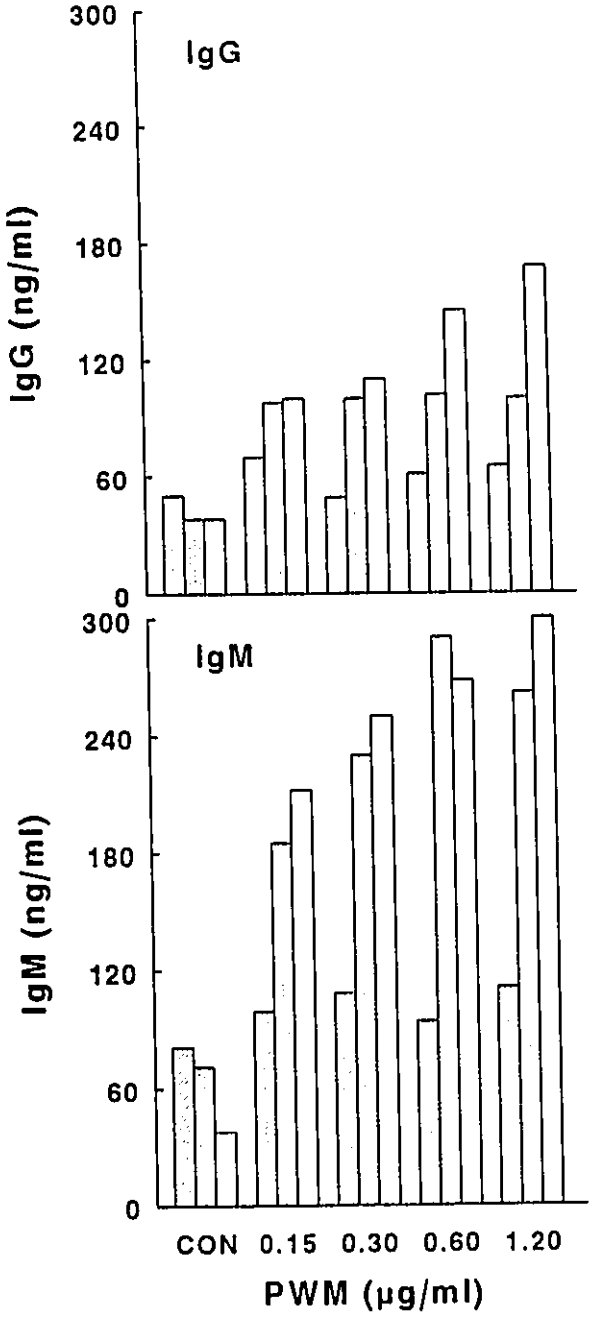

IgG
IgG (ng/ml)
300
240
180
120
60
0
IgM
IgM (ng/ml)
300
240
180
120
60
0
CON
0.15
0.30
0.60
1.20
PWM (μg/ml)

FIGURE 3. PWM-INDUCED IGG AND IGM PRODUCTION. EFFECT OF PWM DOSE AND DURATION.

PBMC ($4x10^5$) were incubated with media (CON), or various doses of PWM (.15-1.2 μg/ml) for 3 [▨], 5 [::], and 7 [ ] days. A 100 μl aliquot of supernatant was taken and the amount of IgG was assessed using an ELISA. Immunoglobulin is expressed in ng/ml. (n=5)


IgG
IgG (ng/ml)
300
240
180
120
60
0
IgM
IgM (ng/ml)
300
240
180
120
60
0
CON
0.15
0.30
0.60
1.20
PWM (μg/ml)

incubation with 0.30 μg/ml PWM is required for a maximum response. These culture conditions were used in all future experiments in which immunoglobulin production resulting from PWM stimulation was assessed.

Figure 4 summarizes the results of monoclonal stimulation of PBMC by anti-CD3, anti-CD4 and anti-CD8 as measured by ^{3}H-Thymidine incorporation. The anti-CD3 was more effective for stimulation than was anti-CD4. The anti-CD8 monoclonal did not stimulate PBMC's.

B. Suppression of Mitogenic Responses by TT

The suppression of the PHA and PWM mitogenic responses by Tetanus Toxoid (TT) was achieved by the addition of 1-5 μg/ml TT to PBMC at the time of culture initiation. The effect of soluble TT on the PHA response is expressed in terms of percent (%) suppression in Figure 5. The PHA blastogenic response was depressed significantly (by approximately 60%) with a dose of 2 μg of TT, while 1 μg affected the response in only a marginal fashion. Higher doses of antigen (3 - 5 μg) severely or completely depressed the response.

Similar suppression by TT was observed in the induction of immunoglobulin synthesis by PWM, as determined by an ELISA. Resulting supernatant IgG and IgM concentrations were determined by extrapolation from optical densities of IgG and

FIGURE 4. BLASTOGENIC RESPONSE RESULTING FROM MONOCLONAL ANTI-CD4, ANTI-CD3, AND ANTI-CD8 STIMULATION.

PBMC ($2x10^5$ cells) were cultured with medium (CON), anti-CD4, anti-CD3 or anti-CD8 mAb for 3 days. The amount of ^{3}H-Thymidine incorporation is expressed in counts per minute. (n=12)

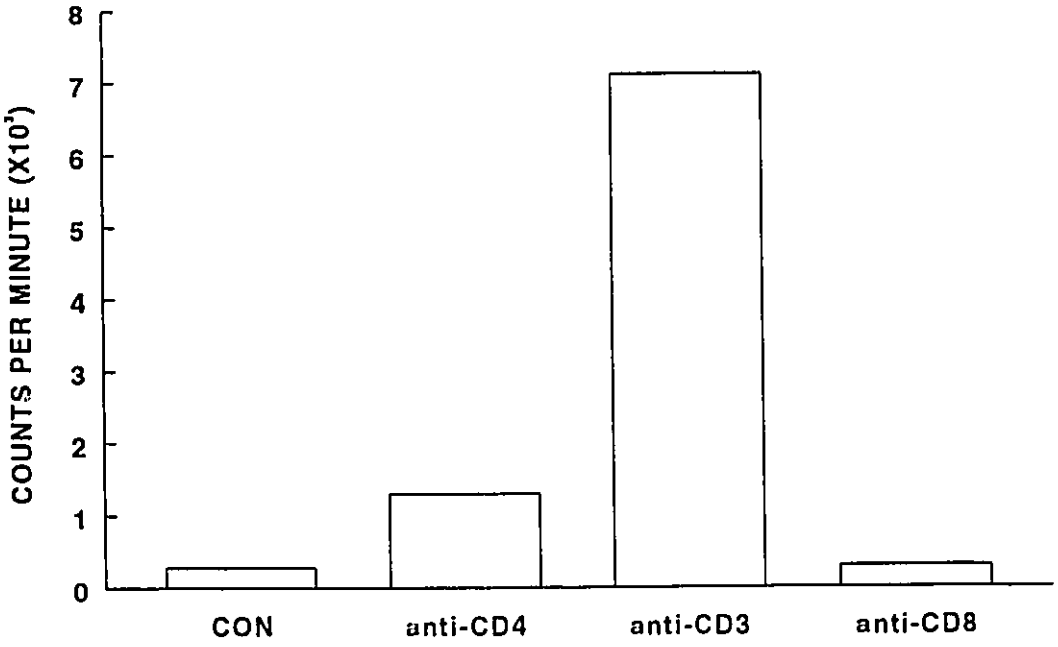
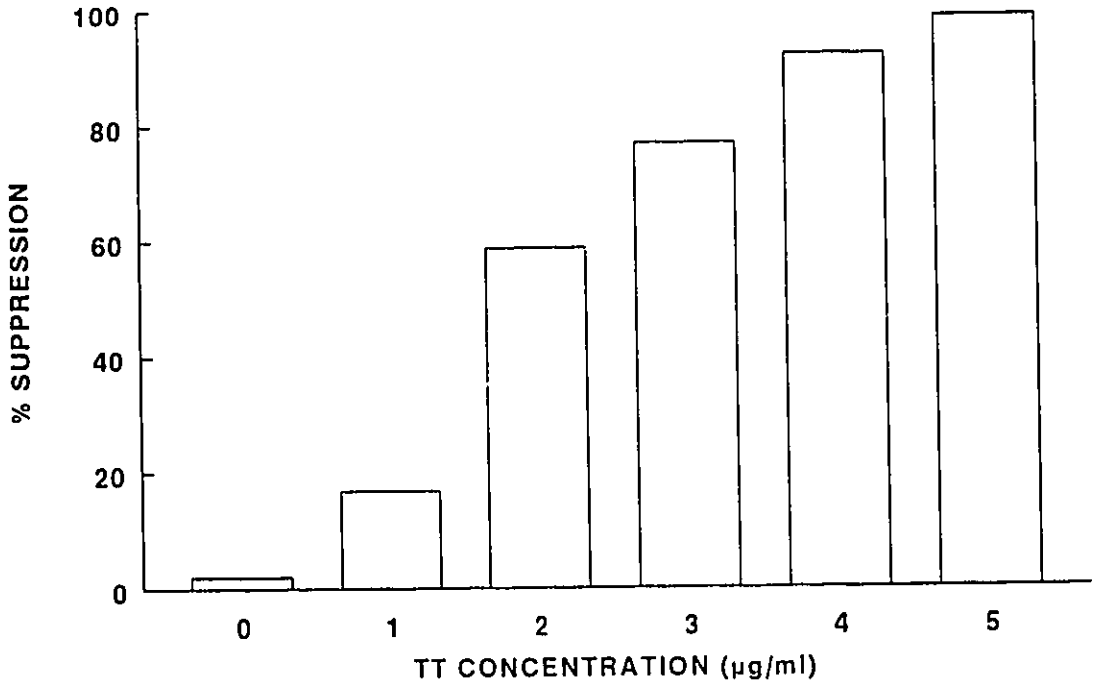

COUNTS PER MINUTE (X10³)
8
7
6
5
4
3
2
1
0
CON
anti-CD4
anti-CD3
anti-CD8

FIGURE 5. SUPPRESSION OF THE PHA BLASTOGENIC RESPONSE BY TT.

PBMC ($2x10^5$ cells) were incubated with various amounts (0-5 μg/ml) of TT. They were stimulated with PHA or given medium for 3 days. The amount of ^{3}H-Thymidine incorporated by unstimulated and stimulated cultures without antigen was 348 and 24,675 counts per minute (CPM) respectively. The CPM of unstimulated, TT-treated cultures was 312 or less. Calculation of % suppression was performed as follows (n=40):

$$\frac{1-[(\text{CPM of cultures}+\text{TT}+\text{PHA})-(\text{CPM of cultures}+\text{TT-PHA})]}{(\text{CPM of cultures}+\text{PHA})-\text{CPM of cultures}+\text{TT}+\text{PHA})} \times 100$$


100
80
60
40
20
0
% SUPPRESSION
0
1
2
3
4
5
TT CONCENTRATION (µg/ml)

IgM standards. The suppression of PWM responses by TT was monitored in the resulting 7-day immunoglobulin production and expressed in terms of % suppression of maximal Ig production. As shown in Figure 6, both the IgM and IgG responses were depressed in a dose-dependent manner (up to a maximum at 4 μg/ml) when the TT was added to the culture. TT was equally suppressive of IgG and IgM synthesis. at the concentrations of TT used. At 1 or 2 μg/ml TT, suppression was 10% and 75% respectively, at 4 μg/ml TT , suppression was complete.

Experiments were performed to determine if TT could also suppress the anti-CD3 or anti-CD4 blastogenic responses. These mAb stimulate T cell proliferation by cross-linking effects on the TCR (Emmrich, 1988, Rudd, 1990). Figure 7 shows both anti-CD3 and anti-CD4 responses being strongly suppressed by 2 μg/ml TT.

The suppression of PHA responses by TT may have been due to binding of TT on the TCR. A monoclonal, anti-CD3 or anti-CD4 was incubated with PBMC and TT (2 μg/ml), and stimulated with PHA. The results of this experiment (Figure 8) are expressed in terms of % suppression. Binding of the TCR area by anti-CD4 was itself slightly suppressive to the PHA response, but no change in the relative % suppression in the TT could be detected. The binding with anti-CD3 had even less effect on the PHA response or on the resulting suppression induced by TT.

FIGURE 6. SUPPRESSION OF PWM-INDUCED IGG AND IGM SYNTHESIS BY TETANUS TOXOID.

PBMC ($4x10^5$ cells) were incubated with various concentrations, (1-5 μg/ml) of TT, in the presence or absence of PWM. Supernatant IgG and IgM immunoglobulin was measured 7 days later. The concentrations of IgG and IgM of the unstimulated or stimulated cultures without TT were 15 and 10, or 80 and 280 ng/ml respectively. The calculation of % suppression was performed as follows: (n=6)

$$\frac{1-[(\text{IgG conc. of cultures}+\text{TT}+\text{PWM})-(\text{IgG conc. of cultures}+\text{TT-PWM})]}{(\text{IgG conc. of cultures}+\text{PWM})-(\text{IgG conc. of cultures}+\text{TT-PWM})} \times 100$$

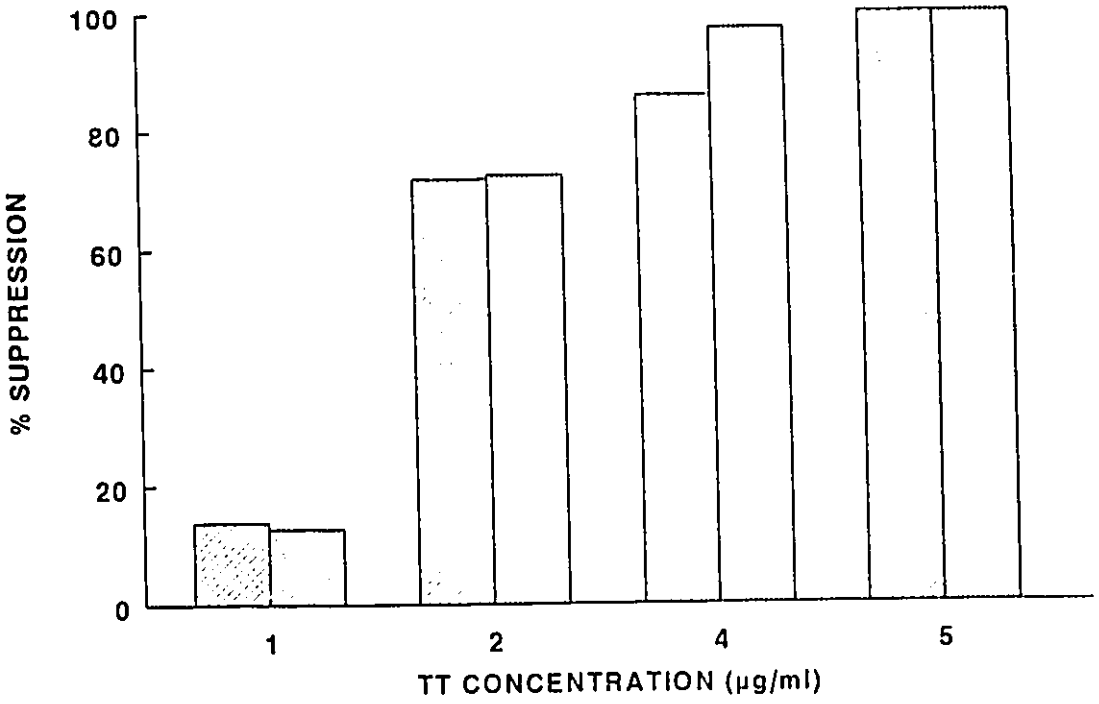
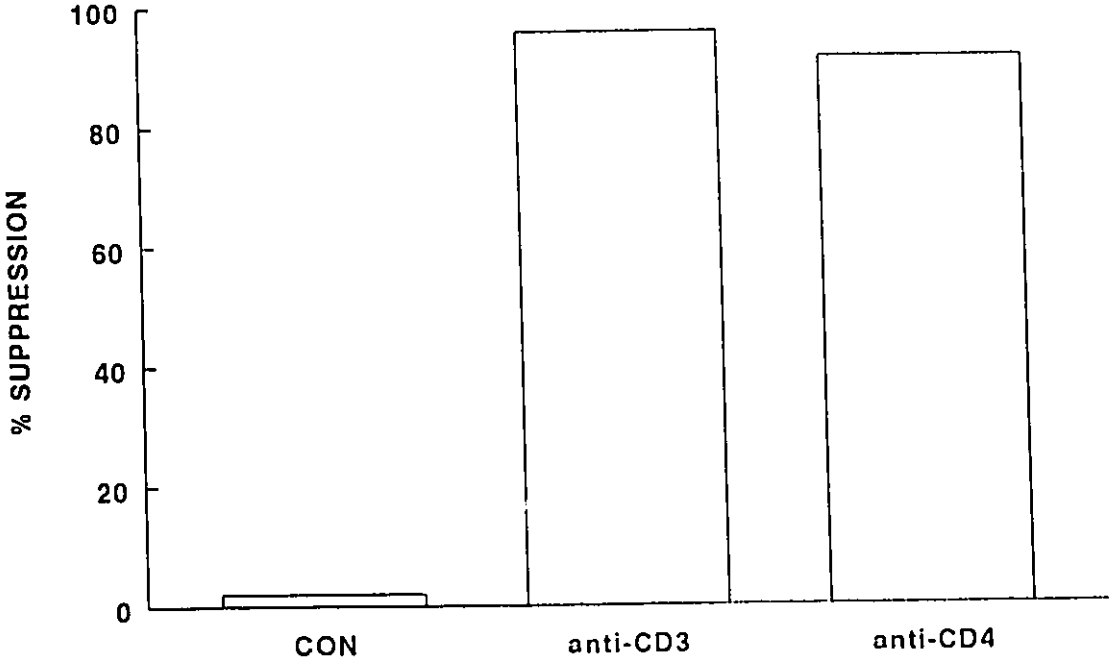

100
80
60
40
20
0
% SUPPRESSION
1
2
4
5
TT CONCENTRATION (µg/ml)

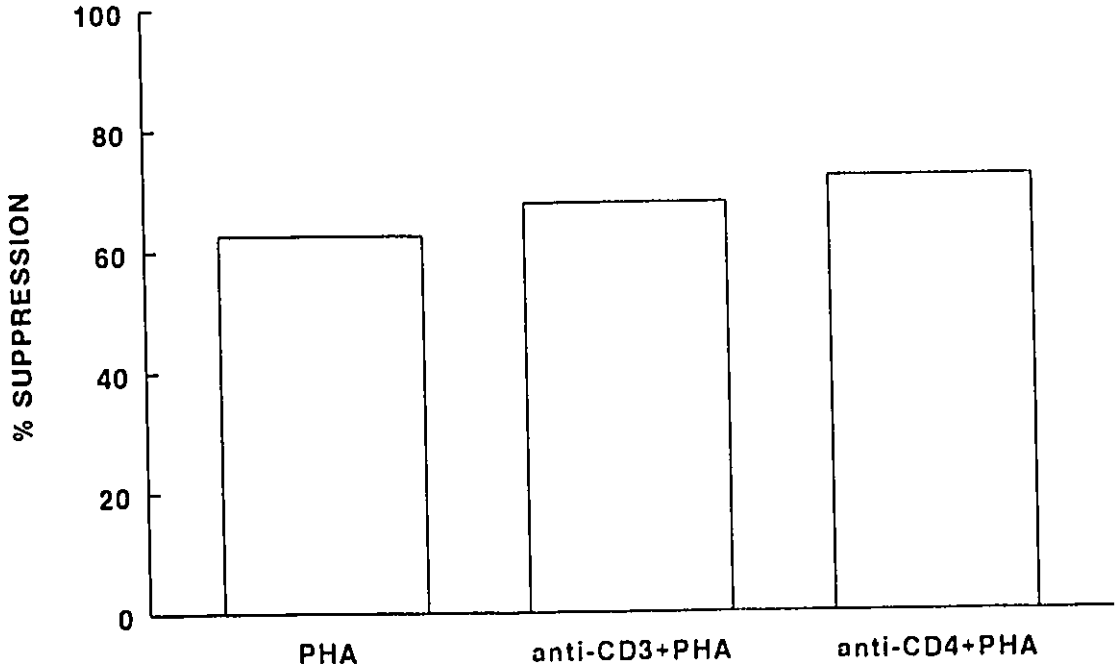
FIGURE 7. SUPPRESSION OF MONOCLONAL ANTIBODY RESPONSES BY TT.

PBMC ($2x10^5$ cells) were cultured with medium, anti-CD3, or anti-CD4 in the presence or absence (CON) of TT for 3 days. The amount of ^{3}H-Thymidine incorporation was measured. Percent suppression of the anti-CD3 and anti-CD4 response was performed as described in figure 5. (n=9)

FIGURE 8. CO-STIMULATION BY MONOCLONALS AND PHA SUPPRESSED BY TT.

PBMC ($2x10^5$ cells) were cultured with medium+PHA, anti-CD3+PHA, or anti-CD4+PHA in the presence or absence of TT for 3 days. The amount of ^{3}H-Thymidine incorporation was measured. Controls containing no PHA and/or no TT were also performed and were below 300 CPM. The calculation of percent suppression was performed as outlined in Figure 5. Suppression of the anti-CD3+PHA and anti-CD4+PHA responses were compared to the suppression of the PHA response. (n=8)


100
80
60
40
20
0
% SUPPRESSION
CON
anti-CD3
anti-CD4


100
80
60
40
20
0
% SUPPRESSION
PHA
anti-CD3+PHA
anti-CD4+PHA

C. Studies on the Mechanism of Suppression Induced by TT

A series of experiments was performed to determine if the TT-induced suppression was due to toxicity of the antigen preparation. If TT were toxic, the incubation of PBMC with it for even a short period of time would be lethal. In addition, the kinetics of induction of suppression would not vary in time if the TT was simply toxic.

PBMC ($2x10^6$) were cultured in the presence or absence of TT (2 μg/ml) for 1, 24, 72 or 120 hr. The percentage of viable cells in each culture was determined by trypan blue dye exclusion. The cells were later washed and stimulated with PHA. TT was not shown to be toxic to the cells. Cell death due to toxicity could not explain the depressed blastogenic response since no difference in cell viability between cells incubated in the presence or absence of TT could be observed over the 120 hr period (Table 1).

TABLE 1. VIABILITY FOLLOWING TT TREATMENT

TIME OF INCUBATION (hours)	% VIABLE CELLS	
	MED	TT
1	99	95
24	76	80
72	71	67
120	54	49

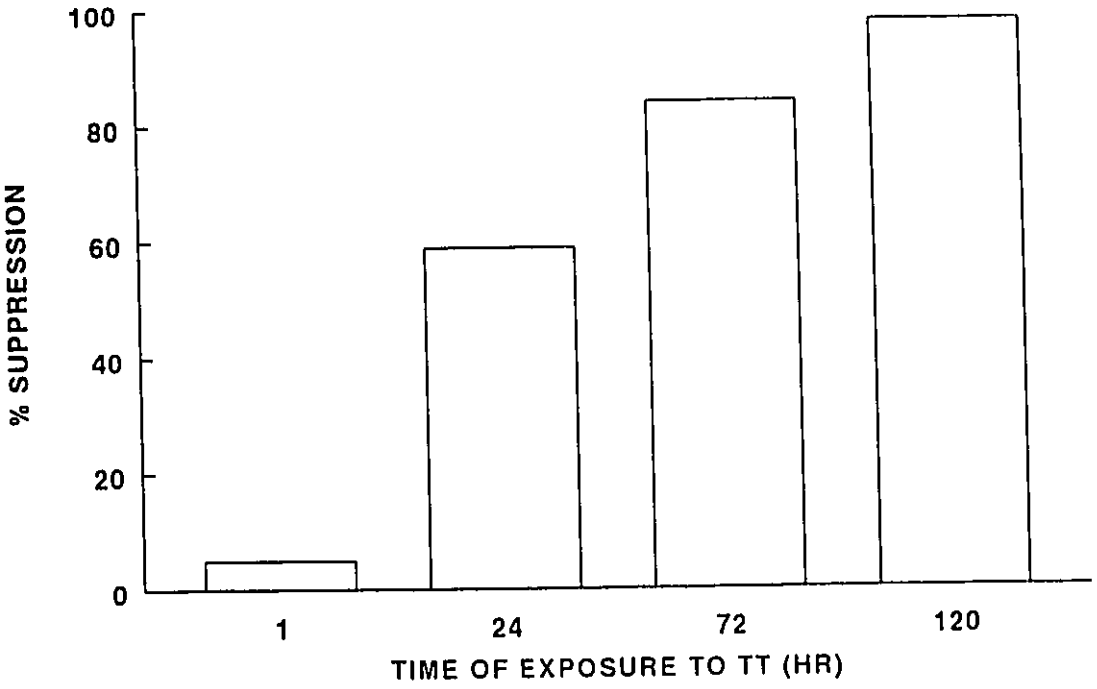
The kinetics of suppression of the blastogenic response following TT treatment are reported in Figure 9 as % suppression. The response of cells incubated for 24 hr with TT and washed prior to stimulation with mitogen was depressed (by 40%), whereas the response of cells incubated with TT for 1 hr was unaffected. The mitogenic response of cells incubated with TT for longer periods of time (72 or 120 hr) and washed was essentially totally suppressed (80-100%).

1. *Effect of Duration of Exposure to TT on Receptor Expression*

The experiments in the above section demonstrate that TT was not toxic to PBMC and that total suppression required more than 24 hr exposure to antigen. The effect of antigen on T cell subpopulations was probed by assaying cell surface marker expression. Following the 1, 24, 72 and 120 hr treatment with TT, PBMC were washed and stained with the monoclonals as outlined in the Methods section. The number of cells expressing the cell surface markers, CD4 and CD8, was not affected by incubation with TT. However, the number of cells expressing the CD25 (IL-2R) activation marker was notably increased in the cultures treated with TT for 24 to 72 hr as compared to cultures exposed to TT for only 1 hr or in the absence of TT altogether (Table 2). CD25 receptor expression at the 120 hr time point is quite low and may be a reflection of the cell death associated with 120 hour incubation in both groups. Since no difference in viability following TT treatment had previously

FIGURE 9. KINETICS OF INDUCTION OF SUPPRESSION BY TT.

PBMC ($1x10^6$ cells) were incubated in the presence or absence of TT (2 μg/ml) for 1, 24, 72 or 120 hrs. The cells were washed and recultured in microtiter plates ($2x10^5$ cells/well) with or without PHA stimulation for 3 days. Calculations were performed as described in Figure 5. (n=16)


100
80
60
40
20
0
% SUPPRESSION
1
24
72
120
TIME OF EXPOSURE TO TT (HR)

been observed, the absence of change in CD4 and CD8 ratios indicates that no obvious preferential selection for these subpopulations was occurring. Maximum expression of CD25 coincided with maximum suppression at 24 and 72 hr initiation of culture with TT, thereby associating TT suppression with an activation of cells.

TABLE 2. EFFECT OF TT ON CD4 , CD8, & CD25 EXPRESSION

TIME IN CULTURE (hours)	%CD4		%CD8		%CD25	
	MED	TT	MED	TT	MED	TT
1	64	65	32	33	10	8
24	65	62	34	35	11	24
72	64	63	32	34	12	29
120	60	61	30	31	11	9

Double fluorescence was used to determine if a preferential increase of CD25 was occurring in a certain population. PBMC were incubated with TT for 1, 24, and 72 hr then harvested. The cells were washed and double-stained for CD25 and CD4 or CD25 and CD8, as described in Methods.

Cells expressing the CD25 antigen were mainly found in the $CD4^+$ population while no significant increase could be detected in the $CD8^+$ subpopulation of cells

(Table 3). Maximum expression of CD25 peaked at 24 hr and was maintained until 72 hr after culture initiation.

TABLE 3. EFFECT OF TT ON CO-EXPRESSION OF CD25, CD4, CD8

TIME IN CULTURE (hours)	%CD4/CD25		%CD8/CD25	
	MED	TT	MED	TT
1	1	1	1	1
24	1	7	1	1
72	2	12	1	1

2. *The Effect of Cytokines and Mediators of Cytokine Production on TT-induced Suppression*

A series of experiments was performed to determine if the suppression induced by TT was due to the lack of production of the cytokines, IL-1, IL-2 and TNF. These products play a role in the blastogenic response (Cantrell *et al.*, 1988; Hamblin, 1988). The role of the arachidonic acid pathway in the suppression was also assessed since the products of this pathway have previously been shown to regulate cytokine production and modulate responses to PHA.

The possible deficiency in IL-1 production by the monocyte was examined by the addition of exogenous IL-1 to alleviate the suppression. IL-1 was added (1 unit

to 20 units) to PHA-stimulated cultures in the presence or absence of TT. The resultsof this experiment are reported in Figure 10. No change in % suppression was observed by the addition of IL-1.

The results of a series of experiments examining the possible deficiency in IL-2 production caused by TT are shown in Figure 11. Interleukin-2 was added to $2x10^5$ PBMC along with TT (1-4 μg/ml) at culture initiation. The suppression of the PHA mitogenic responses by TT could be partially reversed in most cases by 10 units of IL-2 and higher or lower concentrations (1 or 50 units) had no effect on the suppression. However, in some cases, 50 units was the most effective dose. There was much donor variability in the response to IL-2, which could be attributed to different states of baseline activation or to basic immunologic differences between donors. What remained constant throughout was that only the partial suppression (from 2 μg/ml TT in culture or less) could be reversed. Cultures that were completely suppressed could not be induced to proliferate under the culture conditions employed (3 or 4 μg/ml TT).

In addition to the above experiments a combination experiment was performed using both IL-1 and IL-2 to determine if IL-1 could effect a more efficient reversal

FIGURE 10. EFFECT OF INTERLEUKIN-1 ON SUPPRESSION INDUCED BY TT.

PBMC ($2x10^5$ cells) were incubated with various amounts (1 - 4 μg/ml) of TT. They were stimulated immediately with PHA + medium [ ] or with added IL-1 (1 unit/ml) ▨, (5 units/ml) ▨, (20 units/ml) ▩ , for 3 days. Control cultures without antigen were also established. The ^{3}H-Thymidine incorporated for the media control was 372 and 28,542 CPM for the PHA control. Addition of IL-1 did not alter these values. Calculation of % Suppression was performed as outlined in Figure 5. (n=10)

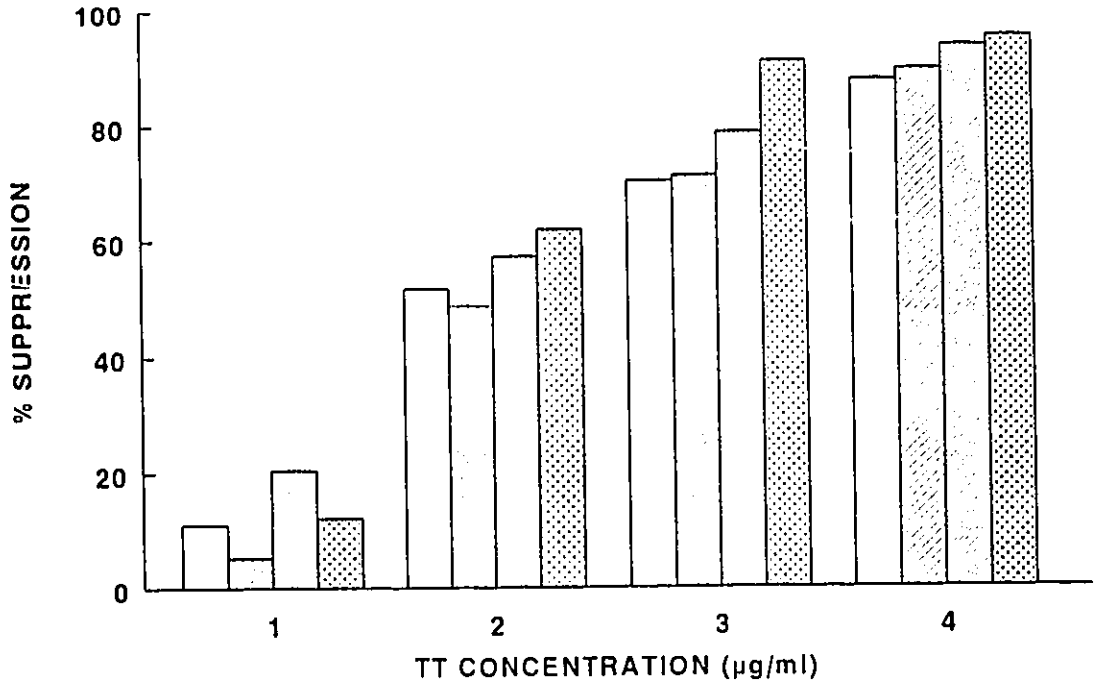
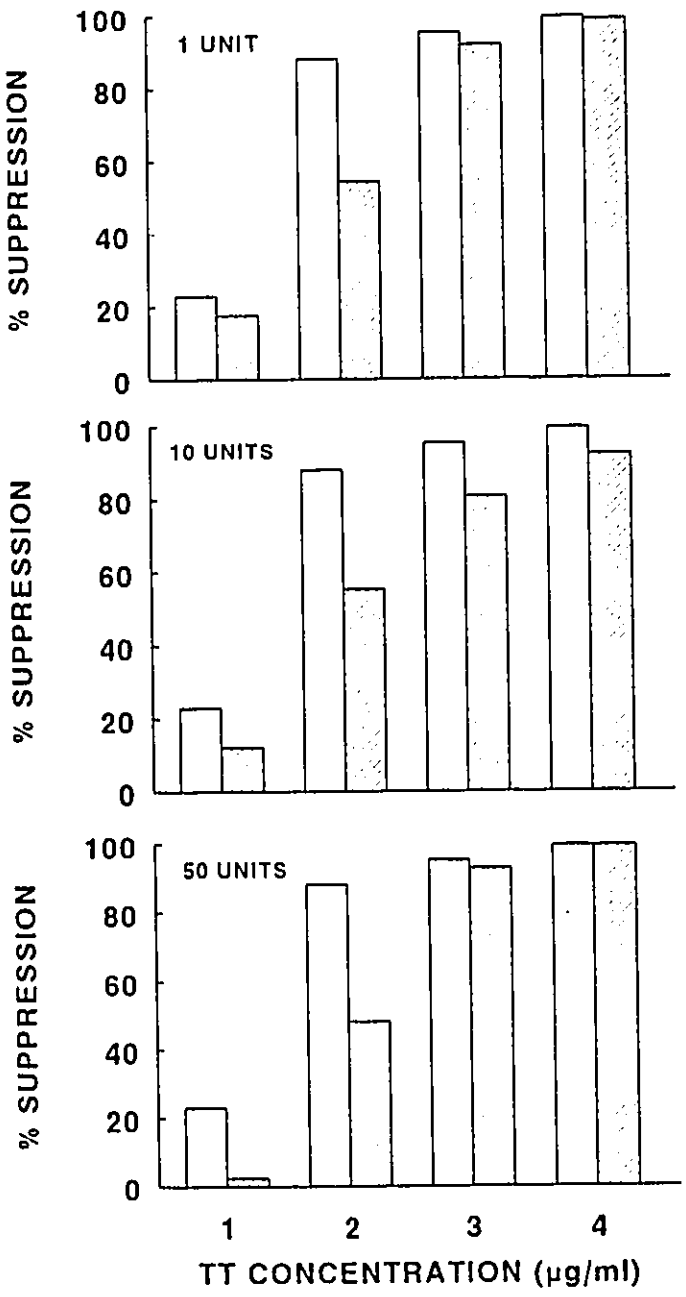

100
80
60
40
20
0
% SUPPRESSION
1
2
3
4
TT CONCENTRATION (µg/ml)

FIGURE 11. EFFECT OF INTERLEUKIN-2 ON TT-INDUCED SUPPRESSION.

PBMC ($2x10^5$ cells) were incubated with various amounts (1 - 4 μg/ml) of TT. They were stimulated immediately with PHA and medium [ ] or in the presence ▨ of various concentrations of IL-2 (1, 10 and 50 units) for 3 days. Control cultures without antigen were established, the ^{3}H-Thymidine incorporated for the media control was 288 and 27,639 CPM for the PHA control. Addition of IL-2 did not itself, alter these values. Calculation of % Suppression was performed as outlined in Figure 5. (n=18)


1 UNIT
10 UNITS
50 UNITS
% SUPPRESSION
100
80
60
40
20
0
1
2
3
4
TT CONCENTRATION (µg/ml)

of suppression by IL-2. PHA-stimulated cultures were suppressed using 2 μg/ml TT, and 1 unit of IL-1 was added with 10 units of IL-2 in an effort to create an enhancement of the response with IL-2 (10 units) alone. Figure 12 shows that IL-1 was not able to enhance the reversal of suppression by IL-2.

A further series of experiments was performed to determine if suppression, once induced, could in any way be reversed by the addition of exogenous IL-2. PBMC ($2x10^6$) were cultured in medium only or in the presence of 2 μg/ml TT for 1, 24, 72, or 120 hr. The cultures were harvested, washed and stimulated with PHA and 10 units of IL-2. The results in Figure 13 demonstrate that IL-2 was not able to reverse the suppression.

Similarly, experiments using recombinant (r) α-TNF were performed in an attempt to reverse suppression as attempted with IL-1 and IL-2. A concentration range of 1-100 ng rTNF was added at culture initiation. Secondary control cultures were also set up using IL-2 so that a comparison between IL-2 and rTNF could be made. The results are shown in Figure 14 and demonstrate that rTNF could not reverse the suppression. Only one dose (of several) of rTNF is reported, but rTNF was ineffective at all doses tested.

FIGURE 12. EFFECT OF IL-2 + IL-1 ON TT-INDUCED SUPPRESSION.

PBMC ($2x10^5$ cells) were incubated with TT (2 μg/ml). They were stimulated immediately with PHA in the presence of medium (CON) , IL-1 (2 units) , IL-2 (10 units) or a combination of IL-2 and IL-1 for 3 days. Calculation of % Suppression was performed as outlined in Figure 5. (n=6)

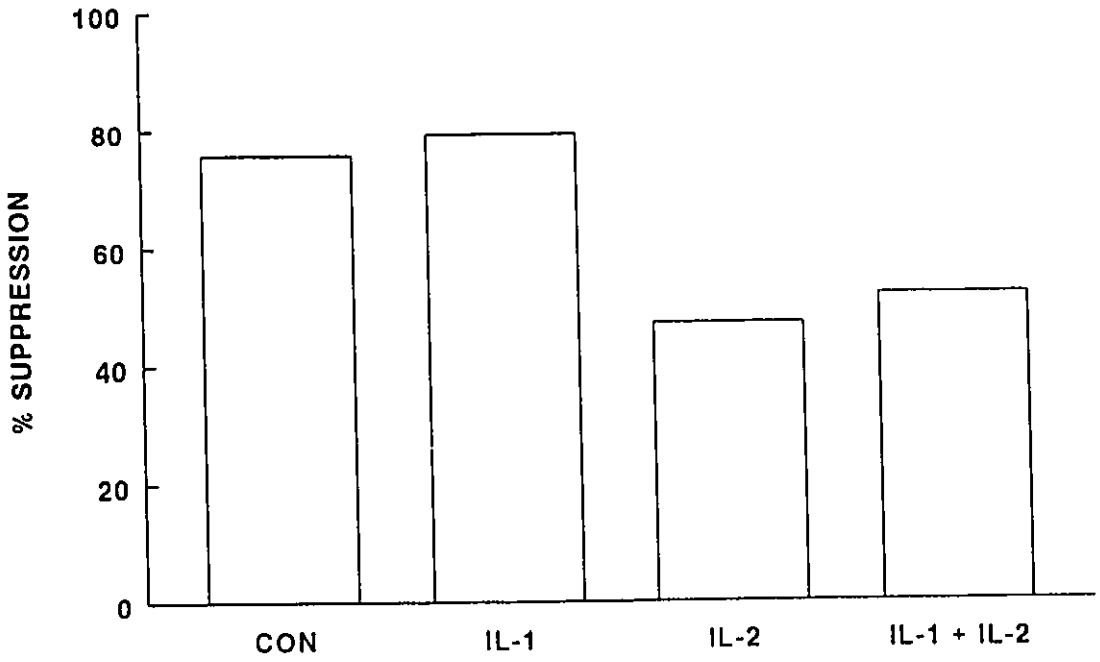

100
80
60
40
20
0
% SUPPRESSION
CON
IL-1
IL-2
IL-1 + IL-2

FIGURE 13. EFFECT OF IL-2 AFTER INDUCTION OF SUPPRESSION BY TT.

PBMC ($1x10^6$ cells) were incubated in the presence or absence of TT (2 μg/ml) for 1, 24, 72 or 120 hr. The cells were washed and recultured in microtiter plates ($2x10^5$ cells/well) and stimulated with PHA in the presence of medium [ ] or IL-2 (10 units) ▨ for 3 days. Calculations for % suppression were performed as outlined in Figure 5. (n=8)

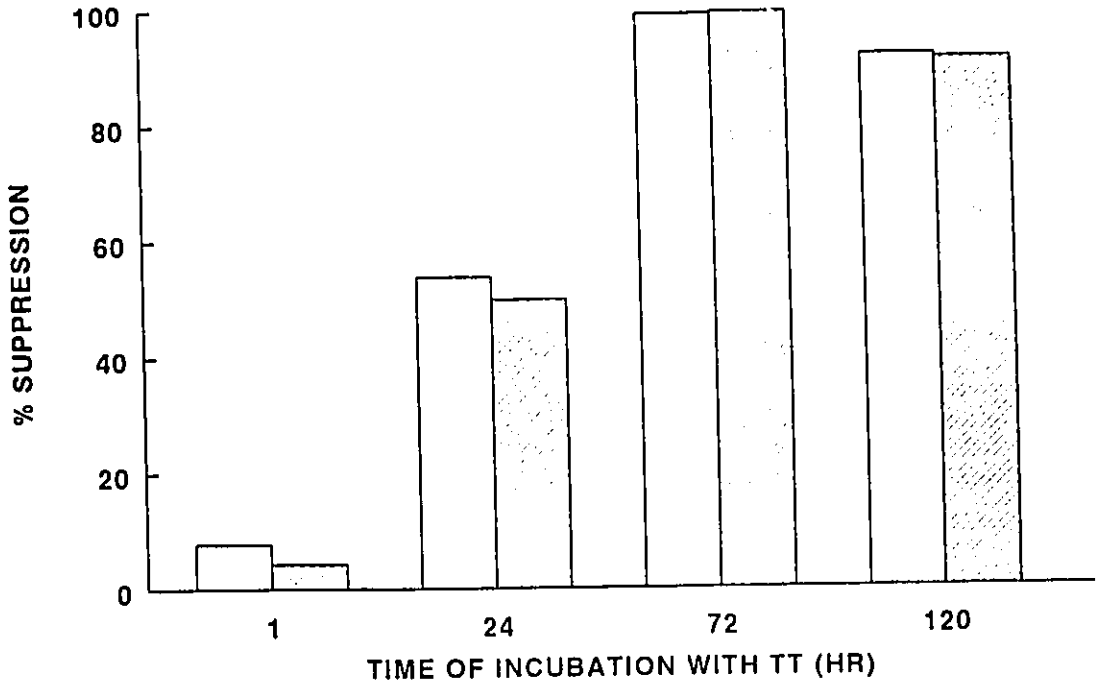
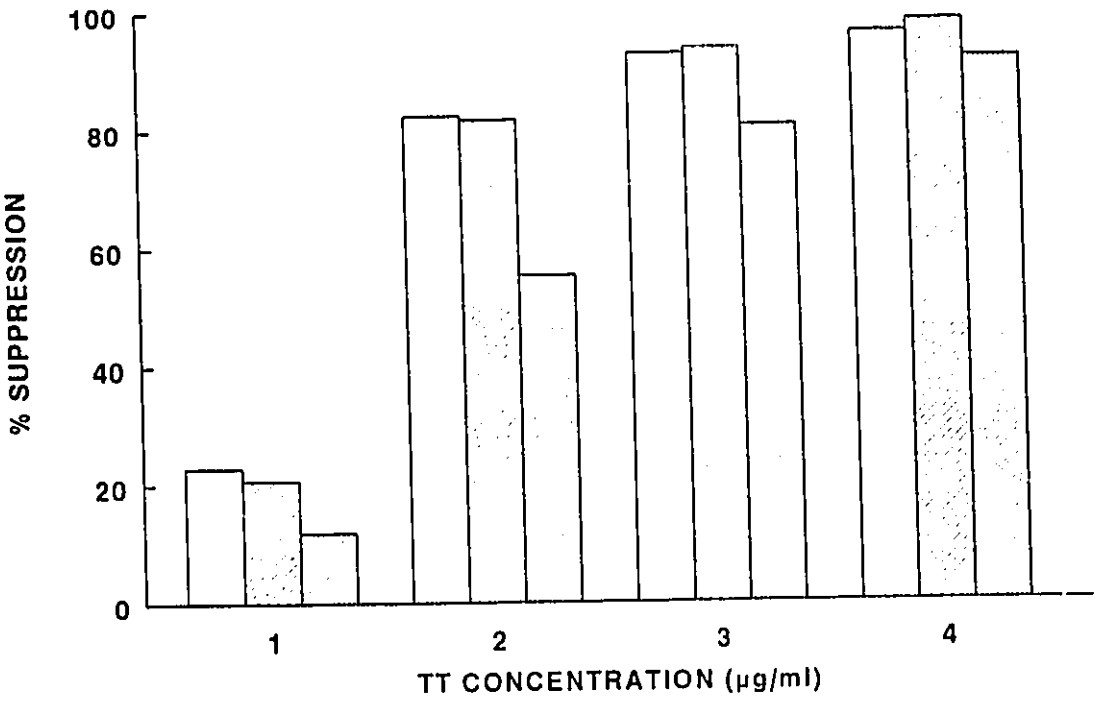

100
80
60
40
20
0
% SUPPRESSION
1
24
72
120
TIME OF INCUBATION WITH TT (HR)

FIGURE 14. COMPARATIVE EFFECT OF TNF AND IL-2 ON THE TT-INDUCED SUPPRESSION OF PHA BLASTOGENIC RESPONSE.

PBMC ($2x10^5$ cells) were incubated with various amounts (1 - 4 μg/ml) of TT. They were stimulated immediately with PHA, and given medium [ ], rTNF-α (50 ng/ml) ▨, or IL-2 (10 units/ml) ▨ for 3 days. Calculation of & Suppression was performed as outlined in Figure 5. (n=5)


100
80
60
40
20
0
% SUPPRESSION
1
2
3
4
TT CONCENTRATION (µg/ml)

Indomethacin, a blocker of arachidonate metabolism, was used to test the possible involvement of prostaglandins in TT-induced suppression. The experiments with indomethacin were performed with 1 - 4 μM indomethacin added at culture initiation. The effect of indomethacin on suppression of the PHA response is demonstrated in Figure 15. Indomethacin at a 1-2 μM concentration was the most efficient for partially decreasing suppression while remaining non-stimulatory to control cultures. A concentration of 4 μM was found to be itself suppressive to the PHA response at low (1μg/ml) TT concentrations.

Similarly, cultures of PBMC were stimulated with PWM in the presence of TT and indomethacin. The effect of 1 μM indomethacin on suppression of PWM stimulation is shown in Figure 16. Results are expressed in terms of % suppression based on IgG or IgM immunoglobulin production. Indomethacin could only partially reverse the observed suppression of the PWM response, as was the case in the PHA response previously (Figure 15). However, it was more effective at alleviating the suppression of PWM response.

3. *Effect of CD4 and CD8 plus Complement on TT-induced suppression*

The role of the T-cell subpopulations bearing CD4 and CD8 surface antigens was examined to determine if the mediation of suppression was population-dependent.

FIGURE 15. REVERSAL OF TT-INDUCED SUPPRESSION OF PHA BLASTOGENIC RESPONSE BY INDOMETHACIN.

PBMC ($2x10^5$ cells) undergoing PHA stimulation were suppressed by TT (1-5 μg/ml) [ ] as outlined in Figure 5. Cultures were additionally established containing indomethacin 1 μM ▨, 2 μM ▨, 4 μM ▨. The calculation of % Suppression was performed as outlined in Figure 5. (n=30)

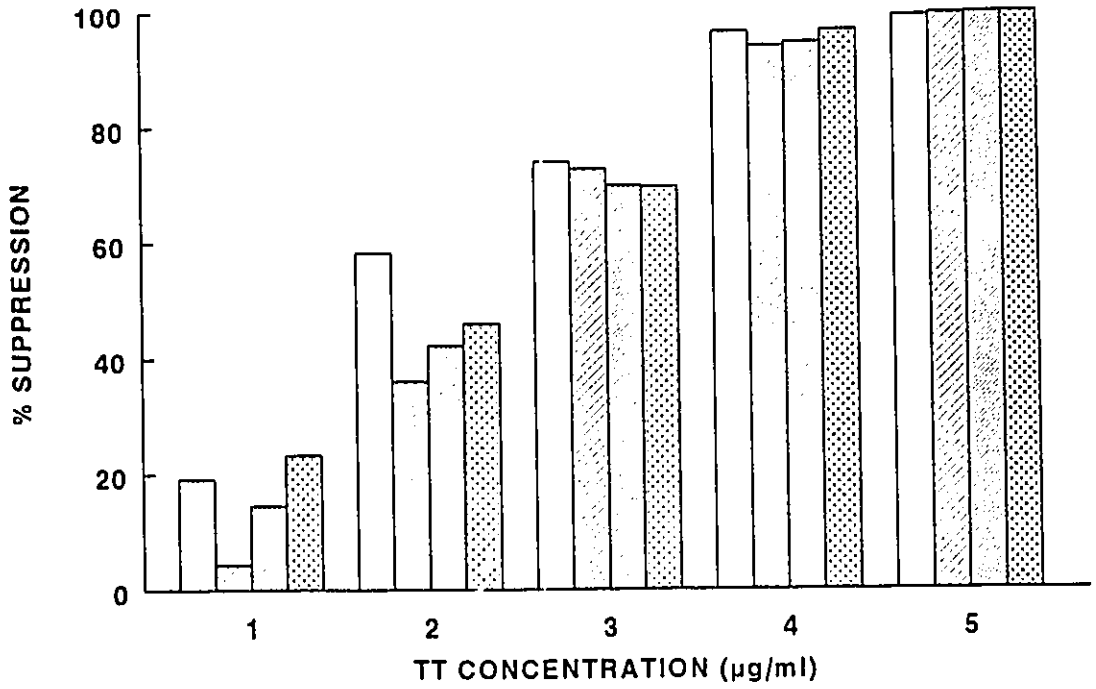
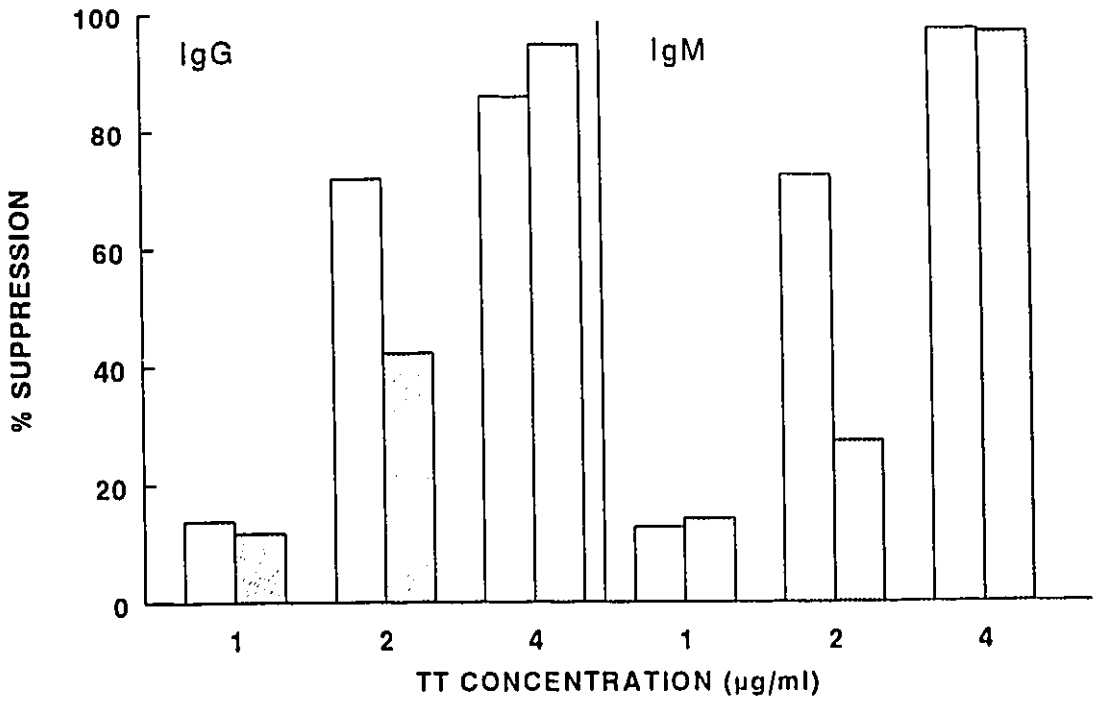

100
80
60
40
20
0
% SUPPRESSION
1
2
3
4
5
TT CONCENTRATION (µg/ml)

FIGURE 16. **EFFECT OF INDOMETHACIN ON SUPPRESSION OF IGG AND IGM SYNTHESIS BY TT.**

PBMC ($4x10^5$ cells) were incubated with various concentrations (1-4 μg/ml) of TT and stimulated with PWM in the presence of medium [ ] or indomethacin (1 μM) [ ]. Supernatant IgG and IgM immunoglobulin was measured 7 days later. The concentrations of IgG and IgM in the unstimulated or stimulated cultures without TT were 15 and 10, or 80 and 280 ng/ml, respectively. (n=6)


100
80
60
40
20
0
% SUPPRESSION
IgG
IgM
1
2
4
1
2
4
TT CONCENTRATION (µg/ml)

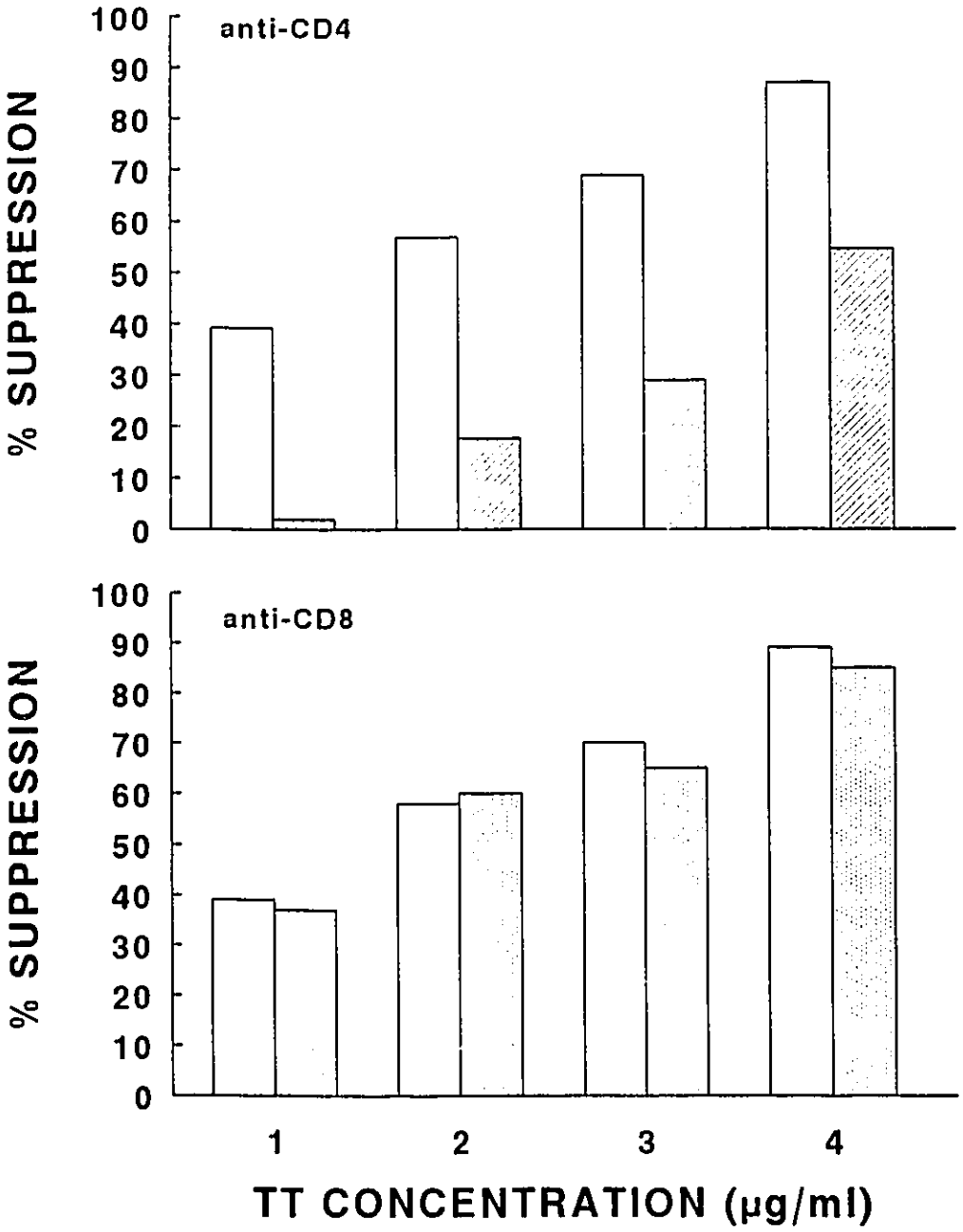
PBMC were treated with the monoclonals anti-CD4 and anti-CD8, and subjected to complement killing (see Methods section). The viability of the cells was determined after killing. Following washing, cells ($2x10^5$/well) were stimulated with PHA in the presence or absence of TT (1 to 4 μg/ml).

Killing of cells using this technique typically resulted in 40-50% cell death when using anti-CD4, and 20 - 30% death for anti-CD8, as measured by trypan blue dye exclusion. This was sufficient to establish that normal $CD4^+$ and $CD8^+$ ratios were disrupted.

The effect of TT on the blastogenic response of cells treated with anti-CD4 mAb + complement or anti-CD8 + complement is depicted in Figure 17. The results indicate a pronounced effect of the anti-CD4 + complement treatment on the reversal of suppression. At 1 μg/ml TT in culture, the reversal is essentially complete (no suppression). At 2-3 μg/ml TT culture there is 40% less suppression in the response; at 4 μg/ml TT the level of suppression is brought down from 87% o 54%. Treatment of PBMC with anti-CD8 + complement had no effect on the suppression induced by TT; the degree of suppression with each dose of TT is almost identical to that of the control.

FIGURE 17. **EFFECT OF ANTI-CD4 OR ANTI-CD8 PLUS COMPLEMENT ON TT-INDUCED SUPPRESSION.**

PBMC were treated with medium [ ], anti-CD4 + complement [▨] or anti-CD8 + complement [::] prior to culture initiation with TT. PBMC ($2x10^5$ cells) were incubated with varied concentrations of TT (1 - 4 μg/ml) and stimulated with PHA for 3 days. The calculation of % suppression was performed as outlined in Figure 5. (n=14)


anti-CD4
% SUPPRESSION
100
90
80
70
60
50
40
30
20
10
0
anti-CD8
% SUPPRESSION
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
TT CONCENTRATION (µg/ml)

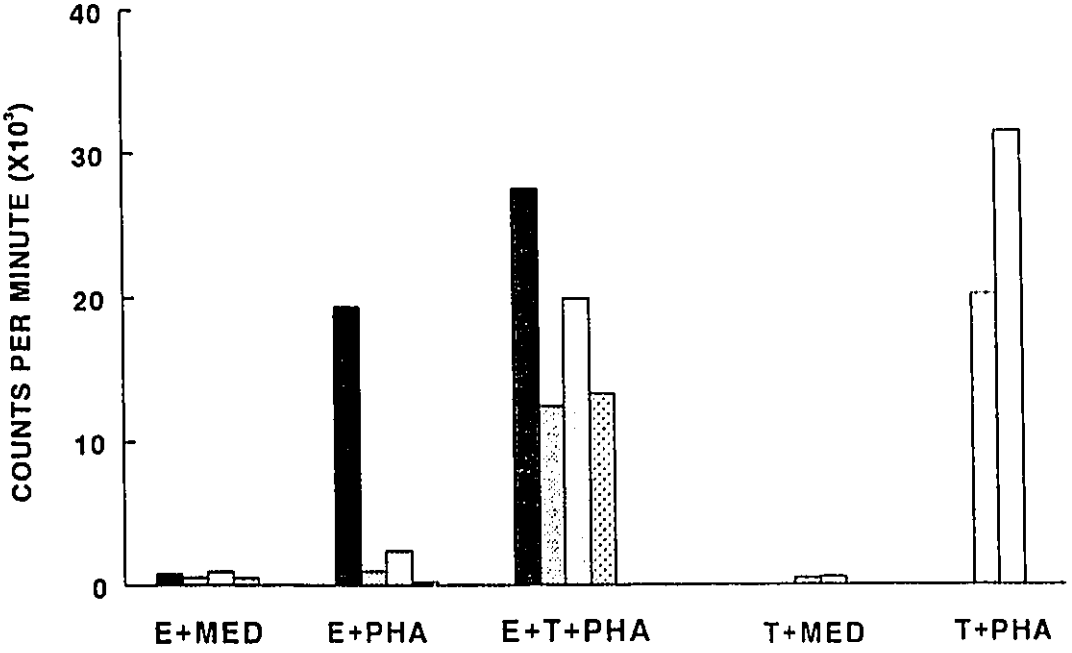
4. ***Transfer of the Suppressive State with Cells Treated with TT***

Experiments were performed to determine if the suppression induced *in vitro* could be transferred and could down-regulate the PHA blastogenic response of fresh autologous PBMC (target cells). PBMC were incubated in the presence or absence of TT for 5 days (effector cells), washed, and in initial experiments, treated with mitomycin C (or not) prior to addition of fresh autologous PBMC. The target cells were obtained by a second venipuncture, 5 days following the primary sample. The effector cells and target cells were combined in a 1:1 ratio with the final cell number totalling $2x10^5$ cells/culture. The cultures were stimulated with PHA for 3 days. Results of the co-culture experiment are shown in Figure 18.

Co-cultures of target and TT-treated effector cells produced a response that was 50% lower than cultures combined with untreated effector cells (lacking TT pre-treatment). This latter response was similar in magnitude to that seen in cultures which had no effector cells added to them. The responses of the control or TT-treated cultures alone displayed normal or depressed responses to mitogenic stimulation as expected. No difference in the transfer of suppression by TT was seen among the Mitomycin C treated groups and non-treated groups.

FIGURE 18. TRANSFER OF SUPPRESSIVE ACTION DURING CO-CULTURE.

Cultures of PBMC ($1x10^5$) were incubate for 5 days in the presence of TT (2 μg/ml) to induce a state of suppression and in medium as a control. Half of each population was treated with mitomycin C or given medium, resulting i 4 pre-incubated groups: MED [▦], TT ▩, MED+mitomycin C ▨, and TT+mitomycin C ▩. These pre-incubated effector cells (E) were recombined in a 1:1 ratio with fresh autologous target cells (T) and incubated ($2x10^5$ cells/culture) and stimulated with PHA for 3 days. T+MED and T+PHA represent the controls for the fresh cells plated at $1x10^5$ ▨ or $2x10^5$ [ ] cells. The amount of ^{3}H-Thymidine incorporation is expressed in counts per minute. (n=8)


40
30
20
10
0
COUNTS PER MINUTE (X10³)
E+MED
E+PHA
E+T+PHA
T+MED
T+PHA

VI. DISCUSSION

We have demonstrated that Tetanus Toxoid (TT) can be immunosuppressive to human cellular mitogenic responses *in vitro*. Many other bacterial, viral, fungal antigens and their toxins have been shown to have suppressive effects *in vivo* and *in vitro* (Wainberg and Mills, 1985; Margolick *et al.*, 1986; Rouse and Horohov, 1986; Cuff *et al.*, 1989; Taub *et al.*, 1989). The complexity of immune interactions allows for the possibility that antigen may modulate the immune system in a negative fashion rather than up-regulating it, which may otherwise be necessary to eliminate pathogen (Asherson *et al.*, 1986). Triggering a suppressive pathway could reflect a shift in a group of cells reacting directly with antigen or indirectly as a result of factors produced by other cells encountering antigen (Aune, 1987). We have attempted to delineate the mechanism by which TT, encountering cells from peripheral blood, could be immunosuppressive.

Experimental parameters were set up by which normal mitogenic responses to PHA and PWM could be semi-quantified. TT clearly suppresses the blastogenic response to PHA as well as IgG and IgM secretion following PWM stimulation (Figures 5 & 6). The suppression is dose-dependent and is complete at higher doses of antigen. TT has not been shown to have any direct toxic effects on cell culture

since viability counts performed by trypan blue dye exclusion remain unchanged (Table 1).

PHA is believed to activate cells by non-specific cross-linking of surface molecules which triggers an activation of the TCR/CD3-Ti (also TCR/CD3/CD4) complex. This type of activation can be achieved more precisely by the use of anti-CD3 or anti-CD4 monoclonal antibodies, whose binding sites cause stimulation of the TCR/CD3-Ti complex by cross-linkage (Rudd, 1983; Emmrich, 1988; Ledbetter *et al.*, 1988; Janeway, 1989). Monoclonal antibody-induced blastogenesis was strongly suppressed by TT (Figure 7). On the other hand, PBMC were stimulated by both PHA and mAb (Figure 8). TT was equally suppressive to PHA and monoclonal stimulation combined as to PHA alone. Occupancy of the CD4 site has been proposed as the mechanism of suppression of PHA responses resulting from gp120 (Shalaby *et al.*, 1987; Mann *et al.*, 1987). A mechanism of suppression that involves blockage of CD3-Ti is discounted by the fact that binding of anti-CD3 or anti-CD4 prior to PHA stimulation does not alter the degree of suppression following TT treatment.

TT may act through disruption of the cellular immune regulatory pathways in some way. Since it is unlikely that TT would first encounter T cells or B cells specific for it in peripheral blood of normal, un-boosted individuals, antigen may

affect the system non-specifically. Previously in this laboratory, HBsAg was shown to be suppressive to PHA responses in both HBsAg-vaccinated and non-vaccinated individuals (Filion and Saginur, 1988; Filion *et al.*, 1989). This would support the view that antigen-related suppression may induce a specific and non-specific phenomenon, and would explain why a broad range of antigens have been able to exert a negative modulation to a similar degree. Suppression in normal individuals has been demonstrated resulting from culture with gp 120, PT, and TA *in vitro* (Shalaby *et al.*, 1987; MacIntyre *et al.*, 1988; Luft *et al.*, 1988). Antigen normally triggers antigen-specific suppressor cells through a feedback mechanism during infection following the induction of appropriate antibody responses. This begins to peak at the time the pathogen is cleared (Roitt, 1989). This is quite distinct from non-specific suppression which occurs prior to and irrespective of antibody production or DTH reactions.

A good candidate for mediation of responses in mitogenic reactions would be a phagocytic cell like the monocyte, which could take up antigen non-specifically, process it and become activated. Upon this activation, other cells could be triggered by the monocyte or factors produced by it (Goodwin *et al.*, 1979; Goldyne *et al.*, 1984; Unanue and Allen, 1987). MΦ have been known to elaborate a wide variety of substances including leukotrienes, HETE's and other metabolites of arachidonic acid, many of which have been shown to have immunomodulatory effects (Goodwin

and Ceuppens, 1983; Goldings, 1986). PGE_2 is one of the end-products of the cyclooxygenase metabolic pathway and has long been associated with immunosuppression and immunomodulation (Goodwin and Webb, 1980; Awara *et al.*, 1986; Vercammen and Ceuppens, 1987; Plescia and Raeis, 1988). This biochemical pathway is not unique to monocytes; it occurs in many cell types (Plescia and Raeis, 1988), but production of PGE_2 is substantial in the MΦ and less in others (Aussel *et al.*, 1987). Among the cells obtained from peripheral blood, MΦ would be the best source for prostaglandins and exceed the quantity produced by T cells, B cells and platelets (Goldyne, 1988). The low ratio of granulocytes as compared to MΦ from normal peripheral blood would not make them a significant source. PGE_2 release from MΦ can increase up to 6-fold following 8-hour treatment of PBMC with another bacterial component, lipopolysaccharide (endotoxin) from *E. coli* (Browning and Ribolini, 1987). The possibility that TT- induced suppression is mediated through prostaglandins was tested by using indomethacin, a known inhibitor of the cyclooxygenase pathway that has often been effective in reversing suppressed mitogenic responses (Vercammen and Ceuppens, 1987). Indomethacin was added at the initiation of culture with TT and PHA or PWM. The use of indomethacin to block prostaglandin production had no effect in increasing control responses as has occasionally been reported in the past (Goodwin and Webb, 1980); thus, endogenous production of PGE_2 may be expressed at low levels in our system. In TT-treated cultures, however, treatment with indomethacin diminished the suppression greatly,

but a complete reversal was never seen (Figures 15 & 16). This points to the possible involvement of the cyclooxygenase pathway within the monocyte as being involved in suppression (Scheuer *et al.*, 1987). Because reversal of suppression was never complete, and varied according to the degree of suppression conferred by TT, it is likely that additional mechanisms of suppression could be involved. These mechanisms may involve inappropriate co-stimulatory signals delivered by the monocyte, or an inability of the T cell to respond to them or to generate its own growth factors.

IL-1 is an important component in the activation of T cells during antigen presentation and mitogenesis. There are two forms of IL-1, cloned and identified as IL-1α and IL-1β (Malkovsky *et al.*, 1988), both sharing a common receptor. IL-1α was initially identified by its ability to co-stimulate thymocyte proliferation in the presence of lectins. It is expressed on the surface of activated monocytes and is membrane-bound. It can also be expressed on the surface of B cells, but the stimuli that trigger it may be different (Paul, 1984; Dinarello, 1986). Its effects are localized and depend upon the proximity of T cells and monocytes for stimulation. IL-1β is released by the monocyte into circulation; it has wide systemic effects and was initially identified as the fever-causing agent, endogenous pyrogen. It is unclear whether all T cells require IL-1 (Bottomly, 1988), but IL-1 acts on cells in two ways: it induces receptors for IL-2, which allows the T cell to become responsive to IL-2,

and it triggers the production of further IL-2. IL-1 not only acts on T cells or cells of lymphoid origin but it also affects many organ systems pleiotropically.

Normally, expression of MHC-antigen complexes non-specifically promotes cell adhesion; this allows the APC (MΦ) to provide co-stimulatory activity for T lymphocyte stimulation. The possibility that TT interferes with the monocyte's ability to deliver an appropriate IL-1 signal was explored by the addition of exogenous IL-1 (Figure 10). Preliminary experiments indicated no reversal of TT-induced suppression could be achieved by its addition. Therefore TT apparently does not interfere with normal IL-1 production or release.

TNF-α, another monokine, shares many functions with IL-1 and has been identified as an up-regulator of immune responses and an enhancer of T-cell proliferation (Scheurich *et al.*, 1987; Dohlsten *et al.*, 1988; Yokota *et al.*, 1988). Deficiency in TNF-α production was tested by the addition of exogenous TNF-α to PHA cultures being suppressed by TT. TNF did not appear to be involved, since it had no effect on the degree of suppression (Figure 14).

Both IL-1 and IL-2 signals are normally required for resting T cells to undergo blastogenesis following PHA stimulation. The possible role of T cells in suppression of this was examined by the addition of exogenous IL-2 at the initiation of culture

(Figure 11). A decrease in the level of suppression was observed most consistently with an optimal amount of IL-2 added (10 units). Addition of sub-optimal IL-2 doses had no effect. Supra-optimal amounts had, on occasion no effect and seemed to possibly suppress the response further. This could be attributed to "freezing" the cells through saturation of receptors, a period where T cells become refractory to further stimuli (Gullberg and Smith, 1986).

Reversal of suppression by exogenous IL-2 may indicate that antigen-induced suppression acts through the blockade of endogenous IL-2 production. Monocyte production of IL-1 may be intact since only IL-2 (alone) was required to reverse suppression. The simultaneous addition of IL-1 and IL-2 resulted in no enhancement by IL-1 on IL-2 activity. The combination diminished the suppression by the same degree that IL-2 alone would have (Figure 12). This suggests that, following interaction with antigen and PHA, monocytes secrete enough IL-1 to potentiate activation of T cells and render them responsive to IL-2.

The necessity for exogenous IL-2 may have been due to a decrease in IL-2 receptors rather than to a deficiency in IL-2 secretion. Immunofluorescence assays were performed to detect the presence of IL-2 receptors. Cells treated with TT did indeed express IL-2 receptors; in fact, the overall percentage of receptors increased with TT treatment, as seen in Table 2. The antigen effect allowed the expression of

IL-2 receptors but did not allow an equivalent or appropriate production of IL-2 that would normally occur and drive the system to proliferation. This finding is consistent with that of Walker *et al.* (1983) who found that PGE_2 inhibits IL-2 production and thereby suppresses blastogenesis. They note that PBMC achieve G_0-G_1 transition and show signs of activation (*eg.* expression of IL-2 receptors), but do not progress to mitosis unless exogenous IL-2 is provided.

Further examination of TT-treated cells by double immunofluorescence to detect any population differences in the expression of IL-2 receptors revealed a marked increase in IL-2R in the $CD4^+$ population concurrent with the exposure to TT (Table 3). No increase in the expression of IL-2 receptors was noted in the $CD8^+$ cells nor in the medium controls.

The increase in IL-2 receptor expression would suggest an activation of a subpopulation among the $CD4^+$ cells, but this increase in IL-2R is associated with a later lack of response to PHA. This could signal a preferential activation of a subpopulation within the $CD4^+$ group that may be involved in mediating the suppression. The existence of a "suppressor-inducer" subpopulation of cells which are part of the $CD4^+$ population has been extensively documented (Thomas *et al.*, 1982; Damle *et al.*, 1984; Beverly *et al.*, 1988; Bottomly, 1988; Powrie and Mason, 1988).

To test the possibility that a $CD4^+$ cell was involved in the induction of suppression by TT, PBMC cells were treated with anti-CD4 and complement to disrupt the balance of $CD4^+$ cells prior to induction of suppression (Figure 17). The results strongly support the idea that the TT-associated suppression is at least partially mediated by cells of the $CD4^+$ phenotype. Elimination of some of these cells prior to treatment by TT dramatically prevented the suppression from occurring as it would in undepleted cultures. A similar depletion of the $CD8^+$ phenotype was performed to rule out effects stemming from $CD8^+$ cells. Disruption of this population had no effect on the suppression as these cultures performed very similarly to control cultures treated with TT (Figure 17).

The triggering of suppression seems to be an early event since IL-2 or indomethacin needs to be present with TT at culture initiation to inhibit the induction of suppression. If suppression has already been induced, IL-2 or indomethacin cannot reverse it (Figure 13). This finding is also consistent with the type of kinetics necessary for the reversal of PGE_2-induced suppression to take place. PGE_2 must be added within a 4-hour time period prior to PHA, and reversal of suppression by indomethacin is possible only if it is also added at this initial period (Awara *et al.*, 1986).

Kinetics experiments designed to determine the time of exposure to TT that is necessary for suppression to be detected, show that suppression occurs between 1 hour (when no suppression is detected) and 24 hours (when suppression is pronounced, at 60%) (Figure 9). Similar findings have been observed in Pertussis Toxin (PT)-induced suppression of CD3-Ti or PHA responses (MacIntyre *et al.*, 1988). A 2-hour period was not sufficient to induce suppression by pertussis toxin. This is consistent with a requirement for processing and activation of secondary cell types, since most biochemical inhibitors that act on metabolism are effective within minutes to an hour. For example, IL-1 binding to the IL-1 receptor stimulates DAG and phosphorylcholine production from phosphatidylcholine in Jurkat cells within five seconds (Rosoff *et al.*, 1988). Further experiments to pinpoint the exact time that suppression is initiated could be performed by exposing the cells to TT for narrow time intervals following addition of PHA, and monitoring the effects on blastogenesis. Alternatively, TT could be added at the start and washed out at different times to determine the extent of exposure to TT required.

To determine if the nature of the suppression was purely metabolic or resulted from the induction of a tangible suppressor cell, TT-suppressed cells were combined with fresh autologous cells during PHA stimulation (Figure 18). Transfer of a suppressive state by TT-treated cells to fresh autologous cells supports the idea of an active mechanism of suppression through the activation of a suppressor cell. The

phenotype of that suppressor cell has not yet been ascertained, but it may be $CD8^+$ even though our earlier experiments showed that decreasing the amount of $CD4^+$ cells in culture prior to exposure to TT did decrease the suppression. The $CD4^+$ cell may be important in the induction of suppression, but the $CD8^+$ cell is probably the effector cell. The necessity for the interplay between both these cell types has been documented (Morimoto *et al.*, 1982 & 1984; Damle *et al.*, 1984; Holly *et al.*, 1988). Furthermore, the effector cell may even be a monocyte, as "suppressor" monocytes have been documented (Griffin *et al.*, 1987; Luft *et al.*, 1988). The individual cell types involved in the induction and effector phases of the response could be further dissected and studied by cell separation or enrichment prior to TT exposure and following TT exposure, respectively.

The possible effect of active replication of cells or active secretion of suppressor factors was investigated by the addition of mitomycin C to feeder cells prior to combination with fresh autologous cells (Figure 18). The results show that the actual transfer of suppression is not due to active replication of the suppressor cells, since mitomycin C treatment (which blocks cellular activity) does not change the effective transfer of TT-induced suppression. Mitomycin C blocks replication of DNA by alkylation and cross-linking of double-stranded DNA. Steric hindrance prevents separation of strands and transcription (Szybalski and Lyer, 1967). Cells

remain metabolically inactive, but retain their ability to provide antigenic stimulus (Ratcliffe and Lamb, 1988).

The suppression resulting from TT may be part of a more general mechanism of suppression that may be triggered by many antigens. TT can be studied along with a wide panel of antigens and compared for stimulatory and inhibitory potential. Interesting work has already been done with pertussis toxin (PT), another bacterial antigen which like TT is part of the common vaccination procedure. PT shows mitogenic properties *in vitro* at low doses (ng/ml), but is also suppressive to mitogenic responses at higher concentrations (μg/ml) (Rosoff *et al.*, 1987). Very recently, Modesto *et al.* (1991) have studied effects of PT on the human transformed Jurkat T cell line. Concentrations of PT, in the same range as that for TT in this study, deliver an activating signal to cells that results in a rise in intracellular Ca^{++} but a down-regulation of CD3/TCR complex within 2 hours. The cells become refractory towards further stimulation by CD3 mAb or PHA. In our experimental system TT may have a similar effect on PBMC: a comparable rise in intracellular Ca^{++} could lead to an up-regulation of IL-2 receptors (as we have observed), but would render cells refractory to stimulation with PHA, PWM, anti-CD3 or anti-CD4 mAb. If their experiments were to be duplicated using TT or other antigens, a common pathway of suppression might be revealed. Related structures common to these pathogens might be triggering the suppressor mechanism. Genetic manipulation

of these structures could be attempted to eliminate detrimental effects encountered during subunit vaccine production.

VII. CONCLUSIONS

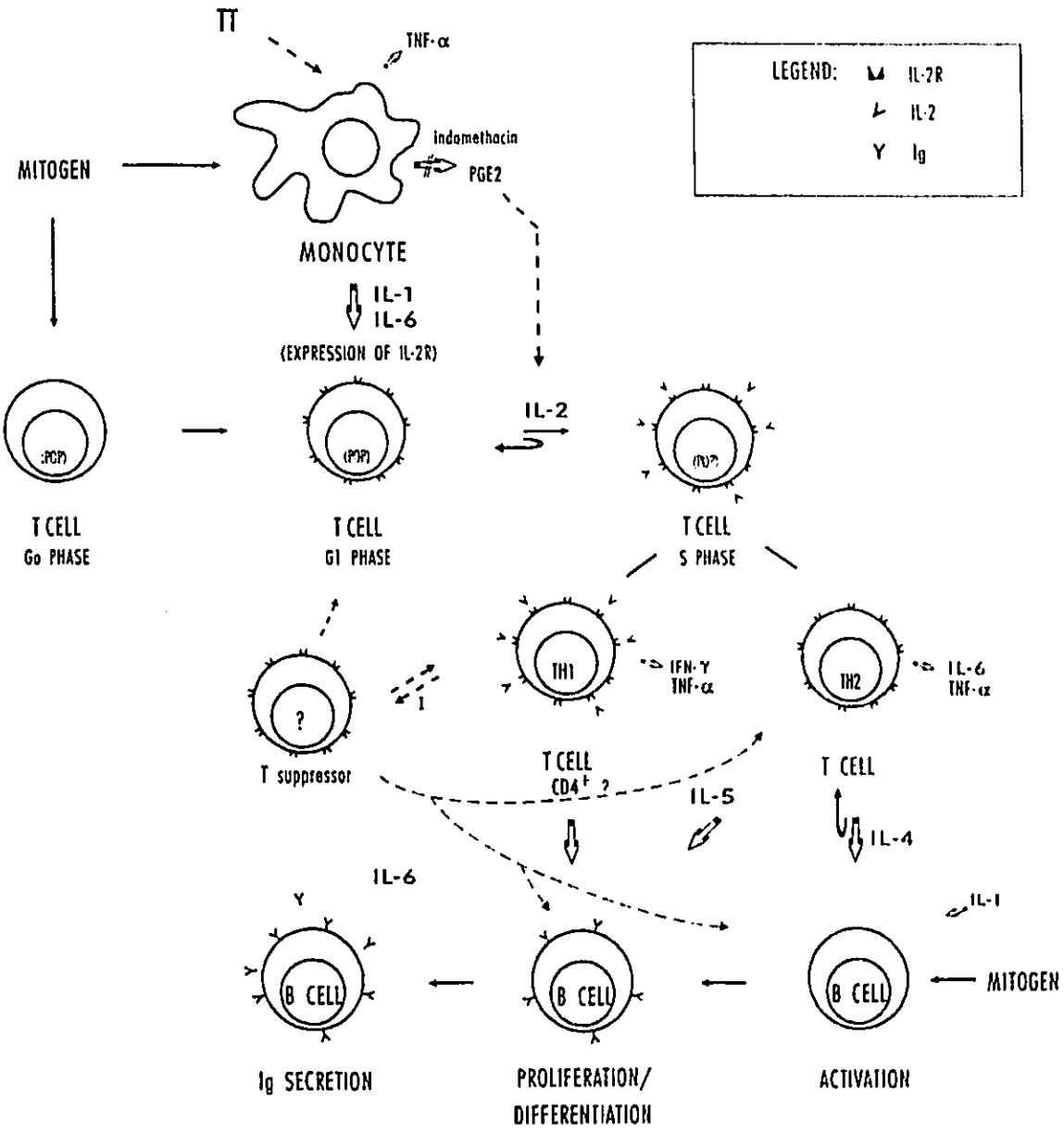
The overall mechanism of suppression can be assessed as follows: it is an early event which occurs within 24 hr of incubation with TT, possibly originating from the MΦ and involving PGE_2. Figure 19 represents a model by which TT might suppress the blastogenic response of PBMC undergoing stimulation by mitogen. TT can trigger the production of PGE_2 (from the MΦ) which in turn could act on T cells to down-regulate IL-2 production but not IL-2 receptor expression. The lack of production of IL-2 does not appear to stem from a lack of IL-1 signal from the MΦ nor from an inability of MΦ to deliver a costimulatory signal in the presence of IL-2. The addition of IL-2 could diminish the suppression, and concomitant addition of IL-2 and IL-1 did not result in an enhanced response to IL-2. Thus, a mechanism where IL-2R expression occurs in the apparent absence of IL-2 production, and where suppression is alleviated by indomethacin, is highly consistent with PGE_2-induced suppression of mitogenesis. To confirm this, culture supernatants could be assayed for the presence and/or increase of PG using HPLC or ELISA.

TT is suppressive to both PWM responses and PHA responses, possibly at the same step since PWM-induced immunoglobulin production involves the cooperation of MΦ, T cells and B cells. Normally T_H1-type cells, once activated, send signals to T_H2-type cells which require priming in order to secrete IL-4 and IL-5 which are

FIGURE 19. A MODEL FOR TT INDUCTION OF SUPPRESSION.

Mitogen stimulates monocytes, T cells, and B cells (solid arrows preceded by Mitogen). The resting T cell population (pop) progresses from G_0 to G_1 phase following mitogenic stimulation and MΦ signals in the form of IL-1/IL-6. G_1 is characterized by expression of IL-2R. The progression from G_1 to S phase is characterized by the production of IL-2 which acts on T cells in an autocrine fashion. TT may trigger PGE_2 production in the MΦ, which prevents IL-2 production. Indomethacin is a blocker of the cyclooxygenase pathway from which PGE_2 is derived, and partially reverses the suppression. In the absence of sufficient IL-2, T cells do not reach S phase. The T cell (pop) is comprised of T_H 1 and T_H 2 subpopulations. In the absence of IL-2 T_H2 cells do not provide help to B cell in the form of IL-4 and IL-5; T_H1 cells do not provide IL-2 signals to B cells, consequently Ig production is suppressed as is the measurable blastogenic response. Additionally, TT may induce a $CD4^+$ suppressor inducer population (probably T_H1). This inducer cell can induce a suppressor cell which can deliver negative signals at various stages in the system: T_H1, T_H2, MΦ or B cell.

Symbols:
Large white arrow - elaboration of major factors
Small white arrow - elaboration of minor factors
Curved arrow - factor involved in autocrine growth
Solid arrow - developmental progression
Broken arrow - suppression
Broken arrow/"I" - induction of suppression
Solid line - branches of subpopulation (T_H1 and T_H2)
// - blocker


TT
TNF-α
LEGEND:
IL-2R
IL-2
Ig
MITOGEN
indomethacin
PGE2
MONOCYTE
IL-1
IL-6
(EXPRESSION OF IL-2R)
IL-2
T CELL
Go PHASE
T CELL
G1 PHASE
T CELL
S PHASE
TH1
IFN-γ
TNF-α
TH2
IL-6
TNF-α
T suppressor
T CELL
CD4+ ?
T CELL
IL-5
IL-4
IL-6
IL-1
MITOGEN
B CELL
B CELL
B CELL
Ig SECRETION
PROLIFERATION/
DIFFERENTIATION
ACTIVATION

required by B cells for differentiation and proliferation. If the initial activation of the T cell which produces IL-2 is blocked, all subsequent signals may be blocked as well, including help to B cells (Figure 19).

A CD4 molecule or cell bearing the CD4 marker is primarily involved during the induction phase of the suppression. A partial depletion of the $CD4^+$ cells using complement, prior to induction of suppression by TT, greatly diminished suppression. In view of the extensive documentation regarding the suppressor-inducer population, the inducer cell is most probably $CD45R^+$ (T_H1, in Figure 19). This could be confirmed using a CD45R mAb to probe and deplete the PBMC prior to TT treatment instead of using $CD4^+$ which is less selective. Suppressor cell involvement is favoured because of the active transfer of suppression to fresh autologous cells, even when the "suppressors" are treated with mitomycin C. Whether that cell is $CD4^+$, $CD8^+$ or a monocyte could be determined by cell separation experiments.

The mechanism for the induction of the $CD4^+$ suppressor-inducer cell is not known. TT may directly interact with an antigen-specific $CD4^+$ inducer cell which would account for the increase of IL-2 receptors in the $CD4^+$ population following TT treatment. However, blood donors in this study were not recent vaccinees for TT, and specific interaction of TT with $CD4^+$ T cell would be unlikely. Alternatively, TT could stimulate the T cell indirectly through the MΦ which would

eliminate the need for specificity, while inducing IL-2R expression in the $CD4^+$ population. This could be tested by separation of MΦ from T cell population, treatment of T cells with TT and observing whether presence of MΦ is required for activation of IL-2R by TT; elimination of other subpopulations ($CD8^+$, $CD45R^+$, B cells *etc.*) would further narrow the requirements for induction of the $CD4^+$ cell.

Which antigens can trigger suppressor pathways over helper ones, and the mechanism by which suppression is achieved remain to be elucidated, and that knowledge would be useful in the treatment of many disease states, as well as in vaccination practices.

VIII. REFERENCES

Adams, D.O., T.A. Hamilton. 1987. Molecular transductional mechanisms by which IFN gamma and other signals regulate macrophage development. *Immunol. Rev.* **97**: 5-27.

Aderem, A.A., D.S. Cohen, D. Wright, Z.A. Cohn. 1986. Bacterial lipopolysaccharides prime macrophages for enhanced release of arachidonic acid metabolites. *J. Exp. Med.* **164**: 165-179.

Akahoshi, T., J.J. Oppenheim, K. Matsushima. 1988. Induction of high-affinity interleukin 1 receptor on human peripheral blood lymphocytes by glucocorticoid hormones. *J. Exp. Med.* **167**: 924-936.

Arora, P.K., R.D. Sekura, E.E. Hanna. 1987. Suppression of the cytotoxic T-Lymphocyte response in mice by pertussis toxin. *Cell. Immunol.* **110**: 1-13.

Asherson, G.L., V. Colizzi, M. Zembala. (1986) An overview of T-suppressor cell circuits. *Ann. Rev. Immunol.* **4**: 37-68.

Aune, T.M. 1987. Role and function of antigen non-specific suppressor factors. Review. *CRC critical reviews in immunology*. 7: 93-130.

Aussel, C., D. Mary, M. Fehlmann. 1987. Prostaglandin synthesis in human T cells: Its partial inhibition by lectins and anti-CD3 antibodies as a possible step in T cell activation. *J. Immunol.* **138**: 3094-3099.

Awara, W., K. Hillier, D. Jones. 1986. Kinetics of prostaglandin E_2 and thromboxane A_2 synthesis and suppression of PHA-stimulated peripheral blood mononuclear leucocytes. *Immunol.* **59**: 557-562.

Babiuk, L.A., M.J.P. Lawman, P. Griebel. 1989. Immunosuppression by bovine herpes virus-1 and other selected herpesviruses. Chapter 8. In *Virus-induced immunosuppression.* Edited by S. Specter, M. Bedinelli, H. Friedman; Plenum Press, New York.

Bagnasco, M., J. Nunes, M. Lopez, C. Cerdan, A. Pierres, C. Mawas, D. Olive. 1989. T cell activation via the CD2 molecule is associated with protein kinase C translocation from the cytosol to the plasma membrane. *Eur. J. Immunol.* **19**: 823-827.

Barnaba, V., A. Musca, C. Cordova, M. Levrero, G. Ruocco, V. Albertini-Petroni, F. Balsano. 1983. Relationship between T cell subsets and suppressor cell activity in chronic hepatitis B virus (HBV) infection. *Clin. Exp. Immunol.* **53**: 281-288.

Benjamin, D.C., J.A. Berzofsky, I.J. East, F.R.N. Gurd, C. Hannum, S.J. Leach, E. Margoliash, J. Michael, A. Miller, E.M. Prager, M. Reichlin, E.E. Sercarz, S.J. Smith-Gill, P.E. Todd, A.C. Wilson. 1984. The antigenic structure of proteins: A reappraisal. *Ann. Rev. Immunol.* **2**: 67-101.

Beverly, P.C.L., M. Merkenshlager, L. Terry. 1988. Phenotypic diversity of the CD45 antigen and its relationship to function. *Immunology*. **Supplement 1**: 3-5.

Bottomly, K. 1988. A functional dichotomy in CD4$^+$ T lymphocytes. *Immunol. Today*. **9**: 268-274.

Briacle, T.J., V.L. Briacle. 1991. Antigen presentation: structural themes and functional variations. *Immunol. Today*. **12**: 124-129.

Brondz, B.D., E.S. Kahn, A.V. Chervonsky, V.R. Isakova, S.G. Apasov, Z.K. Blandova. 1987. Differential genetic requirements for *in vivo* and *in vitro* induction of T-killer and T-suppressor cells in the mutant H-2Kb system and the cross-reactivity of the T-killer clones. *Exp. Clin. Immunogenetics*. **4**: 211-221.

Brown, J.H., T. Jardetzky, M.A. Saper, B. Samraoui, P.J. Bjorkman, D.C. Wiley. 1988. A hypothetical model of the foreign antigen binding site of class II histocompatibility molecules. *Nature*. **332**: 845-850.

Browning, J.L., A. Ribolini. 1987. Interferon blocks interleukin 1-induced prostaglandin release from human peripheral monocytes. *J. Immunol.* **138**: 2857-2863.

Cantor, H. 1984. T lymphocytes. In *Fundamental Immunology*. Edited by E. Paul, Raven Press, N.Y. pp 57-69.

Cantrell, D.A., M.K.L. Collins, M.J. Crumpton. 1988. Induction of IL-2 and IL-3 receptors. *Immunology*. **65**: 343-349.

Casali, P., M. Nakamura. 1989. Immunosuppression by measles virus. Chapter 18. In *Virus-induced immunosuppression*. Edited by S. Spectre, M. Bedinelli, H. Friedman; Plenum Press, New York.

Ceuppens, J.L., M.L. Baroja, K. Lorre, J. Van Damme, A. Billiau. 1988. Human T cell activation with phytohemagglutinin. The function of IL-6 as an accessory signal. *J. Immunol.* **141**: 3868-3874.

Croll, A.D., K.W. Siggins, A.G. Morris, J.M. Pither. 1987. The induction of IFN-γ production and m-RNA's of interleukin 2 and IFN-γ by phorbol esters and a calcium ionophore. *Biochem. and Biophys. Research Comm.* **146**: 927-933.

Cuff, C.F., D.D. Taub, J. Rogers. 1989. The induction of T suppressor cells with a soluble extract of *Candida albicans*. *Cell. Immunol.* **122**: 71-82.

Czop, J.K., K.F. Austen. 1985. Generation of leukotrienes by human monocytes upon stimulation of their B-glucan receptor during phagocytosis. *Proc. Natl. Acad. Sci. USA*. **82**: 2751-2755.

Damle, N.K., N. Mohagheghpour, E.G. Engleman. 1984. Soluble antigen-primed inducer T cells activate antigen-specific suppressor T-cells in the absence of antigen-pulsed accessory cells: phenotypic definition of suppressor-inducer and suppressor-effector cells. *J. Immunol.* **132**: 644-650.

Damle, N.K. , A.L. Childs, L.V. Doyle. 1987. Immunoregulatory lymphocytes in man. Soluble antigen-specific suppressor-inducer T lymphocytes are derived from the $CD4^+CD45R^-p80^+$ subpopulation. *J. Immunol.* **139**: 1501-1508.

Dinarello, C.A. 1986. Interleukin-1: amino acid sequences, multiple biological activities and comparison with tumor necrosis factor (cachectin). In *The year in immunology 1985-86*. Edited by J.M. Cruse, R.E. Lewis. S. Karger AG, Basel. pp.68-89.

Dorf, M., B. Benaceraf. 1984. Suppressor cells and immunoregulation. *Ann. Rev. Immunol.* **2**: 127-158.

Emmrich, F. 1988. Cross-linking of CD4 and CD8 with the T-cell receptor complex: Quaternary complex formation and T cell repertoire selection. *Immunol. Today*. **9**: 296-299.

Escobar, M.R. 1989. Immunomodulation by hepatitis B related viruses. Chapter 2. In *Virus-induced immunosuppression*. Edited by S. Specter, M. Bedinelli, H. Friedman; Plenum Press, New York.

Fernandez-Botran, R., V.M. Sanders, K.G. Oliver, Y.W. Chen, P.H. Krammer, J.W. Uhr, E. S. Vitetta. 1986. Interleukin 4 mediates autocrine growth of helper T cells after antigenic stimulation. *Proc. Natl. Acad. Sci. USA* 83: 9689-9693.

Filion, L.G., R.L. McGuire, L.A. Babiuk. 1983. Non-specific suppressive effect of bovine herpesvirus type 1 on bovine leukocyte functions. *Infect. Immun.* **42**: 106-112.

Filion, L.G., R. Saginur. 1988. Induction of the *in vitro* anti-HBs response by hepatitis B surface antigen. *Clin. Exp. Immunol.* **74**: 321-325.

Filion, L.G., R. Saginur, N. Szczerbak. 1988. Humoral and cellular immune responses by normal individuals to hepatitis B surface antigen vaccination. *Clin. Exp. Immunol.* **71**: 405-409.

Filion, L.G., R. Saginur, C.A. Izaguirre, 1989. Phytohemagglutinin mitogenic response of normal individuals vaccinated with Hepatitis B vaccine. *J. Infec. Dis.* **160**: 398-404

Fiorentino, D.F., A. Zlotnik, T.R. Mosmann, M. Howard, A. O'Garra. 1991. IL-10 inhibits cytokine production by activated macrophages. *J. Immunol.* **147**: 3815-3822.

Gay, D., P. Maddon, R. Sekaly, M.A. Talle, M. Godfrey, E. Long, G. Goldstein, L. Chess, R. Axel, J. Kappler, P. Marrack. 1987. Functional interaction between human T-cell protein CD4 and the major histocompatibility complex HLA-DR antigen. *Nature*. **328**: 626-629.

Goldings, E.A. 1986. Regulation of B cell tolerance by macrophage-derived mediators: Antagonistic effects of prostaglandin E_2 and interleukin 1. *J. Immunol.* **136**: 817-822.

Goldyne, M.E. 1988. Lymphocytes and arachidonic acid metabolism. *Prog. Allergy* **44:** 140-152.

Goldyne, M.E., G.F. Burish, P. Poubelle, P. Borgeat. 1984. Arachidonic Acid metabolism among human mononuclear leukocytes: Lipoxygenase-related pathways. *J. Biol. Chem.* **259:** 8815-8819.

Goodwin, J.S., A. Wiik, M. Lewis, A.D. Bankhurst, R.C. Williams Jr. 1979. High affinity binding sites for prostaglandin E on human lymphocytes. *Cell. Immunol.* **43**: 150-159.

Goodwin, J.S., D.R. Webb. 1980. Regulation of the immune response by prostaglandins. *Clin. Immunol. Immunopath.* 5: 106-122.

Goodwin, J.S., J. Ceuppens. 1983. Special article: Regulation of the immune response by prostaglandins. *J. Clin. Immunol.* 3: 295-315.

Griffin, D.E., R.T. Johnson, V.G. Tamashiro, T.R. Moench, E. Jauregui, I. Lindo de Soriano, A. Vaisberg. 1987. *in vitro* studies of the role of monocytes in the immunosuppression associated with natural measles virus infections. *Clin. Immunol.* **45**: 375-383.

Gullberg, M., K.A. Smith. 1986. Regulation of T cell autocrine growth: $T4^+$ cells become refractory to IL-2. *J. Exp. Med.* **163**: 270-274.

Hamblin, A.S. 1988. Lymphokines and interleukins. *Immunology.* **Supplement 1.**: 39-41.

Hawrylowicz, C.M., E.R. Unanue. 1988. Regulation of antigen-presentation-I. IFNγ induces antigen-presenting properties on B cells. *J. Immunol.* **141**: 4083-4088.

Holly, M., Y.S. Lin, T.J. Rogers. 1988. Induction of suppressor cells by staphylococcal enterotoxin B: Identification of a suppressor cell circuit in the generation of suppressor-effector cells. *Immunology.* **64**: 643-648.

Holsti, M.A., D.H. Raulet. 1989. IL-6 and IL-1 synergize to stimulate IL-2 production and proliferation of peripheral T cell. *J. Immunol.* **143**: 2514-2519.

Howie, S.E.M., J.A. Ross, M. Norval, J.P. Maingay. 1987. In vivo modulation of antigen presentation generates Ts rather than Tdh in HSV-1 infection. *Immunology.* **60**: 419-423.

Hutchings, D.L., M. Campos, L. Qualtiere, L. A. Babiuk. 1990. Inhibition of antigen-induced and interleukin 2-induced proliferation of bovine peripheral blood leucocytes by inactivated bovine herpes virus 1. *J. Virol.* **64**: 4146-4151.

Imboden, J.B., A. Weiss, J.D. Stobo. 1985. Transmembrane signalling by the T cell antigen receptor complex generates inositol phosphates and releases calcium ions from intracellular stores. *Immunol. Today.* 6: 328-331.

Janeway, C.A. 1989. The role of CD4 in T cell activation: Accessory molecule or co-receptor?. *Immunol. Today.* **10**: 234-238.

Kakumu, S., K. Yata, T. Kashio. 1980. Immunoregulatory T-cell function in acute and chronic liver disease. *Gastroenterology*. 79: 1122-1129.

Kilbourne, E.D., C.P. Cerini, M.W. Khan, J. W. Mitchell, P.L. Ogra. 1987. Immunologic response to the influenza virus neuraminidase is influenced by prior experience with the associated viral hemagglutinin: I. Studies in human vaccinees. *J. Immunol.* **138**: 3010-3013.

Kishimoto, T. 1985. Factors affecting B-cell growth and differentiation. *Ann. Rev. Immunol.* **3**: 133-157.

Koerner, T.J., T.A. Hamilton. 1987. Suppressed expression of surface Ia on macrophages by lipopolysaccharide: Evidence for regulation at the level of accumulation of mRNA. *J. Immunol.* **139**: 239-243.

Ledbetter, J.A., C.H. June, P.S. Rabinowich, A. Grossmann, T.T. Tsu, J.B. Imboden. 1988. Signal transduction through CD4 receptors: Stimulatory vs. inhibitory activity is regulated by CD4 proximity to CD3/ T cell receptor. *Eur. J. Immunol.* **18**: 525-532.

Lorre, K., J. Van Damme, J. Verwilghen, M.L. Baroja, J.L. Ceuppens. 1990. IL-6 is an accessory signal in the alternative CD2-mediated pathway of T-cell activation. *Eur. J. Immunol.* **20**: 1569-1575.

Luft, B.J., P.W. Pedrotti, J.S. Remington. 1988. *in vitro* generation of adherent mononuclear suppressor cells to *Toxoplasma* antigen. *Immunology*. **63**: 643-648.

MacIntyre, E.A., P.E.R. Tatham, R. Abdoul-Gaffar, D.C. Linch. 1988. The effects of pertussis toxin on human T lymphocytes. *Immunology*. **64**: 427-432.

Maizel, A.L., S. Mehta, R.J. Ford. 1979. T-lymphocyte/monocyte interaction in response to phytohemagglutinin. *Cell. Immunol.* **48**: 383-397.

Malkovsky, M., P.M. Sondel, W. Strober, A.G. Dagleish. 1988. The interleukins in acquired disease. Review. *Clin. Exp. Immunol.* **74**: 151-161.

Mann, D.L., F. Lasane, M. Popovic, L.O. Arthur, W.G. Robey, W.A. Blattner, M.J. Newman. 1987. HTLV-III large envelope protein gp(120) suppress PHA-induced lymphocyte blastogenesis. *J. Immunol.* **138**: 2640-2644.

Margolick, J.B., D.J. Volkman, T.M. Folks, A.S. Fauci. 1987. Amplification of HTLV-III/LAV infection by antigen-induced activation of T cells and direct

suppression by virus of lymphocyte blastogenic responses. *J. Immunol.* **138**: 1719-1723.

Mathes, L.E., R.G. Olsen, L.C. Hebebrand, E.A. Hoover, J.P. Schaller. 1978. Abrogation of lymphocyte blastogenesis by a feline leukaemia virus protein. *Nature.* **274**: 687-689.

Mathes, L.E., R.G. Olsen, L.C. Hebebrand, E.A. Hoover, J.P. Schaller, P.W. Adams, W.S. Nichols. 1979. Immunosuppressive properties of a virion polypeptide, a 15,000-dalton protein, from feline leukemia virus. *Cancer Res.* **39**: 950-955.

Meuer, S.C., S.F. Schlossman, E.L. Reinherz. 1982. Clonal analysis of human cytotoxic T lymphocytes $T4^+$ and $T8^+$ effector T cells recognize products of different major histocompatibility complex regions. *Proc. Natl. Acad. Sci. USA.* **79**: 4395-4399.

Modesto, J., J.P. Breittmayer, N. Grenier-Brossette, M. Fehlmann, J.L. Cousin. 1991. Pertussis toxin-sensitive G-proteins are not involved in activation of T-lymphocytes. *Cellular Signalling.* **3**: 25-33.

Morimoto, C., J.A. Distaso, Y. Borel, S.F. Schlossman, E.L. Reinherz. 1982. Communicative interactions between subpopulations of human T lymphocytes required for generation of suppressor-effector function in a primary antibody response. *J. Immunol.* **128**: 1645-1650.

Morimoto, C., L.L. Norman, A.W. Boyd, M. Hagan, H.M. Brown, M.M. Kornacki, S.F. Schlossman. 1985. The isolation and characterization of the human helper-inducer T cell subset. *J. Immunol.* **134**: 3762-3769.

Mossmann, T.R., H. Cherwinski, M.W. Bond, M.A. Giedlin, R.L. Coffman. 1986. Two types of murine helper T cell clones. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136**: 2348-2357.

Pantaleo, G., D. Olive, A. Poggi, W.J. Kozumbo, L. Moretta, A. Moretta. 1987. Transmembrane signalling via the T11-dependent pathway of human T cell activation. Evidence for the involvement of 1,2, diacylglycerol and inositol phosphates. *Eur. J. Immunol.* **17**: 55-60.

Paul, W.E. 1984. Fundamental Immunology. Edited by W.E. Paul. Raven Press, New York.

Petit, C., M. Tersmette, F.G. Terpstra, R.E.Y. deGoede, R.A.W. vanLier, F. Miedema. 1988. Decreased accessory cell function by human monocytic cells after infection with HIV. *J. Immunol.* **140**: 1485-1489.

Pimsler, M., T.A. Sponsler, W.M. Meyers. 1988. Immunosuppressive properties of the soluble toxin from *Mycobacterium ulcerans*. *J. Infect. Dis.* **157**: 577-580.

Plescia, O.J., and Racis, S. 1988. Prostaglandins as physiological immunoregulators. *Prog. Allergy*. **44**: 153-171.

Powrie, F., D. Mason. 1988. Phenotypic and functional heterogeneity of $CD4^+$ T cells. *Immunol. Today*. **9**: 274-277.

Prpic V., J.E. Weiel, S.D. Somers, J. DiDuiseppi, S.L. Gonias, S.V. Pizzo, T.A. Hamilton, B. Herman, D.O. Adams. 1987. Effects of bacterial lipopolysaccharide on the hydrolysis of phosphatidylinositol-4,5-biphosphate in murine peritoneal macrophages. *J. Immunol.* **139**: 526-533.

Ratcliffe, M.J.H., J.R. Lamb. 1988. Lymphocyte purification, growth, cloning, functional assays. Chapter 12. In *The Handbook of Experimental Pharmacology: The pharmacology of lymphocytes, vol. 85.* Ed. M.A. Bray and J. Morley, Springer-Verlag, New York

Reinherz, E.L., S.F. Schlossman. 1981. The characterization and function of human immunoregulatory T lymphocyte subsets. *Immunol. Today*. **2**: 69-75.

Richter, M., C.K. Naspitz. 1968. The effects of varying concentrations of phytohemagglutinin and conditions of incubation on the enhanced survival of human peripheral lymphocytes *in vitro*. *Blood*. **32**: 134-139.

Roberts, N.J., M.E. Diamond, R.G. Douglas, R.L. Simmons, R.T. Steigbigel. 1980. Mitogen responses and interferon production after exposure of human macrophages to infectious and inactivated influenza viruses. *J. Med. virol.* **5**: 17-23.

Roberts, N.J., F. Domurat. 1989. Virus-induced immunosuppression. Influenza virus. Chapter 16. In *Virus-induced immunosuppression.* Edited by S. Specter, M. Bedinelli, H. Friedman; Plenum Press, New York.

Roitt, I.M. 1989. Immunology-2nd Edition. Edited by J. Brostoff, D. K. Male. Gower Medical Publishing, London.

Rola-Pleszynski, M. 1985. Immunoregulation by leukotrienes and other lipoxygenase metabolites. *Immunol. Today*. **6**: 302-307.

Rosoff, P.M., R. Walker, L. Winberry. 1987. Pertussis toxin triggers rapid second messenger production in human T lymphocytes. *J. Immunol.* **139**: 2419-2423.

Rosoff, P.M., N. Savage, C.A. Dinarello. 1988. Interleukin-1 stimulates diacylglycerol production in T lymphocytes by a novel mechanism. *Cell.* **54**: 73-81.

Rouse, B.T., D.W. Horohov. 1986. Immunosuppression in viral infections. *Rev. Infec. D.* **8**: 850-873.

Royer, H.D., E.L. Reinherz. 1987. The human T cell receptor for antigen: Structure, ontogeny, and gene expression. *Behring Inst. Mitt.* **81**: 1-14.

Rudd, C.E. 1990. CD4, CD8 and the TCR-CD3 complex: A novel tyrosine kinase receptor. *Immunol. Today*. **11**: 400-406.

Rudd, C.E., C. Morimoto, L.L. Wong, S.F. Schlossman. 1987. The subdivision of the T4 (CD4) subset on the basis of differential expression of L-C/T200 antigens. *J. Exp. Med.* **166**: 1758-1773.

Sabath, D.E., M.B. Prystowsky. 1990. Molecular basis of IL-2 action. In *Lymphokines and the immune response*. Edited by S. Cohen, CRC press, Boca Raton. pp. 183-197.

Scheuer, W.A., Hobbs, M.V., and Weigle, W. 1987. Interference with tolerance induction in vivo by inhibitors of prostaglandin synthesis. *Cell. Immunol.* **104:** 409-418.

Scheurich, P., B. Thoma, U. Ucer, K. Pfizenmaier. 1987. Immunoregulatory activity of recombinant human tumor necrosis factor (TNF-α): induction of TNF receptors on human T cells and TNF-α-mediated enhancement of T cell response. *J. Immunol.* **138**: 1786-1789.

Sercarz, E., U. Krzych. 1991. The distinctive specificity of antigen-specific suppressor T cells. *Immunol. Today*. **12**: 111-118.

Shalaby, M.R., J.F. Krowka, T.J. Gregory, S.E. Hirabayshi, S.M. McCabe, D.S. Kaufman, D.P. Stites, A.J. Ammann. 1987. The effects of human immunodeficiency virus recombinant envelope glycoprotein on immune cell function *in vitro*. *Cell. Immunol.* **110**: 140-148.

Sopori, M.L., Y.L. Hurt, S. Cherian, A.M. Kaplan, T. Diamantstein. 1987. Differential requirement for accessory cells in polyclonal T-cell activation. *Cell. Immunol.* **105**: 174-186.

Suzuki, N., T. Sakane. 1988. Mechanism of T-cell derived helper factor production upon stimulation with pokeweed mitogen in humans. *Clin. Exp. Immunol.* **71**: 343-349.

Swain, S.L., D.T. McKenzie, R.W. Ditton, S.L. Tonkonogy, M. English. 1988. The role of IL-4 and IL-5: Characterization of a distinct helper T cell subset that makes IL-4 and IL-5 (T_H2) and requires priming before induction of lymphokine secretion. *Immunol. Rev.* **102**: 78-105.

Szybalski, W., V.N. Lyer. (1967) The mitomycins and porfiromycins. In *Antibiotics vol.I: Mechanism of Action*. Edited by. David Gottlieb and Paul D. Shaw, Springer-Verlag, New York. pp. 211-245.

Takeuchi, T. C.E. Rudd, S.F. Schlossman, C. Morimoto. 1987. Induction of suppression following autologous mixed lymphocyte reaction: role of a novel 2H4 antigen. *Eur. J. Immunol.* **17**: 97-103.

Taub, D.D., Y.S. Lin, S.C. Hu, T.J. Rogers. 1989. Immunomodulatory activity of staphylococcal Enterotoxin-B: The induction of I-J restricted suppressor factor. *J. Immunol.* **143**: 813-820.

Thomas, H.C., D. Brown, G. Routhier, G. Janossy, P.C. Kung, G. Goldstein, S. Sherlock. 1982. Inducer and suppressor T-cells in Hepatitis B virus-induced liver disease. *Hepatology* **2**: 202-204.

Unanue, E.R., P.M. Allen. 1987. Basis for immunoregulatory role of macrophages and other accessory cells. *Science* **236**: 551-557.

Van Bleek, G.M., S.G. Nathenson. 1990. Isolation of an endogenously processed immunodominant viral peptide from the class I H-2Kb molecule. *Nature*. **348**: 213-216.

Von Pirquet, C. 1908. Das Verhalten der Kutanen Tuberkulin-Reaktion wahrend den Masern. *Dtsch. Med. Wochenschr.* **34**: 1297-1310.

Vercammen, C., J.L. Ceuppens. 1987. Prostaglandin E_2 inhibits human T-cell proliferation after crosslinking of CD3-Ti complex by directly affecting T cells at an early step of the activation process. *Cell. Immunol.* **104**: 24-36.

Vydelingum, O., J. Ilonen, R. Salonen, R. Marusyk, A. Salmi. 1989. Infection of human peripheral blood mononuclear cells with a temperature-sensitive mutant of measles virus. *J. Virol.* **63**: 689-695.

Waage, A., O. Bakke. 1988. Glucocorticoids suppress the production of tumor necrosis factor by lipopolysaccharide-stimulated human monocytes. *Immunology*. **63**: 299-302.

Wainberg, M.A., E.L. Mills. 1985. Mechanisms of virus-induced immune suppression. *Can. Med. A. J.* **132**: 1261-1267.

Walker, C., F. Kristensen, F. Bettens, A.L. deWeck. 1983. Lymphokine regulation of activated (G_1) lymphocytes: I. Prostaglandin E_2- induced inhibition of interleukin 2 production. *J. Immunol.* **130**: 1770-1773.

Warren, H.S., A. Benzos. 1987. Heterogeneity in the activation requirements of T cells stimulated by phytohemagglutinin. *Immunology*. **61**: 167-172.

Weaver, C.T., C.M. Hawrylowicz, E.R. Unanue. 1988. T helper subsets require the expression of distinct costimulatory signals by antigen-presenting cells. *Proc. Natl. Acad. Sci. USA*. **85**: 8181-8185.

Weaver, C.T., E.R. Unanue. 1990. The costimulatory function of antigen-presenting cells. *Immunol. Today*. **11**: 49-54.

Wierenga, E.A., M. Snoek, H.M. Jansen, J.D. Bos, R.A.W. van Lier, M.L. Kapsenberg. 1991. Human atopen-specific Types 1 and 2 helper cell clones. *J. Immunol.* **147**: 2942-2949.

Wong, G.G., S.C. Clark. 1988. Multiple actions of interleukin 6 within a cytokine network. *Immunol. Today*. **9**: 137-139.

Yokota, S., T.D. Geppert, P.E. Lipskey. 1988. Enhancement of antigen- and mitogen-induced human T lymphocyte proliferation by tumor necrosis factor- α. *J. Immunol.* **140**: 531-536.