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**SUPPRESSION OF MURINE SPLENIC MONONUCLEAR
CELL RESPONSE TO MITOGEN BY
IRRADIATION AND TETANUS TOXOID:
A STUDY OF POSSIBLE MECHANISMS**

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

In Partial Fulfilment Of The Requirements For The Degree Of
Master Of Science
Department Of Physiology
Faculty Of Medicine

By

Noel P. Harrington



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ABSTRACT

This study examines the possible mechanisms by which radiation and the bacterial toxin, Tetanus toxoid (TT), suppress the murine splenic mononuclear cell (SMNC) response to mitogen. Previously, it has been shown that certain antigens can down-regulate immune responses both *in vitro* and *in vivo*. This study demonstrates that the lymphocyte proliferation response of SMNC to the mitogenic lectin PHA can be suppressed by TT in a dose-dependent manner *in vitro*, without affecting the viability of the cells, in the anti-proliferative concentrations used (0.5-5 µg/ml). SMNC pre-incubated with TT could suppress the PHA blastogenic response of fresh autologous cells during co-incubation suggesting the involvement of activated suppressor cells. Flow cytometric analysis demonstrated that TT does not produce an alteration in the cellular balance, indicating that the suppression would appear to be dependent upon a change in T cell function. TT down-regulated the expression of class II MHC antigens on antigen-presenting cells which may represent an inappropriate costimulatory signal required for T cell activation.

Whole-body irradiation has been reported to induce active immune suppression. In the present study, ionizing radiation (0-700 cGy) produced decreased spleen cellularity and decreased ability of surviving SMNC to respond to mitogen. There was no evidence, however, to indicate that irradiation (100 cGy) activated suppressor cells during the first 7 days post-irradiation. Similarly, radiation did not seem to interact with TT to increase the amount of TT-induced suppression. Flow cytometric analysis demonstrated a

dramatic alteration in the relative composition of the constituent splenic cell populations following irradiation, with selective enrichment of NK cells and CD4⁺ T lymphocytes. The observed increase in NK cells corresponds with a previously reported increase in natural suppressor cells following total-lymphoid irradiation. The relative balance between NK cells and CD4⁺ T lymphocytes following irradiation may reflect or influence the degree of hematopoietic recovery. A dose and time-dependent expression of CD71, the transferrin receptor, was found following whole-body irradiation. Taken together with changes in the proportion of particular cell sub-types after irradiation, this suggests the potential application of flow cytometric analysis as a biological dosimeter of radiation damage.

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LIST OF ABBREVIATIONS

Ab	antibody or immunoglobulin
APC	antigen-presenting cell
Ca⁺⁺	calcium ion
CD	cluster of differentiation
BFU-E	burst -forming units erythroid
CFU-E	colony-forming units erythroid
CFU-GEMM	colony-forming units multipotent
CFU-GM	colony-forming units granulocyte-macrophage
Con A	concanavalin A
CPM	counts per minute
Cs	cesium
CTL	cytotoxic (cytolytic) T lymphocyte
DAG	diacylglycerol
DC	dendritic cell
DNP	dinitrophenol
D₀	radiation dose that reduces cell survival to 37%
DTH	delayed-type-hypersensitivity
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
Fc	crystallizable fragment of Ig
FCA	flow cytometric analysis

FITC	fluorescein isothiocyanate
g	gram
<i>g</i>	gravity
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
Gy	Gray = 100 rad
³H-TdR	tritiated thymidine
HBV	hepatitis B virus
HETE	hydroxyeicosatetraenoic acids
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IgG	immunoglobulin G
IgM	immunoglobulin M
IFN-γ	interferon gamma
IL	interleukin
IMDM	Iscoe's modified Dulbecco's medium
IP₃	inositol 1, 4, 5-trisphosphate
LAK	lymphoid-activated killer (cells)
LFA	lymphocyte function-associated antigen
LT	lymphotoxin
mAb	monoclonal antibody
MCF	mean channel fluorescence

MΦ	macrophage
M-CSF	macrophage colony-stimulating factor
MHC	major histocompatibility complex
mg	milligram
ml	millilitre
MLR	mixed leukocyte reaction
mM	millimolar
NS	natural suppressor cell
NK	natural killer (cell)
PBS	phosphate-buffered saline
PE	phycoerythrin
PG	prostaglandin
PHA	phytohemagglutinin
PIP₂	phosphatidylinositol 4, 5-bisphosphate
PKC	protein kinase C
PMA	phorbol 12-myristate-13 acetate
PMT	photomultiplier tube
PT	Pertussis toxin
PWM	pokeweed mitogen
RNI	reactive nitrogen intermediates
RT-PCR	reverse transcriptase - polymerase chain reaction
SF	surviving fraction

SEM	standard error of the mean
SRBC	sheep red blood cells
TA	Toxoplasma antigen
Tc	T cytotoxic cell
TCR	T cell receptor
TLI	total-lymphoid irradiation
Th	T helper cell
TNF	tumor necrosis factor
Ts	T suppressor cell
TSRC	anti-theta sensitive regulatory cell
TT	Tetanus toxoid
µg	microgram
µl	microlitre
UVR	ultraviolet radiation
WBC	white blood cell
WBI	whole-body irradiation

I. INTRODUCTION

One of the controversial areas in the field of immunology today is immune suppression and the involvement of so-called "suppressor cells". The focus of this study is to examine potential mechanisms of suppression as induced by Tetanus Toxoid and irradiation on murine blastogenic responses to mitogen. Prior to a specific discussion of immune suppression a general working of the immune system and its role in preventing infection will be outlined.

The immune system has evolved to protect the host from infection and to eliminate pathogens. The external environment, with its variety of infectious agents including fungi, bacteria, parasites and viruses, poses a constant challenge to individuals. Healthy individuals protect themselves against such microbes by a variety of different mechanisms. Some of these defences are present prior to exposure, they are not enhanced by such exposures, and they do not discriminate among foreign substances. These mechanisms are the components of innate or natural immunity and include physical barriers such as the skin, biochemical defences such as the complement proteins, and cells in the blood and tissues such as phagocytes and natural killer (NK) cells (Abbas *et al.* 1991; Roitt, 1989).

There are, however, other defence mechanisms which are induced or stimulated by exposure to foreign substances. They are exquisitely specific for distinct macromolecules, and increase in magnitude with each successive exposure to a particular macromolecule. Constituting what is called acquired or specific immunity, these defences are comprised of cells and factors which allow the system to react quickly and specifically

to an invading organism. The specific immune system "remembers" each encounter with an antigen so that subsequent encounters stimulate increasingly effective defence mechanisms. Furthermore, the specific immune response amplifies the protective mechanisms of natural immunity, directs or focuses these mechanisms to the sites of antigen entry, and thus makes them better able to eliminate foreign antigens.

A. CELLS OF THE IMMUNE SYSTEM

The cells of the immune system are normally present as circulating cells in the blood and lymph, as anatomically defined collections in lymphoid organs, and as scattered cells in virtually all tissues. The anatomic organization of these cells and their ability to circulate and exchange among the blood, lymph, and tissues are of critical importance for the generation of immune responses. All blood cells originate from pluripotent (self-renewing) stem cells which give rise to 2 main lineages of immune cells, the lymphoid and the myeloid lineages. The differentiation of the lymphoid lineage produces the lymphocytes which are the only cells in the body capable of specifically recognizing and distinguishing different antigenic determinants. The myeloid line gives rise to a variety of cells which include mononuclear phagocytes, polymorphonuclear leukocytes (granulocytes), platelets, and erythrocytes. Antigen-presenting cells such as monocytes/macrophages and dendritic cells arise from this branch of development.

The lymphocytes are cells that specifically recognize and respond to foreign antigens. There are two distinct populations of lymphocytes, the T and the B cells. Although they are morphologically similar, they have different functions and

developmental patterns. The T lymphocytes arise in the bone marrow and then migrate to and mature in the thymus ("T" referring to thymus-derived). B cells were originally recognized as cells that differentiated in the Bursa of Fabricius in birds. In mammals, B cells differentiate initially in the fetal liver, and later in the spleen and bone marrow of adult mammals (Abbas *et al.* 1991; Paul, 1989).

Following production and maturation in the generative lymphoid organs of the thymus and bone marrow, lymphocytes migrate to peripheral lymphoid tissues such as lymph nodes, the spleen, and Peyer's patches. Localization in anatomically defined tissues or organs optimizes the cellular interactions necessary for specific immune responses. Moreover, in the peripheral lymphoid tissues, T and B cells are generally located within separate compartments. T cells are largely restricted to the paracortical or interfollicular areas of the lymph nodes or tonsils, and to the periarteriolar sheath in the spleen. In contrast, B cells are concentrated in lymphoid follicles. Mature lymphocytes maintain such a compartmentalization while continually migrating from one lymphoid organ to another via the bloodstream and the lymphatic vessels where they can respond to foreign substances.

The physiological role of the recirculating lymphocyte pool is to patrol the body and search for invading pathogens. The circulation pathway of lymphocytes allows efficient cellular communications between the blood, lymph, peripheral sites of antigen entry, and lymphoid tissues where immune responses develop. Pathogens and antigens tend to be trapped in two main sites: blood-borne pathogens are removed by the spleen,

whereas pathogens entering extravascular compartments are carried by afferent lymphatic vessels to lymph nodes.

B. HUMORAL AND CELL-MEDIATED IMMUNITY

Lymphoid cells represent approximately 35% of the total leukocyte population. Of this circulating lymphoid pool B cells constitute 10-15% and are classically defined by the presence of surface immunoglobulins or antibodies (commonly written as Ab) which specifically recognize and respond to antigens. In response to antigenic stimulation B cells participate in "humoral immunity" (antibody-mediated) by differentiating into antibody-secreting cells. Any foreign material which is capable of eliciting an antibody response 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. When released from the mature B cell, antibodies can bind to the structure that has elicited them and trigger several of the effector functions of the immune system which promote the antigen's elimination. Humoral immunity is the principal defence mechanism against the extracellular phases of bacterial, viral, and parasitic microbes and their secreted toxins.

In contrast, obligate intracellular microbes such as viruses and some bacteria proliferate inside host cells, where they are inaccessible to circulating antibodies. Defence against such infections depends upon the T lymphocytes and is termed "cell-mediated" or cellular immunity. This type of defence functions by inducing and promoting the intracellular destruction of microbes or the lysis of infected cells. B cells release their

antigen-specific receptors in soluble form, whereas T cell receptors are membrane molecules (called the T cell receptor, TCR) distinct from, but structurally related to, antibodies. Since the TCR is membrane bound, its identification has been more difficult than the soluble immunoglobulin molecules released from B cells after stimulation. In fact, until 1982-83, virtually nothing was known about the nature of the TCR. Then, in a very brief period, the TCR was isolated and sequenced, and the genes for the TCR were cloned (Sprent, 1989). The typical TCR molecule expressed on mature T cells consists of a disulfide-linked heterodimer consisting of two chains, α and β . A small population (approximately 2%) of T cells expresses a rather different TCR molecule composed of γ and δ chains. Curiously, these γ - δ TCR molecules are found in large numbers in skin epidermis (Koning *et al.* 1987) and gut epithelium (Goodman & Lefrancois, 1988) but at this time their role remains obscure. Both the α - β and γ - δ bearing TCR molecules are linked to a cluster of cell surface molecules termed the CD3 complex. These molecules appear to have an important role in T cell activation (Paul, 1989).

C. T CELL SUBSETS AND LYMPHOCYTE MARKERS

T lymphocytes can be subdivided into functionally distinct populations. The identification and analysis of T cell subsets has been made possible with the discovery that functionally distinct populations express different membrane proteins. With the advent of hybridoma technology during the last ten years, monoclonal antibodies (mAbs) have become the principal tool for analyzing surface molecules of the immune system. Functionally and developmentally distinct classes of lymphocytes can be distinguished

based upon their surface markers. Some markers are specific for cells of a particular lineage or maturational pathway, while the expression of others varies according to the state of activation or differentiation of the same cells.

A uniform nomenclature system has been adopted for the identification of surface markers. According to this system, a surface marker which identifies a particular lineage or differentiation stage, which has a defined structure, and which is recognized by a group of antibodies, is called a cluster of differentiation (CD). International cooperative efforts were instrumental in co-ordinating this development and in studying all antibody-defined structures at the molecular level with all available reagents. This effort has been accomplished by conducting a series of international workshops. Today over 130 different entities have been assigned CD designations (Schlossman *et al.* 1994a). Although CD designations were initially adopted for human leukocytes, it is now common practice to refer to homologous markers in other species by these same designations.

Classifying lymphocytes using CD antigens is of enormous value. It has allowed the identification of cells which participate in various immune responses and provided the opportunity to individually analyze their specificities, response patterns, and effector function. For example, T cells are defined by the surface expression of CD3 molecules. CD3 is complexed with the TCR and those cells bearing the $\alpha\beta$ heterodimers of TCR can be further subdivided into populations expressing either the CD4 or CD8 antigen. Cell surface expression of CD4 and CD8 molecules divides mature T cells into two distinct subsets, with the expression of these molecules being mutually exclusive. Thus, mature T cells express either CD4 or CD8 markers, but rarely both. It is now generally agreed that CD4⁺ and CD8⁺ cells are discrete lineages (Paul, 1989). The ratio of CD4⁺ to CD8⁺

cells is usually 2:1, although this ratio can vary considerably, especially with disease. Many other determinants have also been and continue to be defined by monoclonal antibodies and may include receptors for cytokines, adhesion molecules, or may not yet have an assigned function.

D. CD4⁺/CD8⁺ T CELLS AND MHC RESTRICTION

Unlike B cells, T cells generally do not react to free (native) antigens but to fragments of antigen complexed to major histocompatibility complex (MHC) molecules expressed on the surface of other cells (accessory cells). T cells are thus said to display "MHC restriction". Although the TCR repertoire of CD4⁺ and CD8⁺ cells would appear to be very similar, these cells have very different patterns of MHC restriction, that is, CD4⁺ and CD8⁺ cells interact with different classes of MHC molecules. CD8⁺ cells almost invariably recognize antigens complexed to MHC class I molecules, whereas CD4⁺ cells react to antigens bound to MHC class II (also called Ia for I region-associated) molecules (Gay *et al.* 1987; Meuer *et al.* 1982).

Class I molecules are expressed on virtually all cell types and consist of two non-covalently associated polypeptide chains, a highly polymorphic MHC-encoded heavy (α) chain and a nonpolymorphic non-MHC-encoded light (β) chain, β_2 -microglobulin. Class II molecules are expressed on only a few cell types, in particular, B cells and typical antigen-presenting cells (APCs) such as dendritic cells and macrophages ($M\Phi$). The class II molecules consist of two non-covalently linked chains, α and β , each chain being MHC encoded.

It has long been argued that native antigens have to be "processed" so as to allow

small fragments of antigen to come into close proximity with MHC molecules (Unanue, 1981). The recent description of the three-dimensional structure of class I molecules, however, has been enormously useful in deciding how antigen and MHC epitopes are displayed for recognition by T cells (Bjorkman *et al.* 1987). It was found that the peptide binding cleft was 7-10 amino acid residues. It is now generally accepted that the T cell recognition of antigen involves a trimolecular interaction between the TCR, MHC molecules, and peptides held in the MHC cleft (Davis & Bjorkman, 1988). The TCR makes joint contact with the peptide held in the MHC cleft and with the borders of the cleft (which contain polymorphic residues).

Studies in the early 1970's indicated that the MHC-restricted specificity of T cells tends to be directed to "self" rather than "allo" (foreign) MHC molecules (Katz *et al.* 1973; Zinkernagel & Doherty, 1974), that is, to MHC molecules which are encountered in the thymus during ontogeny. The selection of the T cell repertoire in the thymus is a complex process in which small numbers of mature functional T cells are tailored to the extrathymic environment and are drawn from a large pool of immature T cells.

There is now evidence that T accessory molecules physically bind to MHC molecules. CD8 molecules bind to class I molecules and CD4 molecules bind to class II molecules (Davis & Bjorkman, 1988; Goldstein & Mescher, 1988). Such binding is believed to be a device to increase the overall avidity of T-APC interaction. The binding of T accessory molecules is directed to nonpolymorphic sites on MHC molecules and is unique for each class of MHC molecule.

E. CLONAL SELECTION AND EXPANSION

The T and B cells express specific receptors for the great variety of determinants that they may encounter. This has been referred to as the T or B cell "repertoire". Cells bearing receptors which bind strongly to a given determinant are stimulated to multiply. The stimulation results in the formation of a clone of thousands of daughter cells specific for that determinant. This clonal selection hypothesis, first suggested by Jerne in 1955 and more clearly enunciated by Burnet in 1957, has since been substantiated to be correct. Thus, the presence of antigen-specific clones of lymphocytes occurs prior to and independent of exposure to antigen.

The cells constituting each clone have identical antigen receptors, which are different from the receptors on the cells of all other clones. Clonal selection occurs both for B lymphocytes through their surface immunoglobulin and for T lymphocytes through their T cell receptor. Antigen may bind to the limited number of cells in circulation which recognize it and induce the cells to proliferate, such 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. 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 to an overall understanding of pathogenesis, immune response to antigen, and clinical manipulation of these responses to our advantage.

F. CELL FUNCTIONS AND NETWORKS

The specific immune system is remarkable for its complexity and diversity. Immune responses require the co-ordinated and precisely regulated interplay of many different cells and secreted cell molecules. The T cell population is heterogeneous and functions via interactions between several cell types including antigen-presenting (accessory) cells, B cells, T helper, T cytolytic, and T suppressor cells.

Most CD4⁺ cells are T helper cells (Th) while most CD8⁺ cells are T cytolytic (or cytotoxic) cells (Tc). In the late 1970's studies indicated that activated T cells release soluble factors which influence the growth and differentiation of other cells (Smith & Ruscetti, 1981). These protein hormones are now termed "lymphokines", "interleukins", or the more general term, "cytokines". In response to antigenic stimulation, T helper cells secrete cytokines whose function is to promote the proliferation and differentiation of the B cells. This causes B cells to proliferate and differentiate into plasma cells which secrete antibodies. Th cells can also deliver signals to other cells, including cytolytic T lymphocytes (CTLs), the effector cells of cellular immunity. CTLs can lyse antigen-bearing target cells, tumors, and foreign tissue through direct cell contact. T lymphocytes can also act on other cell types such as macrophages and induce them to eliminate a foreign body. This type of cellular response is referred to as delayed-type hypersensitivity (DTH).

Studies conducted in the late 1960's and 1970's suggested the possible existence of an additional subset of T cells called T suppressor cells (Ts) which, under certain conditions, can downregulate the immune response (Green *et al.* 1983; Dorf & Benaceraf, 1984; Gershon *et al.* 1972). Ts are responsible for inhibiting immune responses by means of a feedback mechanism which serves to limit clonal expansion and effector cell activity in response to an antigenic stimulus. This regulatory system is thought to ensure that unnecessary immune responses do not persist following clearance of antigen from the host and to play a role in preventing immune responses to self-antigens. The influence of Ts cells has been seen in many different types of immune responses, including delayed-type hypersensitivity, transplantation immunity, and T cell-B cell interaction leading to antibody production. The notion that some T cells can inhibit the function of other T cells is now widely accepted, however, it remains unclear whether these cells are of a separate lineage. The inhibition seen with Ts cells is often antigen-specific, but Ts cells have rarely been successfully cloned *in vitro* as have the other subtypes.

A variety of cell types, including T cells, B cells (Katz *et al.* 1974), and monocytes/macrophages (Baird & Kaplan, 1977; Oehler *et al.* 1977) have been cited as mediators of immune suppression. In the overwhelming majority of situations, however, a critical role for T lymphocytes has been identified. The use of antibodies specific for T cell surface markers has allowed selective depletion or enrichment of T cells from test populations and suggested the T cell identity of suppressor cells. Furthermore, it was found that CD8⁺ T cells play a dominant role in mediating suppression (Cantor & Boyse, 1975) and this has been generalized to a large number of experimental settings. The

nature of the MHC recognition specificity expressed by CD8⁺ cells is unclear, and the functional role that might be played by the CD8 molecule on suppressor cells is similarly unknown. The requirement of the CD8 phenotype, however, is not absolute and several instances have been characterized in which some CD4⁺ T cells mediated suppression in the absence of any CD8⁺ cells (Bottomly *et al.* 1983).

Soon after the existence of T-cell-mediated suppression was appreciated, several lines of experimental evidence suggested that interactions among two or more distinct T cell subpopulations might be involved. Suppressor networks of cells in the mouse have recently been described (Dorf & Benaceraf, 1984) involving Ts1 which serves as an inducer cell, and Ts2 as an effector cell. In addition, a 3-cell system has been described with Ts1 as an inducer, and Ts2 as a transducer cell which amplifies the interaction between the inducer and the effector cell, denoted by Ts3. Each of these cell types is thought to elaborate its own suppressor factors, which removes the need for actual contact between the T cells. A number of investigators have characterized the inducer cell as CD4⁺ and the effector cell as CD8⁺ (Cantor *et al.* 1976; Germain & Benaceraf, 1981)

G. T CELL ACTIVATION

Pathogens are either removed by the spleen or are carried by afferent lymphatic vessels to lymph nodes. In the lymphoid tissues antigens are presumably broken down into peptides and associate with MHC molecules on APCs. When recirculating T cells encounter antigen-bearing APCs in the T-dependent areas of the lymphoid tissues, antigen-reactive T cells bind to APCs and become temporarily sequestered. This process is quickly followed by extensive proliferation and subsequent effector functions of T cells.

In vivo systems, however, are unable to provide detailed information on T cell induction and proliferation. Such information has been derived largely from *in vitro* systems in which T cells can be stimulated in a controlled manner, and their responses can be measured accurately. Much of our current information on the requirements for stimulation of unprimed T cells has come from studies with MHC alloantigens and artificial ligands such as mitogens and anti-TCR monoclonal antibodies.

1. *Experimental Model: Lectins*

It has been known for many years that polymeric plant proteins called lectins such as phytohemagglutinin and concanavalin A are strongly mitogenic for unprimed T cells (Ling & Holt, 1967; Simons *et al.* 1969). These compounds stimulate T cells to proliferate regardless of specificity by binding to TCR and TCR-associated molecules and subsequently inducing rapid DNA synthesis. Similar strong polyclonal proliferative responses of T cells can be induced with anti-TCR or anti-CD3 mAbs (Weiss & Imboden, 1987). Such polyclonal activation of T cells eliminates the difficulties encountered in studying small numbers of antigen specific responding cells within a resting T cell population. T cell responses to artificial ligands have been shown to generally require the presence of other cell types, which are termed "accessory" cells, such as monocytes (Maizel *et al.* 1979; Rosenstreich *et al.* 1976). These cells, through non-specific binding, can present the ligand to the T cell in a cross-linked form which is a prerequisite of T cell triggering. In addition, it is believed that accessory cells synthesize certain soluble or cell-bound molecules which play a critical role in inducing or potentiating T cell

activation (Unanue & Allen, 1987; Springer, 1990; Ceuppens *et al.* 1988).

2. Biochemical Events

When antigen is presented to a T cell or when the TCR is bound by an activating antibody or lectin, a series of membrane and cytoplasmic events rapidly occur. These changes which occur during the first few seconds to minutes after stimulation include increases in cytoplasmic free calcium, pH changes, protein phosphorylations, and changes in cyclic nucleotides. These events are hypothesized to be causally related to the more distal events of T cell activation, such as cytokine production and mitosis. These early "signal" events are similar to those that have been described in the stimulus-response physiology of a variety of non-lymphoid cells, such as mast cells, platelets, endocrine secretory epithelial cells, and muscle.

Within seconds of the binding of ligands to the TCR, there is an increased rate of phospholipase C-catalyzed hydrolysis of plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2). This results in increased cytoplasmic levels of PIP_2 breakdown products: diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP_3). PIP_2 breakdown is followed by a rapid rise in the cytoplasmic ionized calcium concentration which is thought to be a result of the IP_3 -stimulated release of membrane-sequestered intracellular calcium stores (Imboden *et al.* 1985). A sustained increase in cytoplasmic calcium is often maintained for over an hour and is dependent upon the influx of extracellular calcium. Protein kinase C (PKC) is activated as a result of the increases in DAG and calcium which have been shown to activate purified PKC (Imboden

et al. 1985). Elevated calcium concentrations also favour the formation of complexes with the ubiquitous calcium-dependent regulatory protein calmodulin. This leads to the activation of calcium-calmodulin-dependent protein kinases which phosphorylate several different T cell membrane and cytoplasmic proteins. Further details regarding events distal to calcium-activated kinases and the relevant substrates of activated PKC are not yet available but are areas of active research.

The relevance of these biochemical changes to the functional activation of T cells is supported by the fact that PKC activators such as phorbol myristate acetate (PMA) and calcium ionophores act synergistically to promote the later differentiative and mitotic events which are normally seen in T cells in response to TCR-binding ligands. The fact that neither PKC activators nor calcium ionophores are sufficient alone for T cell activation (but their combination is) has been interpreted as evidence for a two-signal model of T cell activation. In this model T cells require at least two different signals which are generated by two different cell surface-binding events in order to generate a full response. One signal may be provided by the binding of MHC-associated antigen to the TCR and is transduced by the CD3 complex. The second signal may be triggered by accessory molecules on T cells binding to ligands on APCs, or by costimulators produced by the APCs binding to specific receptors on T cells. Many questions, however, remain unanswered such as which T cell surface proteins generate which signals.

The phosphorylation, via protein kinases, of several different T cell membrane, cytoplasmic, and DNA binding proteins occurs within seconds following ligand binding to the TCR. The net result which begins within minutes is the transcription of a variety of genes (more than 70) whose protein products are assumed or known to be essential for

functional activation to proceed. Three main functional categories of genes which are expressed early during T cell activation are cellular proto-oncogenes, cytokine genes, and cytokine receptor genes. The protein products of several genes may be expressed on the cell surface of the T cell during the events associated with the activation, differentiation, and proliferation of T cells, thereby serving as activation markers/indicators.

3. *The Role of IL-2 and Proliferation of T Cells After Activation*

One of the most important events which occurs during a primary immune response to antigen is the proliferation of clones of antigen-specific cells. This involves the highly regulated production of certain lymphokines which can function as growth factors and regulate the expression of receptors for these lymphokines. Although their release may be the direct result of antigenic stimulation, the effects of lymphokines are not antigen-specific (Dinarello & Mier, 1987). Thus, lymphokines act independently of antigen and are pleiotropic.

Activated T cells produce the cytokine interleukin-2 (IL-2) which acts as a growth factor, and therefore the transcriptional regulation of its gene is essential for the mitotic component of functional T cell activation (Leonard *et al.* 1985). This soluble factor can interact with the very same cell that produced this growth factor (an autocrine effect), or it can interact with any other cell that expresses IL-2 receptors (a paracrine effect). Thus, activated T cells that produce IL-2 can 1) promote their own clonal expansion, 2) promote the proliferation of other T cells that are activated by the same or related antigen but cannot produce IL-2, 3) promote the expansion of previously stimulated cells that express

low levels of the IL-2 receptor (i.e., memory T cells), and 4) promote the growth of non-T cells that express IL-2 receptors (i.e., B cells and NK cells).

Mitotic division of activated T cells results in the expansion of clones of cells with the same antigen specificity and thereby amplifies the immune response to a particular antigen. Along with cytokine assays, the measurement of mitotic activity by ^3H -thymidine incorporation into newly synthesized DNA is one of the most frequently used assays for T cell activation. When T cells are stimulated through the TCR:CD3 complex, mitotic activity can be measured within 48 to 72 hours *in vitro*.

4. Regulation of Effector Function

A principal method for the regulation of immune effector functions involves the control of different T cell subsets which secrete different patterns of cytokines. An example of this is the opposite secretory patterns observed in the mouse Th1 and Th2 clones. Initial experiments revealed two populations of CD4^+ cells which had opposite secretory patterns. Some CD4^+ cells release IL-2, IFN- γ , and Lymphotoxin (LT), but do not synthesize IL-4 or IL-5; other CD4^+ cells release IL-4 or IL-5, but not IL-2 or IFN- γ (Mossmann *et al.* 1986; Fiorentino *et al.* 1989). T cells which show these two patterns of lymphokine release have come to be termed Th1 and Th2 cells, respectively. Generally speaking, Th1 cells appear to be largely responsible for delayed-type hypersensitivity reactions whereas Th2 cells play a major role in antibody-mediated (humoral) responses. Of the two, the response which is mounted after exposure to antigen can determine the survival of the organism. BALB/c mice respond to infection by

Leishmania major with a predominantly humoral response that is ineffective, and consequently the mice die. C57BL/6 mice, however, have a DTH response which ultimately cures the infection (Howard *et al.* 1980). Such observations suggest the possibility of Th1-Th2 cross-regulation. IFN- γ , a product of Th1 cells, inhibits proliferation of Th2 clones *in vitro* (Gajewski & Fitch, 1988). Recently Fiorentino *et al.* (1989) characterized a cytokine (IL-10) that inhibits synthesis of several cytokines by Th1 clones. IL-10 was found to act indirectly on Th1 cells via APCs (Fiorentino *et al.* 1991b), thereby limiting cellular immune reactions.

H. IMMUNOREGULATION AND ACCESSORY CELLS

As previously described, an important characteristic of antigen recognition by T lymphocytes is that T cells recognize and respond to foreign protein antigens only when the antigen is attached to the surfaces of other cells. The advent of cell culture technology established the requirements of T cells to recognize protein antigen molecules by APCs which possess class II MHC molecules. The first experiments showed that T cells from experimental animals immunized with protein antigens did not proliferate in cultures devoid of APCs (Waldron *et al.* 1973). The requirement for APCs was also evident in responses to the plant lectins phytohemagglutinin and concanavalin A, and in the mixed leukocyte reaction (Schwartz *et al.* 1978; Ahmann *et al.* 1979). The important role of class II MHC molecules to the interaction became evident with the profound inhibition of T cell activation by the addition of antibodies to class II MHC molecules (Relinger *et al.* 1975). Those cells bearing class II MHC molecules degrade proteins to

a sufficiently small size and appropriate conformation which can non-covalently attach to the binding clefts of the MHC, a process called antigen processing.

The requisite properties that allow a cell to function as an APC for class II MHC-restricted helper T lymphocytes include the ability to process endocytosed antigens and the expression of class II MHC gene products. The best defined APCs for helper T lymphocytes are: mononuclear phagocytes, B lymphocytes, dendritic cells, Langerhans cells of the skin, and in humans, endothelial cells (Paul, 1989). Each of these populations presents antigen to varying degrees and function to initiate immune responses through the recognition of the peptide antigen/MHC complex by clonally restricted TCR on T cells resulting in their activation.

1. Costimulators and Accessory Molecules

The APC stimulation of T cells is a complex phenomenon involving additional stimuli beyond those initiated by ligand binding to the T cell antigen receptor. These stimuli, referred to collectively as costimulator activities, although incompletely characterized, can be grouped into two major sets: those that promote the physical contact of APCs and T lymphocytes and those that promote the growth/activation of the T cell.

Cell adhesion molecules promote contact between the APCs and the T lymphocytes. The LFA-1 (lymphocyte function-associated antigen-1) molecule promotes the non-antigen-dependent adhesion of the T cells to a variety of lymphoid cells that bear LFA-1's complementary receptor, termed ICAM-1 (intercellular adhesion molecule-1). Monoclonal antibodies to LFA-1 have been shown to inhibit antigen presentation

(Springer *et al.* 1987; Golde *et al.* 1985).

The first and best known example of a costimulator which promotes growth/activation of T cells is interleukin-1 (IL-1). IL-1 was initially defined as a molecule released by macrophages which was essential for the proliferative response of thymocytes to lectins (Gery *et al.* 1972). Despite extensive studies, the precise role of IL-1 as a costimulator has not been elucidated. Mounting evidence suggests that one of its effects may be to enhance the antigen-presenting function of APCs by promoting cell to cell adhesion (Cavender *et al.* 1986). Most recently, Th1 clones have been shown not to require IL-1 whereas Th2 clones have IL-1 receptors and are strictly dependent on the expression of IL-1 by the APCs (Weaver *et al.* 1988; Greenbaum *et al.* 1988). These studies suggest that there is more than one costimulator, and that the presence of one or another influences how T cells are activated. More recently the lymphokine interleukin-6 and mAbs reactive with several distinct non-TCR molecules expressed on T cells (including CD2, CD5, and CD28) have all been able to substitute for the putative accessory stimulus provided by APCs in some systems (Yang *et al.* 1986; Weiss *et al.* 1984; Ledbetter *et al.* 1985). These reagents may have varying effects, some may function as adhesion molecules, augmenting the interaction between the T cell and APC, whereas others may influence or initiate signal transduction events.

2. Monocytes/Macrophages as Accessory Cells

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

immunity. Monocytes arise from the bone marrow and circulate in the blood for about 1 day, after which they are distributed among different tissues where they differentiate further into macrophages. Monocytes and macrophages do not divide under normal circumstances. As they are exposed to both antigenic stimuli and the products of surrounding cells, monocytes progressively acquire new functional capabilities. They acquire changes in their surface phenotype and secrete a series of products which may regulate the activities of other leukocytes including leukotrienes, prostaglandins (PG), HETEs (hydroxyeicosatetraenoic acids), interleukins and reactive oxidative intermediates (Goodwin & Ceuppens, 1983; Goldings, 1986; Rola-Pleszynski, 1985).

In the tissues, macrophages play an integral role in the response to external stimuli. The functions of macrophages ($M\Phi$) are multiple and their diverse repertoire of surface receptors allow the $M\Phi$ to interact with many extracellular molecules, proteins and polysaccharides. The macrophage can internalize and subject these molecules to intracellular metabolic changes. Macrophages are also highly secretory, including such products as proteases, complement proteins, and regulatory factors such as IL-1, IL-6, tumor necrosis factor (TNF) and arachidonate derivatives. These molecules have a profound effect particularly in the context of the anatomy and environment of a given tissue. The macrophage is therefore a key participant in tissue homeostasis. Most of the macrophage-secreted molecules are released following their activation. Activation stimuli primarily include bacterial products such as lipopolysaccharide and lymphokines. Upon activation by lymphocytes, the monocytes/macrophages acquire new properties such as the up-regulation of class II MHC molecules in response to IFN- γ (Adams & Hamilton,

1987; Koerner & Hamilton, 1987). By virtue of their receptors for lymphokines, MΦ are important effector cells in cellular immune reactions characteristic of delayed hypersensitivity.

The amount of class II MHC on the macrophage membrane is key if this cell is to function as an APC. The level of class II MHC expression is under regulation in the macrophage by IFN-γ. Increase in class II MHC expression is rapid, both *in vitro* and *in vivo*, following exposure to IFN-γ. The release of IFN-γ takes place by way of two different cellular pathways. One involves the activation of T cells by antigen presented by the APCs; the second pathway is independent of T cells and operates only with intracellular pathogenic microorganisms which are taken up by macrophages. This process results in the acute release of TNF with the activation of NK cells to release IFN-γ. Class II expression by the macrophage is inhibited by prostaglandins, contact with immune complexes, microorganisms, and α-fetoproteins (Unanue, 1984; Lu & Unanue, 1988).

I. SUPPRESSION OF CELLULAR RESPONSES BY ANTIGEN

As we have seen, the cellular immune response is elicited and regulated by the complex cooperative interactions of the various T cell subpopulations and APCs. Similarly, the humoral response requires active communication among B cells, T cells and APCs. Inactivation of one or more of these cell types may alter immune responses and lead to immunosuppression.

Immunosuppression has been seen in many clinical states and seems to occur as

a result of infection from certain viral, bacterial and fungal agents. This includes clinical states resulting from *Hepatitis B* virus (HBV) (Kakumu *et al.* 1980), *Bordetella pertussis* (Arora *et al.* 1987), *Toxoplasma gondii* (Luft *et al.* 1988), *Mycobacterium ulcerans* (Pimsler *et al.* 1988), and influenza (Roberts *et al.* 1989). The immunosuppression can be the result of a direct cytotoxic effect on the cells of the immune system. For example, viruses such as HIV may infect and destroy specific cells involved in immunity. Moreover, proteins or glycoproteins of these pathogens may be released and may interact directly with specific cells thus influencing 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 the specific antigens which induced suppression or may be a more generalized suppression to a wide variety of antigens or stimuli (Reinherz & Schlossman, 1981).

In vitro examples of immunosuppression have been gathered from agents which have been associated with clinical immunosuppression. In the late 1970's feline leukemia virus was being studied for its immunosuppressive characteristics. 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 (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 and the lack of a host response has been attributed to immunosuppression. Toxin added at culture initiation suppressed Con A proliferation and the mixed leukocyte reaction (MLR) in a dose-dependent manner. A mechanism of action involving different populations of lymphocytes was not, however, examined.

Suppression of mitogen activity can be induced *in vitro* and *in vivo* by the addition of soluble extracts from *Candida albicans*. 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 (sheep red blood cell) responses in a dose-dependent manner. Both suppressor B cells and suppressor T cells have been implicated in their model.

Pertussis toxin (PT), the main toxin produced by *Bordetella pertussis*, has been reported to suppress immune responsiveness (Arora *et al.* 1987). PT was 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 after less than 2 hours of incubation resulted in no suppression. Finally, they concluded that the suppression was being mediated by CD4⁺ and CD8⁺ suppressor T lymphocytes (Arora *et al.* 1987).

Adherent mononuclear cells (enriched in monocytes) have been implicated in the suppression of lymphocyte proliferation resulting from toxoplasmosis (causative agent, *Toxoplasma gondii*) or *in vitro* incubation with *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 revealed that the presence of CD4⁺ cells, but not CD8⁺ cells, was required for monocytes to become immunosuppressive.

Recently *Helicobacter pylori* was demonstrated to suppress the *in vitro* proliferative response of human peripheral blood mononuclear cells to antigens as well as to mitogens without affecting cell viability (Knipp *et al.* 1994). Pretreatment of monocytes with a soluble cytoplasmic fraction of *H. pylori* led to a suppressed proliferation of T cells after PHA treatment. Culturing of isolated T cells with PHA and PMA revealed that the proliferative response of lymphocytes could also be inhibited independently of monocytes. The anti-proliferative effect was associated with a reduction of IL-2 receptor expression as well as an inhibition of blastogenesis.

A cell-free extract of *Salmonella typhimurium* has also been shown to inhibit T cell mitogen-induced proliferation of spleen cells from non-immunized mice (Katsyhiko & Toshihiko, 1994). The suppression of T cell proliferation was associated with the inhibition of IL-2 secretion and this, in turn, was dependent upon the inhibition of the stimulatory activity of protein kinase C on IL-2 secretion.

Clearly, from the above examples, 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. Immunosuppression induced by microorganisms can involve different cell types and cell subsets of the host immune system. As outlined above, immunosuppressive agents can mediate their effects via activation of "suppressor" macrophages (Luft *et al.* 1988; Vandekerckhove *et al.* 1994), activation of T cells with suppressor activity (Arora *et al.* 1987), or direct alteration of cell functions (Beltz *et al.* 1988) including regulation of monocyte accessory cell activity (Tsuyuguchi *et al.* 1990). As early as 1982 it was shown that both CD4⁺ and CD8⁺ populations were necessary for the induction of suppression (Morimoto *et al.* 1982). Communicative interactions between CD4⁺ and CD8⁺ cells were required for the induction of the suppressor-effector function in an *in vitro* primary anti-dinitrophenol (DNP) antibody response. The effector cells of the suppression appeared to be CD8⁺, however, suppression could not be exerted without the initial induction step by CD4⁺ 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 also be of great importance. Conversely, perturbation of this network may lead to serious systemic consequences such as autoreactivity and tissue destruction in addition to suppression.

J. IMMUNE SUPPRESSION BY IONIZING RADIATION

The immune system can also be adversely affected after exposure to ionizing

radiation. Ionizing radiation induces biochemical changes in living material either directly as a result of striking a sensitive target, or indirectly (and primarily) by the action of free radicals. Ionizing radiation is taken to include X and γ rays, alpha and beta particles, electrons, protons, neutrons and cosmic rays. In contrast, ultraviolet and visible light, infrared radiation and radio waves produce no ionization in living matter. Ionization is the process by which a fast moving quantity of energy is transferred to some of the atoms of the material through which it is travelling, leaving them as electrically charged ions. The end result is often the production of free radicals which are electrically neutral, highly reactive atoms or molecules possessing an unpaired electron in their outer orbits. The free radical then has a strong tendency to pair the odd electron with a similar one in another radical or to eliminate the odd electron by an electron transfer reaction with a normal, stable atom. Free radicals can therefore be electron acceptors (oxidizing species) or electron donors (reducing species). Possessing a lifetime generally less than 10^{-10} sec, free radicals are very short-lived.

In living matter water is the most abundant material and is thus the most likely to become ionized. Ionization of water produces the free radicals $\text{OH}\cdot$ and $\text{H}\cdot$ which can react with each other, with other water molecules, or with their own reaction products. These radicals are of considerable energy and can break chemical bonds. In addition, the free radicals can abstract hydrogen from organic molecules thereby producing secondary free radicals, $\text{R}\cdot$. A great part of the radiobiological damage is a consequence of this indirect action, since cells and tissues are composed of 70-90 percent water. Free radicals may also react with molecules of oxygen to produce peroxide radicals, both of hydrogen

and of important organic molecules, some of which have been shown to be involved in the initiation and propagation of free-radical chain reactions which are potentially highly damaging to cells (Riley, 1994).

Although the chain of chemical reactions which result in damage to critical biological molecules may take as little as 10^{-6} seconds, the final expression of biological damage may take hours, days or even years. High doses of radiation can kill cells by the rapid cessation of cellular metabolism and cellular disintegration. This type of death is often termed "non-mitotic" or "interphase death". It has also been demonstrated that radiation can induce the actively regulated mechanism of cell death in mammalian cells, known as "apoptosis" or "programmed cell death" (Searle *et al.* 1975; Hendry *et al.* 1987). During apoptosis, cells shrink and degrade their chromatin to oligonucleosome-length fragments, before loss of membrane integrity. The apoptotic stimulus created by ionizing radiation, however, is not well defined. Radiation at much lower doses can also "kill" cells when they undergo cell division. At the biophysical level, doses of a few Gy can cause DNA strand breakage which results in the inhibition of DNA synthesis and the cessation of mitosis. This loss of a cell's ability to undergo unlimited cell division is termed "reproductive death". Mitosis and cell division are among the most radiation-sensitive processes which a cell performs. Lower doses result in delays in the onset of DNA synthesis, a slowing down of the passage of cells through S phase, and a reduction in the overall amount of DNA synthesis (Anderson, 1976).

The survival of an animal following irradiation depends largely on the recovery of hematopoiesis. The hematopoietic capacity of the bone marrow, the organized

lymphoid tissues, and individual small recirculating lymphocytes are all exquisitely radiosensitive. Significant depression of the lymphocyte count is noted after whole-body exposure to doses as low as 0.25 Gy, and 1 Gy reduces the peripheral lymphocyte count to 25% of normal values within 4 hours of exposure in rats 4-7 months old (Anderson, 1976). Since hematopoiesis provides a renewal source of mature circulating blood cells, those cells which are killed by irradiation or subsequently die as part of their lifespan cannot be replaced until hemopoietic recovery occurs. This leaves the host particularly susceptible to infection.

1. *Activation of Suppressor Cells*

There is a body of evidence to suggest that in addition to the cytorreduction of the lymphoid system, ionizing radiation can also cause active suppression of the immune system through induction of non-specific "suppressor cells". In neonatal rodents several investigators have described a non-specific suppressor cell which inhibits both cellular proliferation and the generation of cytolytic T cells by responder cells in the mixed leukocyte reaction (MLR) (Argyris, 1978; Mosier *et al.* 1977; Ptak & Skowron-Cendrzak, 1977). It has been suggested that the naturally occurring suppressor cells in the neonatal spleen are intimately involved in the induction of tolerance to allogeneic tissues. These suppressor cells inhibit the generation of antigen-specific cytolytic cells and enhance the generation of antigen-specific suppressor cells (Argyris, 1982).

Total-lymphoid irradiation (TLI) has been found to mimic several of the conditions found in the neonate including the ease of tolerization, the marked reduction in the

proportion of T and B cells, and the high proportion of "null" cells (Oseroff *et al.* 1984). For several weeks after TLI the lymphoid tissues were found to contain non-specific suppressor cells (Strober *et al.* 1979; Oseroff *et al.* 1984). Okada *et al.* (1983) demonstrated that spleen cells obtained from TLI mice within a short time after the completion of radiotherapy can inhibit the proliferation of responder cells and the generation of cytolytic cells in the MLR. Inhibition was independent of the mouse strain which was used as the source of responder or stimulator cells. Investigators have also shown that suppressor cells of the MLR are present in the spleen of mice which have been given whole-body irradiation (WBI) (Waer *et al.* 1984). These cells were first identified several days after irradiation, and they did not persist for nearly as long as those observed in the spleen of neonates or TLI-treated mice. The suppressor cells were found to have characteristics in common with natural killer (NK) cell lineage: both are large granular lymphocytes found in the "null" cell population (that is, they do not readily fall within the T lymphocyte, B lymphocyte, or monocyte/macrophage lineages), both lack antigen specificity, and both carry out their effector function without antigenic stimulation. The suppressors, however, appeared to lack natural killer activity as measured by the lysis of YAC-1 cells (Oseroff *et al.* 1984). In view of their characteristics shared with the NK lineage, these cells have thus been called natural suppressor (NS) cells. NS cells secrete soluble suppressor factors which block IL-2 synthesis and inhibit IL-2-dependent T cell proliferation in the presence of excess IL-2 (Moore *et al.* 1992; Van Vlasselaer *et al.* 1992).

The natural suppressor cells appear only briefly during the early maturation of the

lymphoid tissues, but can be induced in adults by manipulation of the lymphoid tissues with certain treatment regimens, such as TLI. Both TLI and neonatal mice have spleens which are known to be primarily hematopoietic organs which contain only a small percentage of T and B cells (Oseroff *et al.* 1984). Similar T and B lymphocytopenia was found in the peripheral blood (Strober *et al.* 1979). Return to normal percentages of T and B cells occurred about 30 days after the end of irradiation. Thus, the regulatory effects of null suppressor cells on splenic T lymphocytes occur at a time when the number of T cells is relatively low.

Do suppressor cells have a role to play in the critical immune suppression after exposure to ionizing radiation? The mechanism by which radiation activates suppressor cells or eliminates a down-regulating control mechanism of such cells is not known, but it may be related to changes in the relative proportions of certain lymphocyte populations. An understanding of the mechanism of induction of suppressor cells in the irradiated host would benefit the treatment of individuals exposed to ionizing radiation from any source. Conversely, the induction of suppressor cells could be manipulated for improved treatment of transplantation and the induction of tolerance to non-self. Suppressor cells may be activated directly by the radiation, or their activity may be related to their relative resistance to radiation damage. Non-dividing cells, such as macrophages and suppressor cells, are not susceptible to reproductive cell death and therefore may be less sensitive to radiation than the other T and B lymphocytes.

It has thus been reported that both radiation and microorganisms may induce

immunosuppression via suppressor cells. There may be an integrative role of specific infections before or after irradiation in enhancing suppression. Exposure to radiation disrupts the gastrointestinal lining by preventing replacement of the epithelial cell layer on the villi. Normally non-threatening gut bacteria or endotoxins are able to invade the blood and due to the body's reduced immunity, an opportunistic infection may arise. Are microbes more apt to induce suppression after radiation? Or are suppressor cells activated due to a lack of control after radiation? Experiments done in germfree mice have shown that the absence of bacteria makes them more resistant to the effects of radiation (Broerse *et al.* 1984). The hypothesis of two signals for the induction of suppression has been supported by other investigators (Ullrich & Magee, 1988) who demonstrated that T suppressor cells were present in the spleens of UV-irradiated antigen-sensitized mice. It was suggested that UVR may alter the ability of APCs to activate helper cells and, at the same time, activate a UVR-resistant APC which induced suppressor cells.

Our laboratory has examined the effect of antigen on the specific and non-specific immune response both *in vivo* and *in vitro*. Work done by Filion *et al.* (1989) demonstrated that the bacterial product Tetanus toxoid (TT) can induce non-specific suppressor cells which down-regulate the immune response of human peripheral blood mononuclear cells *in vitro*. Since immunosuppression induced by either microorganisms or irradiation can involve different cell types and cell subsets of the host immune system, it was our hypothesis that changes in the proportion of certain mononuclear cell populations and the expression of certain surface markers are related to the activation or

unmasking of suppressor cells. It was thus our aim to characterize the various cell subsets of splenic mononuclear cells of the mouse and to determine how they are influenced by Tetanus toxoid from *Clostridium tetani* and whole-body irradiation. Such a characterization and quantification of the various mononuclear cell populations has been made possible with the advent of flow cytometric analysis. In recent years, flow analysis and cell sorting have become standard research tools in immunology and in an expanding range of other disciplines. The power of flow analysis derives from its ability to make rapid quantitative multiparameter measurements on statistically large numbers of cells.

K. A ROLE FOR SOLUBLE MEDIATOR(S) IN IMMUNE SUPPRESSION?

There is evidence indicating that various antigens and radiation are capable of causing immune suppression. The question remains, however, as to what the mechanism is and what the mediators of immune suppression are. Suppressor cells may produce an excess of cytokines with inhibitory function. For instance, transforming growth factor- β (TGF- β) is a powerful inhibitor of T and B cell proliferation, and IFN- γ inhibits the proliferation of B cells *in vitro*. We focused our attention on whether the newly described suppressive cytokine, IL-10 (cytokine synthesis inhibitory factor), has a role to play in the immune suppression induced by radiation and/or TT. IL-10 was originally identified as a Th2 cell product which inhibited the proliferation of Th1 cell clones by downregulating IFN- γ and IL-2 production (Fiorentino *et al.* 1989). Subsequently other cell types including B cells (O'Garra *et al.* 1992), Tc cells (Inoue *et al.* 1993), and monocytes/macrophages (Fiorentino *et al.* 1991a) were shown to be sources of IL-10, and

a spectrum of additional IL-10 activities was identified.

There is some evidence to suggest that IL-10 may have a role in our hypothetical mechanism. Despite the limited ability of UV radiation to penetrate tissue, the suppression seen after exposure to UV radiation is systemic. Although the mechanism is not clear, most of the evidence to date supports the concept that UV-induced soluble suppressive factors are involved. IL-10 from keratinocytes has been demonstrated to play a role *in vivo* in UV-induced immunosuppression by impairment of antigen-presenting cell function (Enk & Katz, 1992; Ullrich, 1994).

Recently the expression of IL-10 mRNA was examined *in vivo* by reverse transcriptase - polymerase chain reaction (RT-PCR) in normal and irradiated mice (Broski & Halloran, 1994). Expression of IL-10 mRNA was increased in the spleen and kidney in a dose-dependent fashion after irradiation. The authors concluded that IL-10 mRNA is detectable in many organs of normal mice and that the increased levels of steady state IL-10 mRNA after irradiation suggest that this molecule plays a general role in the physiology of all tissues and may explain some of the immunosuppressive effects of ionizing radiation.

II. HYPOTHESIS

Immunosuppression induced by microorganisms and irradiation may involve different cell types and cell subsets including the activation of suppressor cells. It is the hypothesis that 1) Tetanus toxoid (TT) and irradiation are capable of actively suppressing murine blastogenic responses *in vitro* and have an integrated role in enhancing immune suppression, 2) changes in the proportion of mononuclear cell populations and the expression of certain cell surface markers are related to the unmasking or activation of suppressor cells, and 3) the suppression is mediated through soluble factors.

III. OBJECTIVES

The work reported in this thesis addresses the potential mechanisms underlying the immunosuppression induced by TT and irradiation on murine T cells. Specifically,

- 1) to determine if Tetanus toxoid can actively suppress murine blastogenic responses,
- 2) to determine if irradiation can actively suppress murine blastogenic responses,
- 3) to determine the combined effect of irradiation and Tetanus toxoid on immunosuppression, and
- 4) to study the mechanism(s) involved in the generation of the suppression by
 - a) identifying the cell types and subsets of mononuclear cells
in the murine spleen using flow cytometric analysis,

- b) determining how cell types and surface markers are influenced by TT and whole-body irradiation, and
- c) investigating the possible involvement of interleukin-10 as an effector of the suppression.

IV. METHODS AND MATERIALS

A. EXPERIMENTAL ANIMALS

Female C57BL/6 mice, 18-20 grams and 6-8 weeks of age from Charles River Laboratories (Montreal, PQ) were used. They were inbred mice so that mononuclear cell populations and cell surface marker expression could be examined using mAbs and compared to treatment conditions with minimum variability. The mice were kept 4 to 6 per cage at room temperature and were supplied with Purina Mouse Chow Pellets and acidified tap water (pH 2.5) *ad libitum*. All experiments received prior approval from the University of Ottawa Animal Care Committee and were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

B. SPLEEN REMOVAL

The mice were sacrificed by cervical dislocation after which their bodies were sterilized by immersion in 70% alcohol. The mice were placed ventral side down and an incision was made along the left flank. The peritoneal mesentery which covers the spleen was cut. The spleen was removed, cleansed of adipose tissue, and placed in a sterile Eppendorf vial containing phosphate buffered saline, pH 7.2 (Gibco Laboratories, Burlington, ON).

C. PREPARATION OF SINGLE CELL SUSPENSIONS

The spleens were transferred to a sterile glass petri dish containing approximately

1 ml of phosphate buffered saline (PBS). The spleens were massaged with sterile 16 gauge needles bent at a 90 degree angle. Incisions were made at the base and apex of the spleen and the cells were gently massaged out of the spleen capsule. The contents of the dish were aspirated and expelled 2-3 times using a 10 cc syringe and a 23 gauge needle in order to produce a single cell suspension. The cell suspension was transferred to a 14 ml polypropylene test tube. The petri dish was rinsed twice with fresh PBS to collect any remaining cells and the rinse was added to the test tube. The suspension was allowed to settle for 5 minutes allowing clumps of cells to settle at the bottom of the tube. The supernatant was removed and added to a 50 ml conical polypropylene centrifuge tube; the last ml of the suspension was discarded. The volume was recorded and an automated nucleated cell count of the suspension (erythrocytes were removed by Zapoglobin II, Coulter Electronics, Burlington, ON) was performed on a Coulter Zm Cell Counter. The viability of the cells was determined using 0.4% Trypan Blue dye exclusion.

D. ISOLATION OF LYMPHOCYTES

The cell suspension was diluted in PBS to a concentration of 1×10^7 cells/ml and the mononuclear cells were isolated from the cell suspension on Lympholyte-M (Cedarlane Laboratories, Hornby, ON) by gradient centrifugation for 20 minutes at 1500 x g and 22°C. The resulting white buffy layers which consisted of splenic mononuclear cells (SMNC) (lymphocytes, NK cells, and monocytes) were collected by means of a plastic 2 ml pipette connected to a Pipet-aid aspirator (Drummond Scientific, Broomall,

PA). The cells were washed twice with PBS and resuspended in complete medium (Iscove's Modified Dulbecco's Medium (Gibco) containing L-glutamine and 225 mM HEPES buffer). Prior to use, the medium was enriched with 20% heat-inactivated bovine calf serum (Gibco), 10 µg/ml gentamicin (Gibco), and 1% of 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Company, St. Louis MI). A cell count and viability were recorded.

E. LYMPHOCYTE PROLIFERATION ASSAY

Murine splenic mononuclear cells (0.25 to 4×10^6) were added to triplicate wells of 96-well flat-bottomed tissue culture plates (CoStar, Cambridge, MA) and incubated (in a 0.2 ml volume) with the required mitogen at 37°C for 72 hours in 5% (v/v) CO₂ and 95% (v/v) air. DNA synthesis was assayed by the addition of 1.0 µCi of tritiated thymidine (³H-TdR) (Amersham Life Science, Buckinghamshire, UK), specific activity 5.0 Ci/mM, to each culture during the final 18-24 hours of the incubation period. Cultures were harvested onto glass fiber filter paper by means of an automated sample harvester (Skatron Inc., Sterling, VA). The filters were washed with water, dried, and placed into a scintillation cocktail. The radioactivity was determined in a liquid scintillation counter (Beckman LS3801, Toronto, ON). Suppression of the mitogenic response was achieved by the addition of Tetanus toxoid (TT) (Institut Armand Frappier, Laval, PQ), at varying concentrations (0.5-5 µg/ml), to culture. The TT was either present throughout the entire culture period or removed by washing at the times indicated.

F. TRANSFER OF THE SUPPRESSIVE EFFECT TO FRESH SYNGENEIC CELLS

SMNC were harvested from mice which had been irradiated (0 to 700 cGy).

These SMNC were cultured in complete medium with or without optimal TT (2.5 µg/ml as determined in Figure 5) for 48 hours at concentrations of 5×10^3 to 2×10^6 cells in a final volume of 0.6 ml per well, using flat-bottom 24-well microculture plates (Corning, Corning, NY). After the 48-hour culture period, the irradiated/TT-treated cells (effector cells) were co-cultured in various numbers with a constant number (2×10^6) of fresh syngeneic SMNC (target cells). The effector and target cells were stimulated with phytohemagglutinin for 72 hours. Cultures were pulsed with 1.0 µCi of tritiated thymidine (^3H -TdR) 18-24 hours prior to culture termination and harvested. Effector cells were treated *in vitro* with 30 Gy prior to co-culture in order to prevent their proliferation.

G. IRRADIATION

Mice were placed in ventilated plexiglass containers and exposed bilaterally to gamma radiation (0-700 cGy) at a dose rate of 1.15 Gy/min from a ^{137}Cs radiation source (Nordion International Inc., Kanata, ON).

H. FLASK CULTURING FOR FLOW CYTOMETRIC ANALYSIS AND MITOGENIC RESPONSES

Cells destined for flask culture were isolated on Lympholyte-M and diluted to a concentration of 4×10^6 cells/ml. Cultures were prepared by adding 5 ml of 4×10^6 cells/ml suspension to 25 ml polystyrene tissue culture flasks (Corning) containing 5 ml complete medium with or without (control) TT (2.5 µg/ml). These flasks (2×10^6 cells/ml) were incubated for 48 hours with 5% CO_2 at 37°C. The caps on the flasks were kept loose to ensure adequate ventilation.

Following the 48-hour incubation period the cells were suspended using a pipette and pooled in 50 ml conical tubes. The flasks were washed with 5 ml of cold complete medium and any adherent cells were removed with a cell scraper (Costar). The flask contents were added to the 50 ml conical tubes and centrifuged at $300 \times g$ for 10 minutes at 22°C . The supernatant was suctioned off leaving approximately 1 ml to resuspend the pellet. The cells were washed in complete medium and centrifuged at $300 \times g$ for 10 min at 22°C . Those cells which were to be used in flow cytometric analysis (FCA) were resuspended in 2 ml of PBS/0.1% sodium azide (NaN_3) (Fisher Scientific, Orangeburg, NY) and counted. The cells were further diluted with PBS/0.1% sodium azide to a concentration of 1×10^7 cells/ml. The viability of the cells was checked by Trypan Blue dye exclusion and the cells were prepared for FCA. Those cells which were to be used for mitogen blastogenic response assays were diluted in complete medium to a concentration of 4×10^6 cells/ml and cultured (in triplicate) with mitogen in a total volume of 0.2 ml of culture medium. The optimal concentration (as determined in Figures 3 and 4) of phytohemagglutinin (PHA) (Gibco) or concanavalin A (Con A) (Sigma) was employed.

L FLOW CYTOMETRIC ANALYSIS OF MURINE SPLENIC MONONUCLEAR CELLS

1. *Immunofluorescent Staining of Cells*

Cells to be analyzed by FCA were diluted with PBS/0.1% sodium azide to a concentration of 1×10^7 cells/ml. Aliquots of 100 μl were placed into 7 ml polystyrene

conical test tubes which contained 100 μ l of rat Ig (10 μ g/ml) to block non-specific Fc receptor binding to splenic mononuclear cells.

TABLE 1. MONOCLONAL ANTIBODIES AND COMBINATIONS USED IN THIS STUDY^a

TUBE	ANTIBODY ^b	ISOTYPE	[OPTIMAL] μ g/10 ⁶ cells	CELL REACTIVITY
1	Auto			
2	Isotype controls	IgG2a-PE IgG2b-FITC	2 0.4	
3	Thy1.2-PE CD45-FITC	IgG2b IgG2b	0.4 0.1	T cells B cells
4	Thy1.2-PE CD8-FITC	IgG2b IgG2a	0.4 2	T cells T _s /T _c
5	Thy1.2-PE CD4-FITC	IgG2b IgG2a	0.4 0.1	T cells T _H /T _{Ind}
6	NK-FITC	IgG2a	0.1	NK cells
7	CD4-FITC Ia-PE	IgG2a IgG2a	0.1 0.5	T _H /T _{Ind} Class II MHC
8	CD4-FITC CD25-PE	IgG2a IgG2b	0.1 0.4	T _H /T _{Ind} T _{act}
9	CD4-FITC CD71-PE	IgG2a IgG2a	0.1 0.2	T _H /T _{Ind} T _{act}
10	CD8-FITC Ia-PE	IgG2a IgG2a	2 0.5	T _s /T _c Class II MHC
11	CD8-FITC CD25-PE	IgG2a IgG2a	2 0.4	T _s /T _c T _{act}
12	CD8-FITC CD71-PE	IgG2a IgG2a	2 0.2	T _s /T _c T _{act}
13	F4/80-PE	IgG2b	0.2	Macrophages
14	CD45-FITC Ia-PE	IgG2b IgG2a	0.1 0.5	B cells Class II MHC

^aA summary of CD markers is given by Clark and Lanier (1989), with more details provided by Schlossman *et al.* (1994b).

^bAll mAbs were obtained from Pharmingen, San Diego, CA, except F4/80-PE (Caltag, San Diego, CA)

The cells were incubated for 10 minutes at 22°C. The appropriate fluorescently labelled

monoclonal antibodies (mAbs), the quantities of which had been previously determined by titration experiments, were added to each tube. The appropriate isotypic controls were also run with each sample. The tubes were vortexed gently and incubated at room temperature for 15 minutes. The cells were then washed with 2 ml PBS/0.1% sodium azide and centrifuged at 1500 x g for 5 minutes at 22°C. The cells were fixed with 2% paraformaldehyde (Sigma) for 10 minutes, rewashed in PBS/0.1%Na azide and resuspended to a final volume of 300 µl. Table 1 lists the antibodies used.

2. Flow Cytometric Analysis by the Coulter Epics Profile II:

Flow cytometric analysis was performed using the Coulter Epics Profile II (Coulter Electronics) which was operated at 15 mW and 488 nm. For further details on the theory behind flow analysis, the reader is directed to Appendix A. Standard Brite Flow Cytometric Fluorescence Intensity Standardization Beads (Coulter) were used to calibrate the fluorescence signals for Fluorescence 1 (green) and Fluorescence 2 (red). By ensuring that the peaks for each fluorochrome fell within the established ranges, as defined by the mean channel numbers for the fluorochromes, it was possible to compare samples that had been run days or even months apart.

Fluorescence signals were directed by a 550 nm long-pass dichroic mirror to the photomultiplier tube (PMT) which was equipped with either a 575 nm interference filter (red) or a 525 nm interference filter (green). Colour compensation to correct for spectral overlap (contamination of FITC fluorescence in the PE channel) was performed using the system software. Approximately 5 000 to 10 000 cells were analyzed per sample and the

resulting histograms were analyzed using the Epics Elite Flow Cytometry Workstation software (Coulter). The software was used to set gates on forward scatter (FS) versus side scatter (SS) dot plots and thus focused analysis only on mononuclear cells, excluding granulocytes and non-cellular debris (Figure 1A). An appropriate isotypic control was used to negate non-specific antibody binding to mononuclear cells in each sample. The cursor was set as in Figure 1B in order to gate out 98 ± 1 non-specific staining. Fluorescence to the left of the cursor was recorded as non-specific staining and to the right, as specific staining.

Prior to using a mAb for a particular surface marker, it was first necessary to determine the optimal concentration, that is, the lowest concentration of mAb which consistently labelled the highest percentage of SMNC and provided the highest level of fluorescence intensity. The mean channel number or the mean fluorescence intensity is a measure of the quantity of antigen bound to an individual cell. In Figure 2 the result of a titration experiment is shown for which 0.04, 0.16, 2 and 10 μg of FITC-labelled anti-CD8 mAb in a volume of 10 μl was added to 1×10^6 cells in 0.2 ml of PBS/0.1% NaN_3 . As the quantity of mAb was increased from 0.04 to 2 μg , the mean channel number of the CD8 positive population increased. This is seen as a shift of this population to the right. The addition of a greater quantity (10 μg) of the mAb did not result in any further binding which demonstrates a saturation effect. However, at higher concentrations of mAb there was greater non-specific binding to other cell types, as indicated by a rightward shift of the unstained cells with respect to the autofluorescence. Therefore, for the anti-CD8 FITC mAb, a concentration of 2 $\mu\text{g}/10^6$ cells was used to

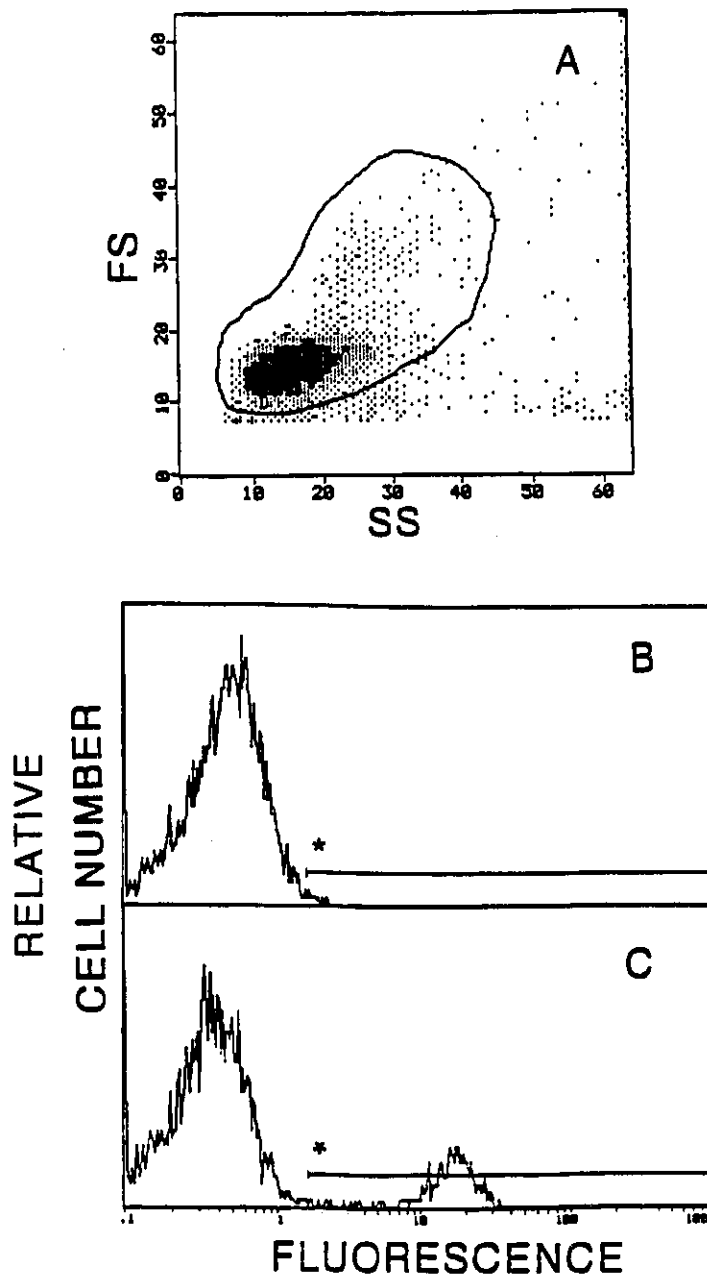


FIGURE 1. CHARACTERIZATION OF SMNC BY FLOW CYTOMETRY AND GATING.

The characterization of SMNC populations was performed using the Coulter Epics Profile II and the Elite software. A dot plot (A) of forward light scatter (FS) versus side light scatter (SS) of processed SMNC was used to set a gate on mononuclear cells as indicated. In (B) a histogram of an IgG2a isotypic control sample gated on mononuclear cells is demonstrated. The cursor (*) was set to gate out $98 \pm 1\%$ of non-specific staining. In a sample labelled with CD8-FITC (C), fluorescence to the right of the cursor (*) was recorded as specific staining.

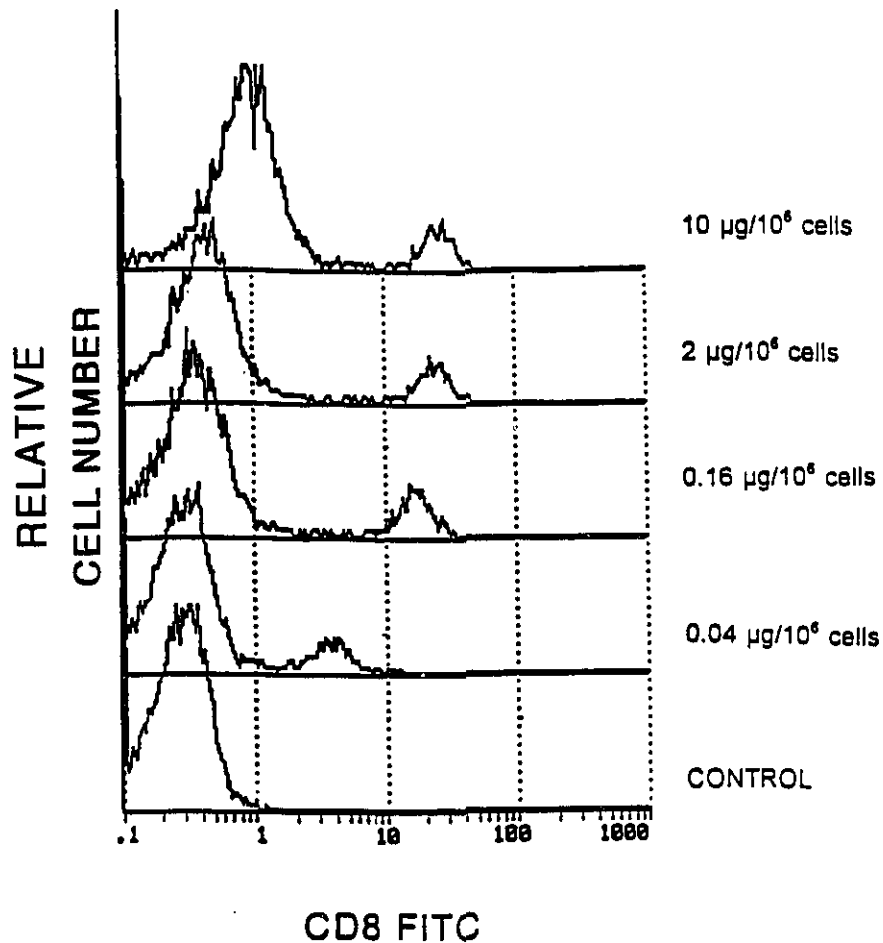


FIGURE 2. TITRATION OF ANTI-CD8 FITC MONOCLONAL ANTIBODY

Expression of CD8 by murine SMNC is demonstrated. The horizontal axis represents the log fluorescence intensity of CD8 expression and the vertical axis represents the number of cells. Anti-CD8 FITC (0.04, 0.16, 2, and 10 μg) was added to 1×10^6 SMNC. Increasing the quantity of anti-CD8 FITC resulted in a greater mean intensity of fluorescence as indicated by a rightward shift of the CD8 positive population. Saturation was observed by 2 μg anti-CD8 FITC/ 10^6 cells, so this concentration was used in all subsequent experiments.

stain SMNC for subsequent FCA. Titrations were performed with each of the antibodies used in this study.

J. 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 (Gibco) 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, whereas non-viable cells were stained blue.

K. ELISA FOR IL-10 CONCENTRATION

The levels of IL-10 in culture supernatant were measured using an ELISA kit (PerSeptive Diagnostics, Cambridge, MA). Briefly, the IL-10 levels in culture supernatants measured by ELISA were performed in the following manner: dilutions of IL-10 standards of known concentration or experimental culture supernatants were added (50 µl/well) into anti-IL-10 antibody-precoated wells and incubated at room temperature for 1 hour. The plates were washed with the kit wash buffer containing tris-buffered saline and surfactant with 0.05% ProClinic-300 added as a preservative using the Bio-Tek EL403 automated microplate washer. Biotinylated rat monoclonal anti-IL-10 antibody (50 µl/well) was added and the plate was incubated for 1 hour at room temperature and washed. A streptavidin-horseradish-peroxidase conjugate was added to the plate (50 µl/well) and incubated at room temperature for 30 minutes. The plates were washed and 50 µl of substrate (3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide) was

added. The reaction was stopped after 15 minutes with 1N HCl and the plates were read at 490 nm employing the Bio-Tek/ELISA plate reader.

The standard curves relating standard IL-10 and optical density were plotted. The concentration of IL-10 in the culture supernatants was calculated from the optical density of 50 µl samples.

L. CALCULATIONS

1. *Percent suppression:*

For each experiment, each condition was performed in triplicate and tritiated thymidine incorporation was measured in counts per minute (CPM). The mean CPM and standard deviation were calculated for each condition. Calculation of percent suppression in all experiments was performed as follows:

$$\text{Percent Suppression} = \frac{1 - (\text{CPM of cultures} + \text{TT} + \text{PHA}) - (\text{CPM of cultures} + \text{medium})}{(\text{CPM of cultures} + \text{PHA}) - (\text{CPM of cultures} + \text{medium})} \times 100\%$$

2. *Absolute cell counts:*

$$\text{Absolute Counts} = \text{Total White Blood Cell Count} \times \% \text{ Mononuclear cells} \\ \times \% \text{ Positively stained Cells}$$

3. *Surviving fraction:*

$$\text{S.F.} = \frac{\text{Number of cells surviving following a given radiation dose}}{\text{Number of cells from unirradiated control mice}}$$

M. STATISTICAL ANALYSIS

All data are presented as mean $\pm$ standard error of the mean (SEM), or $\pm$ standard deviation (SD) where indicated.

The effects of culturing SMNC with TT on PHA blastogenic responses, cell viability, proportions of cell subsets, and mean channel fluorescence (MCF) for surface markers, and the levels of IL-10 in culture supernatants were analyzed using the Student's *t*-test with a two-tailed test of significance (McClave *et al.* 1988). Differences at the $p < 0.05$ level were considered to be statistically significant.

The effects of WBI using doses of 100, 400, and 700 cGy on days 1, 4, and 7 post-irradiation on nucleated cell count, PHA response, and populations (absolute numbers and relative proportions) of SMNC were compared by two-way factorial analysis of variance (ANOVA). If the results from this test were significant ($p < 0.05$), a multiple comparison test (Bonferroni) was applied.

The ^3H -TdR incorporation, cell counts, and flow cytometric analysis results of sham-irradiated mice were compared (Student *t*-test) with non-irradiated mice. No significant differences were found between these groups so the data was pooled for statistical comparisons against irradiated mice.

1. Flow Cytometry

i. Linear Region Statistics

Statistics for a region on a histogram were calculated by the Elite software as follows:

$$\text{Percent} = \frac{\text{Number of cells in the region}}{\text{Total number of cells in the histogram}}$$

$$\text{Mean} = \frac{\sum (\text{channel number} \times \text{count in the channel})}{\text{area}}$$

$$SD = \frac{\sum ((\text{channel number} - \text{Mean})^2 \times \text{count in the channel})}{\text{area}}$$

For Mean and SD, the summations were performed over all the channels that lie within the region.

ii. Log Region Statistics

Log Mean Channel = Intensity (Mean)

Log SD = Intensity (Mean + SD/2) - Intensity (Mean - SD/2)

The Elite software displayed relative intensity numbers on two-parameter log histograms.

The following formula was used to convert relative intensity numbers to log channel numbers:

$$N(\log \text{ channel number}) = 256 \log \frac{(10 \times \text{relative intensity})}{1.024}$$

V. RESULTS

A. CULTURE CONDITIONS FOR MITOGENIC STIMULATION

Experiments were performed to determine the optimal concentrations of cells and mitogens for the induction of blastogenesis. The optimal PHA and Con A responses were determined by varying the cell concentration and the mitogen dose in a 3-day culture (Figures 3 and 4), as measured by ^3H -Thymidine incorporation. A plateau of this response was reached when 2×10^5 cells were cultured with a 1:25 concentration of PHA or 12.5 $\mu\text{g/ml}$ of Con A in a total volume of 0.2 ml. These conditions were chosen so as to limit any response variability due to fluctuations in cell number, while maintaining a sensitivity to any suppressive activity and minimizing the amount of cells necessary to obtain a sufficient blastogenic response.

The higher proliferative response caused by Con A in comparison to that which was caused by PHA was expected, since Con A stimulates both mature and immature T cells, whereas PHA is able to stimulate only mature T cells (Paul, 1989). For this reason Con A was used as a positive control for all mitogenic experiments.

B. SUPPRESSION OF THE MITOGENIC RESPONSE BY TETANUS TOXOID

1. *Effect of TT on the PHA Response*

Murine spleen cells were cultured with Tetanus toxoid (TT) (0.5-5.0 $\mu\text{g/ml}$) in 96-well plates and PHA was added to activate the splenic T lymphocytes. The effect of soluble TT on the PHA response is expressed in terms of percent suppression in Figure

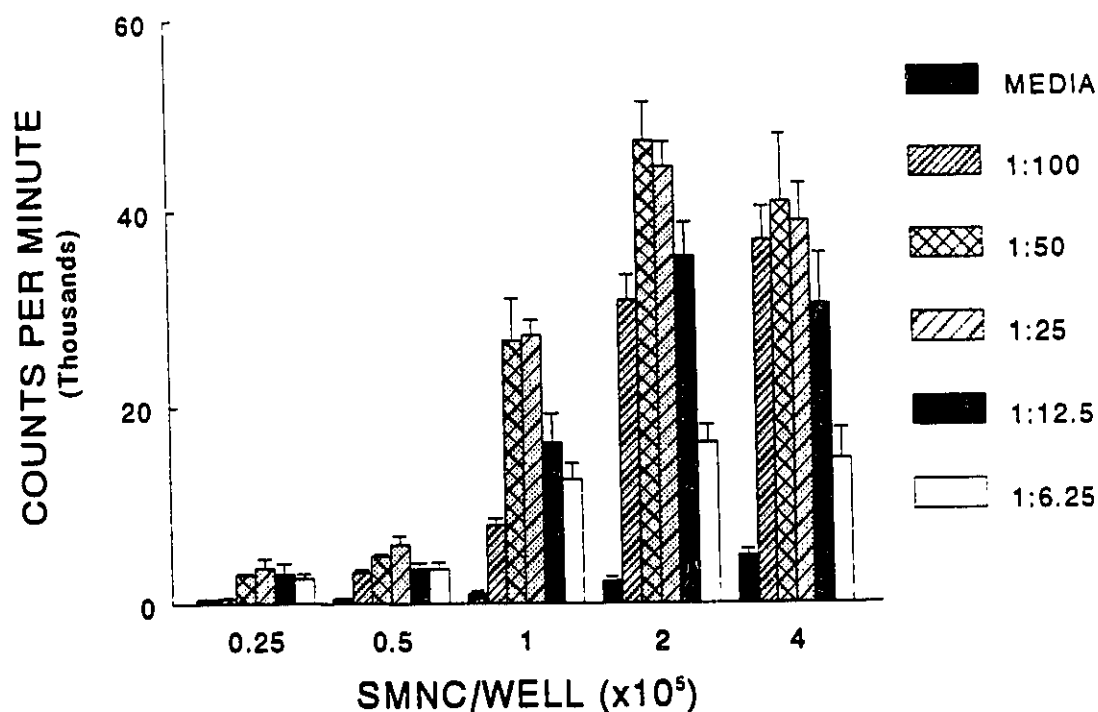


FIGURE 3. PROLIFERATIVE RESPONSE OF MURINE SPLENIC MONONUCLEAR CELLS TO PHA.

SMNC (0.1 ml of 0.25, 0.5, 1, 2, and 4×10^6 cells/ml) were incubated for 72 hours with medium and various dilutions of PHA (final culture dilutions of 1:6.25, 1:12.5, 1:25, 1:50, and 1:100). The final numbers of cells per culture in a final well volume of 0.2 ml were 0.25, 0.5, 1, 2, and 4×10^5 . The amount of ^3H -Thymidine incorporated is expressed in counts per minute. The data ($n=6$) represent the mean and SEM.

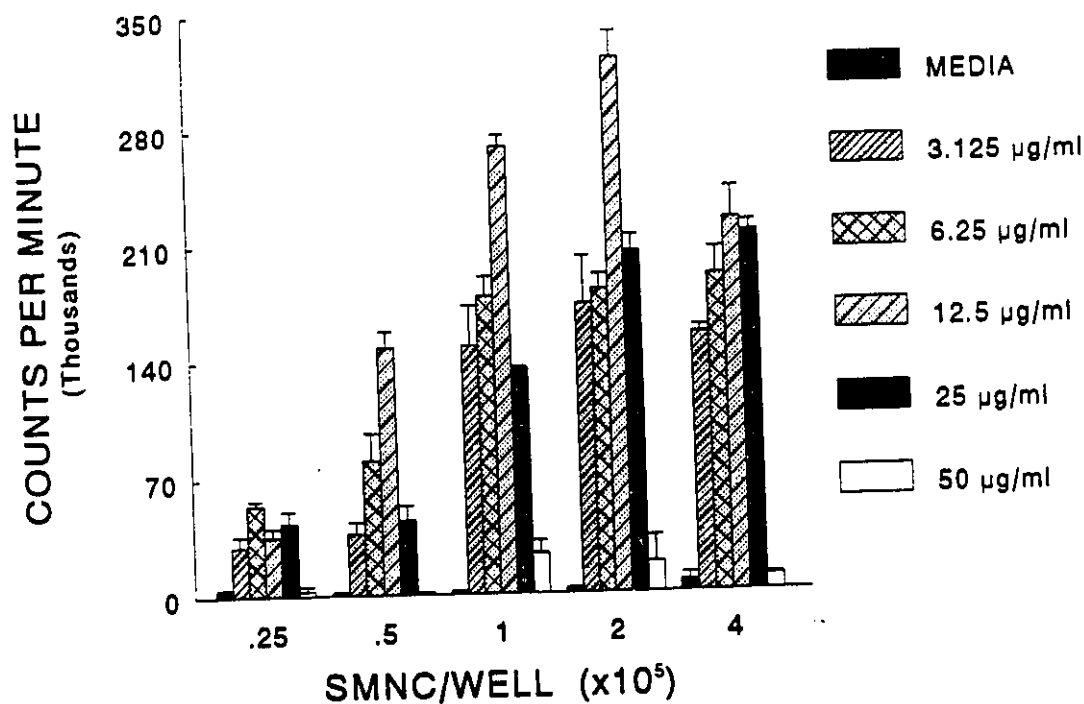


FIGURE 4. PROLIFERATIVE RESPONSE OF MURINE SPLENIC MONONUCLEAR CELLS TO CON A.

SMNC (0.1 ml of 0.25, 0.5, 1, 2, and 4×10^6 cells/ml) were incubated for 72 hours with medium and various dilutions of Con A (final culture dilutions of 3.125, 6.25, 12.5, 25, and 50 $\mu\text{g/ml}$). The final numbers of cells per culture in a final well volume of 0.2 ml were 0.25, 0.5, 1, 2, and 4×10^5 . The amount of ^3H -Thymidine incorporated is expressed in counts per minute. The data ($n=6$) represent the mean and SEM.

5B. Doses of 1.5 to 5.0 $\mu\text{g/ml}$ resulted in a significant suppression of the PHA response as compared to the control SMNC which were activated with PHA in the absence of TT ($p < 0.05$) (Figure 5A). The PHA blastogenic response was depressed by 36% with a dose of 1.5 $\mu\text{g/ml}$ of TT, 64% with a dose of 2 $\mu\text{g/ml}$ of TT; and severely or completely depressed with doses of 3-5 $\mu\text{g/ml}$. A TT concentration of 2.5 $\mu\text{g/ml}$ was chosen for further experiments because the suppressive activity of TT was observable but not so completely suppressive as to prevent the detection of any additional suppression as a result of irradiation.

2. Effect of Duration of Exposure to TT.

The kinetics of induction of suppression were examined in order to determine what culture period would be necessary to study the mechanism of suppression induced by TT (Figure 6). SMNC (2×10^5 cells) were cultured in the presence or absence of TT (2.5 $\mu\text{g/ml}$) for 1, 2, 24, 48, 72, and 120 hours. The cells were washed and stimulated with PHA. The response of cells incubated for 1 hour was unaffected. After 2 hours the PHA response was depressed by 22%, and after 24 hours by 53%. The mitogenic response of cells incubated with TT for longer periods of time (48, 72, or 120 hr) was completely suppressed.

The viability of SMNC was determined after culturing with TT by Trypan Blue dye exclusion in order to determine if the TT-induced suppression was due to toxicity of the antigen preparation. No significant differences in cell viability between cells incubated in the presence or absence of TT could be observed over the 120 hour culture

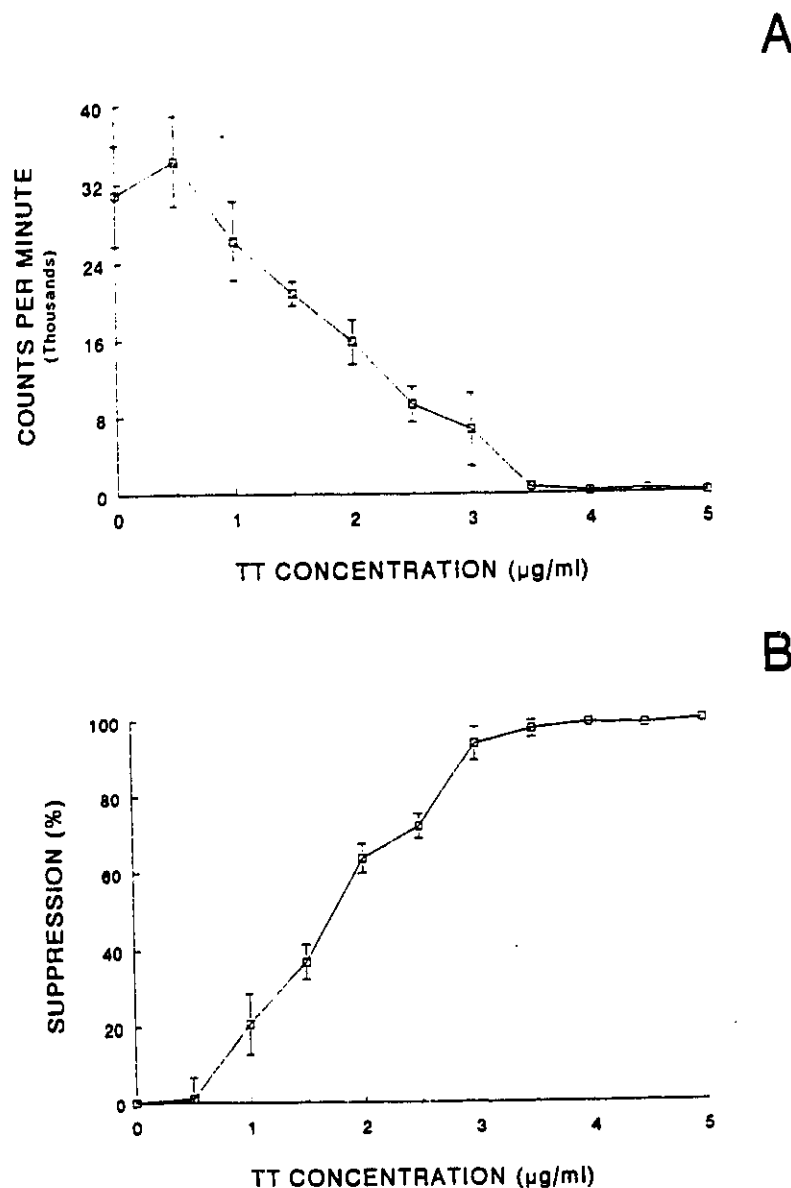


FIGURE 5. SUPPRESSION OF THE PHA BLASTOGENIC RESPONSE BY TETANUS TOXOID.

SMNC (2×10^5 cells) were incubated for 48 hours with various amounts of TT (0-5 µg/ml) and stimulated with the optimal amount PHA (1:25 dilution) or given medium only (no PHA) for 72 hours. A representative experiment of a total of 5 is shown in A. Results are expressed as mean CPM of ^3H -TdR incorporation and SEM for triplicate cultures. Significant inhibition of T cell proliferation by TT was observed at concentrations of 1.5 to 5.0 µg/ml ($p < 0.05$). In B the effect of the TT in 5 experiments is expressed in terms of percent suppression. Calculation of percent suppression was performed as outlined in the Methods.

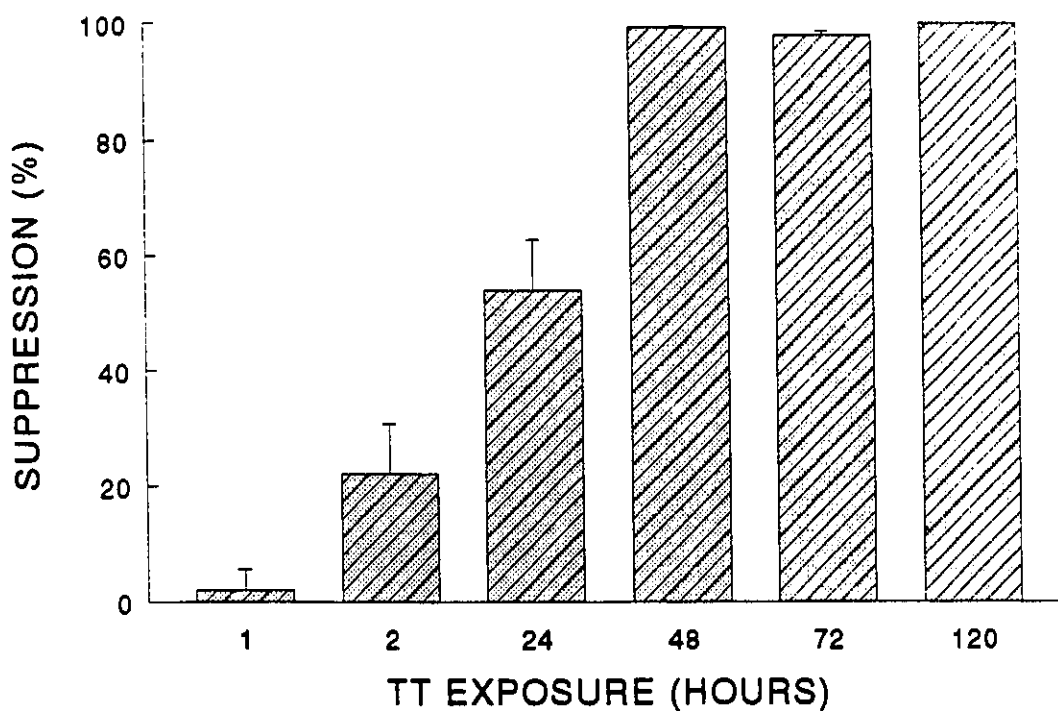


FIGURE 6. KINETICS OF INDUCTION OF SUPPRESSION BY TETANUS TOXOID.

SMNC (2×10^5 cells) were incubated in the presence or absence of TT ($2.5 \mu\text{g/ml}$) for 1, 2, 24, 48, 72, or 120 hours. The cells were washed with PBS and recultured in 96-well microtiter plates (2×10^5 cells in 0.2 ml) with or without PHA stimulation for 3 days. Calculation of percent suppression was performed as described in the Methods. The data represent the mean and SEM for $n=6$.

period (Table II).

TABLE II. VIABILITY FOLLOWING TT TREATMENT*

Time of Incubation (Hour)	% Viable Cells (SEM)	
	Medium	TT
1	99.0 (0.5)	99.0 (0.2)
24	98.5 (0.8)	97.1 (0.4)
48	98.1 (1.0)	95.1 (2.2)
72	85.2 (1.5)	80.3 (3.0)
120	85.0 (2.0)	82.0 (2.8)

*SMNC (2×10^6) were cultured in the presence or absence of TT (2.5 $\mu\text{g/ml}$) for 1, 24, 48, 72, or 120 hr. The percentage of viable cells in each culture was determined by Trypan Blue dye exclusion. The data are the mean and SEM of $n=4$. There was no significant difference at the $p<0.05$ level between culture conditions using a two-tailed Student's *t*-test.

3. Transfer of the Suppressive State with TT-Treated Cells.

The experiments described above demonstrated that TT was not toxic to SMNC and that full suppression required more than a 24 hour exposure to antigen. Experiments were performed to determine if the suppression induced *in vitro* could be transferred to down-regulate the PHA blastogenic response of fresh autologous SMNC (target cells). SMNC were cultured in the presence or absence of TT (2.5 $\mu\text{g/ml}$) for 48 hours (effector cells), were washed, and irradiated (30 Gy) prior to the addition of fresh autologous SMNC (after the 48 hour culture period of the effector cells). Varying doses of effector cells (3×10^3 to 1.2×10^6) were co-cultured with a constant number of target cells (1.2×10^6). The cultures were stimulated with PHA for 3 days. The activity of TT-induced suppressor cells was evaluated by comparing the PHA responses of co-cultures which

contained medium-treated (no TT) effector cells with the responses of co-cultures containing TT-treated effector cells. Results of the co-culture experiments are shown in Figures 7 and 8.

Figure 7 is a representative experiment in which significant inhibition of target T cell proliferation was observed with 0.3 , 0.6 , and 1.2×10^6 effector cells ($p < 0.01$). This suppression was observed in 7 replicate experiments which were pooled in Figure 8, illustrating that varying the dose of TT-treated effector cells resulted in a dose-dependent suppression of the PHA response of the fresh target cells. Co-culture of target and TT-treated effector cells, in a 1:1 ratio, produced a response that was 83% lower than cultures with untreated effector cells (lacking TT pretreatment). This latter response was similar in magnitude to that of PHA-stimulated target cells alone, with no effector cells added (Figure 7). Effector cells alone showed no proliferation in response to PHA as a result of pretreatment with 30 Gy irradiation. These results indicate that TT can induce active suppressor cells which inhibit the PHA response of untreated autologous SMNC.

Additional experiments were carried out to assess the possibility that antigen carry-over might be responsible for the suppressive activity of the TT. Normal SMNC were cultured in the absence of TT for 48 hours, they were harvested, and allowed to incubate with TT ($2.5 \mu\text{g/ml}$) for 1 hour, after which they were washed and co-cultured with target cells and PHA for 3 days. The results in Figure 9 show that SMNC incubated with TT for only one hour do not exhibit significant suppressive activity. The results suggest that the inhibitory activity of TT was probably not due to carryover of TT into the co-culture.

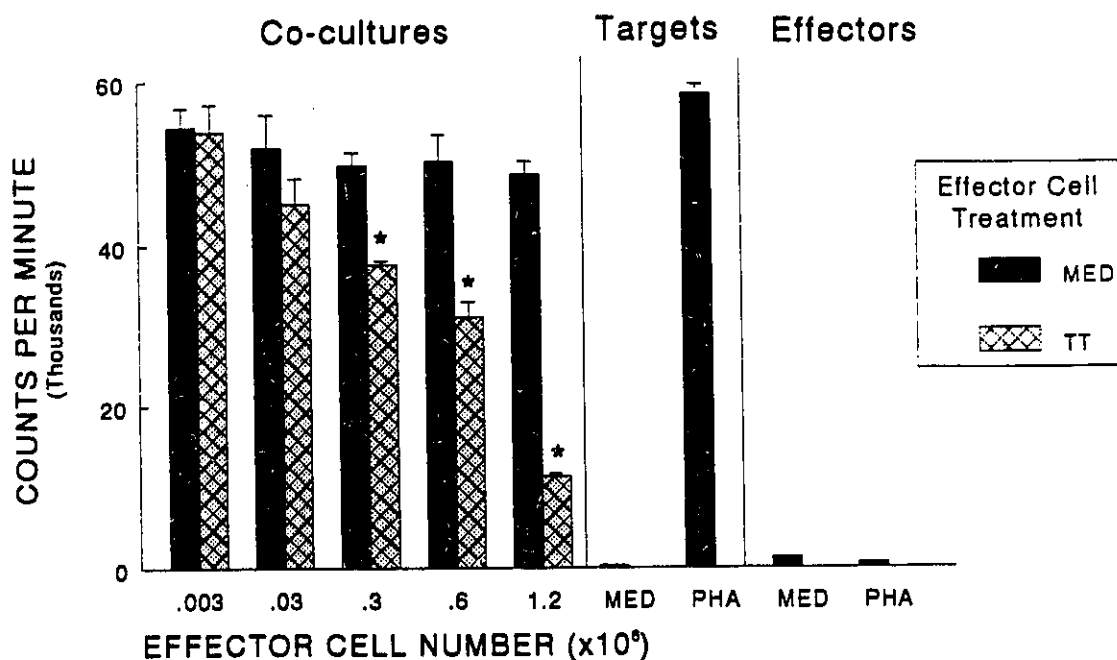


FIGURE 7. TRANSFER OF SUPPRESSIVE ACTIVITY WITH TT-TREATED CELLS.

Cultures of SMNC (3×10^3 , 3×10^4 , 3×10^5 , 0.6×10^6 , and 1.2×10^6) were incubated for 48 hours in the presence or absence of TT ($2.5 \mu\text{g/ml}$) becoming "effector cells". The SMNC were washed and co-cultured with fresh autologous target cells (1.2×10^6) and stimulated with PHA for 3 days. Effector cells were irradiated with 30 Gy prior to co-culture so that any proliferation was due only to the target cells. The TT-treated effector cell numbers that resulted in significant suppression ($p < 0.05$) of the PHA response versus untreated effector cells are indicated by an asterisk. The data is the mean and SEM of triplicate cultures from one representative experiment of a total of 8.

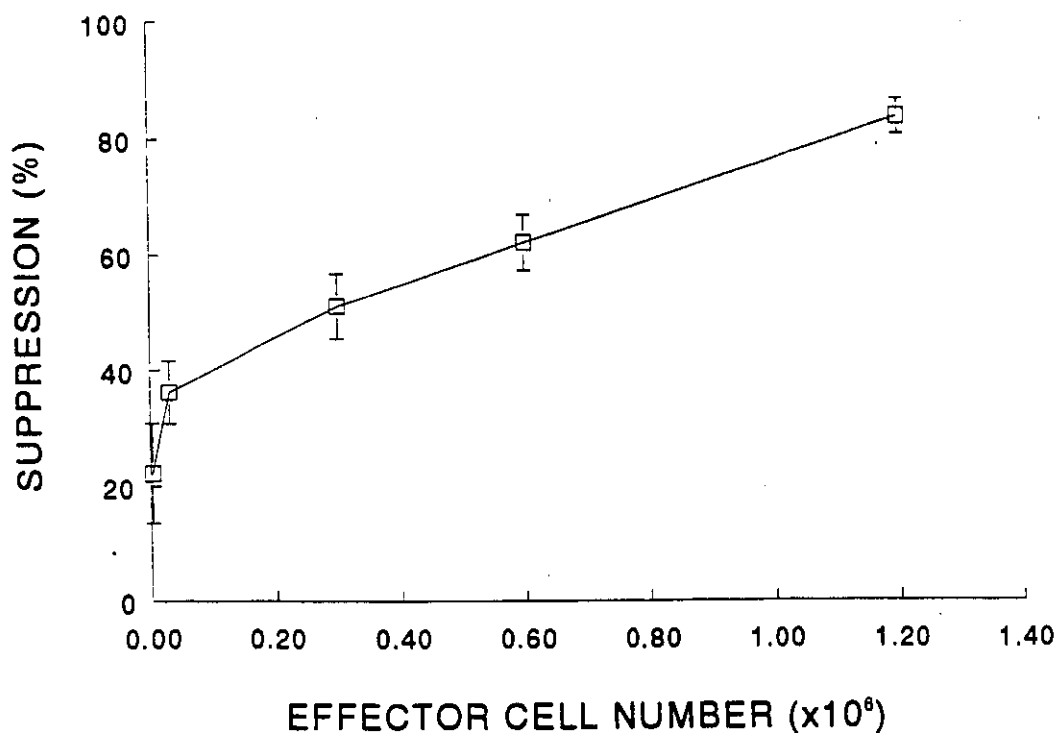


FIGURE 8. INCREASED SUPPRESSION WITH GREATER NUMBERS OF TT-TREATED EFFECTOR CELLS.

The dose-response characteristics of suppression from Figure 7 are demonstrated. Varying numbers of TT-treated effector SMNC (3×10^3 , 3×10^4 , 3×10^5 , 0.6×10^6 , and 1.2×10^6) were co-cultured with a constant number of fresh autologous target SMNC (1.2×10^6). Calculation of percent suppression was performed as outlined in the Methods. Each point represents the mean and SEM of pooled data from 8 experiments.

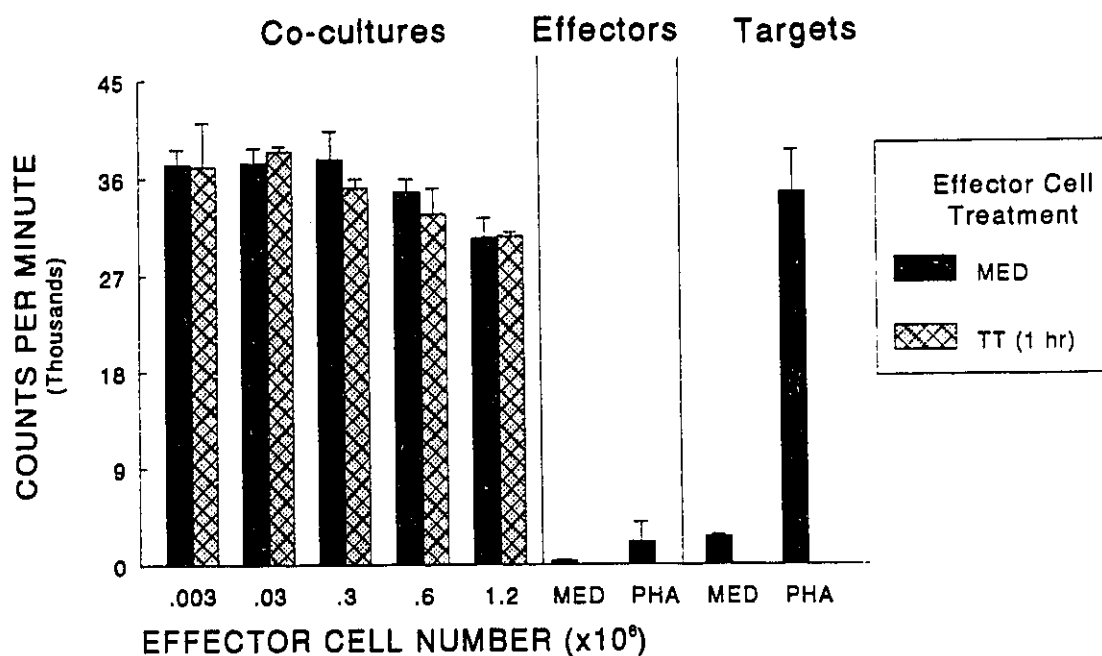


FIGURE 9. NO TRANSFER OF SUPPRESSIVE ACTIVITY WITH CELLS TREATED WITH TT FOR 1 HOUR.

As in Figure 8 except that SMNC were cultured in the absence of TT for 48 hours, harvested, and allowed to incubate with TT (2.5 $\mu\text{g/ml}$) for only 1 hour. Following the incubation, the cells were washed and co-cultured with target cells. Results shown are the mean and SEM of triplicate cultures. The figure shows the results from one of 3 experiments.

C. THE EFFECTS OF WHOLE-BODY IRRADIATION

1. *Effect on Cellularity*

Mice were given whole-body irradiation (WBI) (0-700 cGy) and after 1, 4, and 7 days their spleens were removed and a single cell suspension was made. A nucleated cell count was performed using a Coulter Counter Zm after lysis of red blood cells. Irradiation caused a dose-dependent decline in the cellularity of the spleen (Figure 10). All doses resulted in significantly fewer nucleated cells than 0 cGy (sham) at all time durations ($p < 0.05$). Doses of 400 and 700 cGy resulted in fewer cells than 100 cGy ($p < 0.05$), however, there was no significant difference between 400 and 700 cGy. There was no significant difference in the absolute number of cells during the 7 day post-irradiation period at each whole-body dose. The total number of nucleated cells per spleen was used in combination with flow cytometric analysis in order to determine the absolute number of the various SMNC populations as described in the Methods.

2. *Effect on the PHA Response of SMNC*

Not only was there decreased cellularity of the mouse spleen following irradiation, but the surviving SMNC also had a decreased ability to respond to mitogen. This was demonstrated by examining the PHA response after WBI of 100, 400, and 700 Gy (Figure 11). The dose-response curve indicated a clear radiation-induced depression of SMNC proliferative capabilities over this range. The proliferation of SMNC isolated on days 1 and 4 post-WBI showed a significant decline from controls after 100 and 400 cGy and a further decline from controls after 700 cGy ($p < 0.05$). Proliferation of SMNC isolated day 7 post-WBI showed a significant reduction with each increase in radiation dose

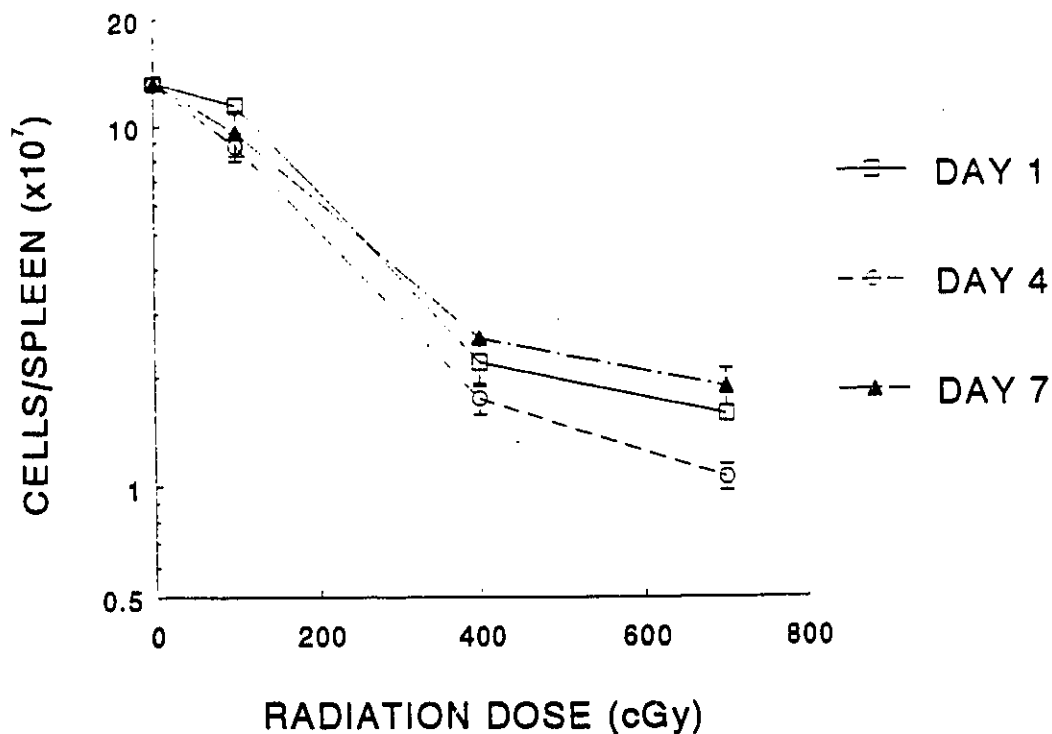


FIGURE 10. DOSE-DEPENDENT DECLINE IN THE TOTAL NUMBER OF NUCLEATED CELLS BY IRRADIATION

Mice were irradiated with 0-700 cGy and their spleens were removed on days 1, 4, and 7 post-irradiation. The surviving nucleated cells were counted using a Coulter Cell Counter. The total number of nucleated cells/spleen declined in a dose-dependent fashion after irradiation. The data represent the mean and SEM of 5 experiments with cells pooled from the spleens of 2-10 mice per experiment depending on the radiation dose used; 2 mice were used per experiment for doses of 0 and 100 cGy, 8 mice for 400 cGy, and 10 mice for 700 cGy. A two-way ANOVA determined that there was a significant decline in cell number with all doses of radiation and at all time points versus control mice. Although doses of 400 and 700 cGy resulted in fewer cells than 100 cGy ($p < 0.05$), there was no significant difference between 400 and 700 cGy.

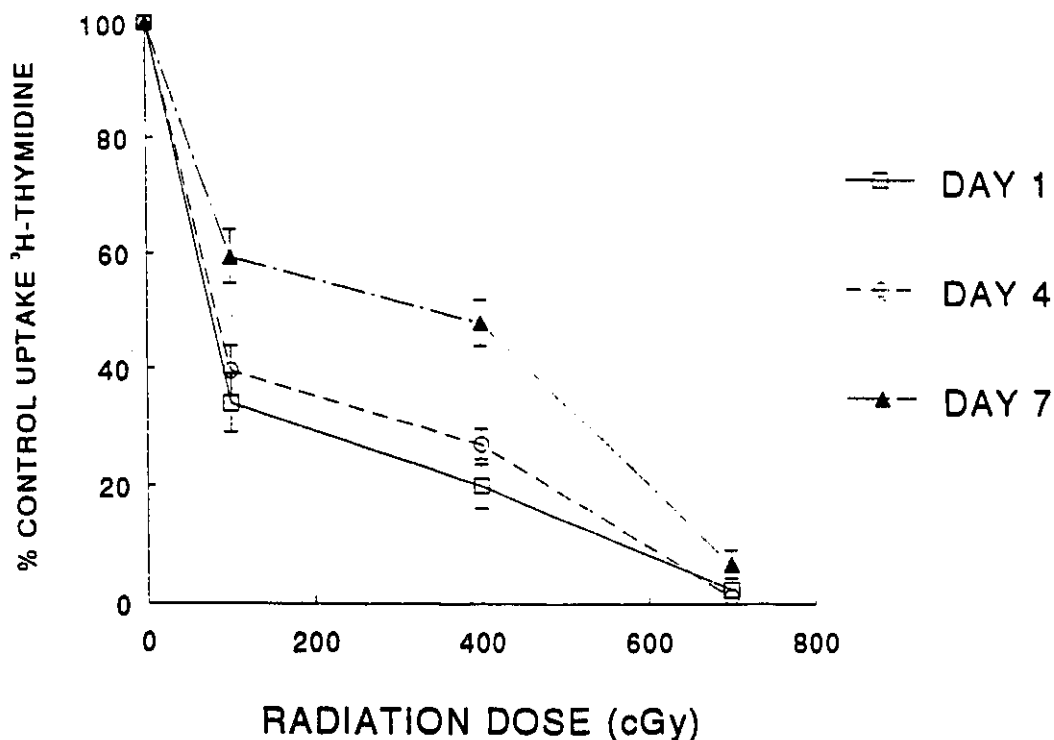


FIGURE 11. PHA RESPONSE OF SMNC ISOLATED FROM MICE EXPOSED TO WHOLE-BODY-IRRADIATION

Mice were exposed to WBI 1, 4, and 7 days prior to removal of their spleens and isolation of SMNC. The SMNC (2×10^6 cells) were cultured and stimulated with an optimal dilution (1:25) of PHA or remained unstimulated for 72 hours. The amount of ^3H -TdR uptake in cultures from irradiated mice is expressed as a mean percentage ($\pm$ SEM) of corresponding control uptake. The data represent the pooled results of 4 experiments. The mean values of uptake by unstimulated and stimulated control cultures were 1095 and 28 787 counts per minute (CPM) respectively. A two-way ANOVA determined that there was a significant decline in the proliferative capabilities of SMNC isolated from mice for all doses and harvested on all days post-WBI as compared to the control mice ($p < 0.05$). SMNC isolated on day 7 post-WBI showed significantly less proliferation with each increase in radiation dose. SMNC from mice which received doses of 100 and 400 cGy and isolated 7 days post-WBI showed significantly greater proliferation than SMNC isolated on days 1 and 4 ($p < 0.05$).

($p < 0.05$). At doses of 100 and 400 cGy, however, the proliferation was significantly greater than that of SMNC isolated on days 1 and 4 post-WBI ($p < 0.05$).

3. Transfer of Suppressive State with SMNC from WBI mice.

To determine if irradiation could produce active suppression of the murine blastogenic response, experiments were performed in which "effector" cells from WBI mice were added to fresh autologous cells to see if they could down-regulate the PHA response of these "target" cells (analogous to the TT co-culture experiments). Mice were given 100 cGy of WBI and their SMNC were harvested on days 1 and 7 post-irradiation becoming the effector cells. Varying doses of effector cells (3×10^3 to 1.2×10^6) were co-cultured with a constant number of fresh untreated target cells (1.2×10^6). The cultures were stimulated with PHA for 3 days. The results of the co-culture experiment are shown in Figure 12. Co-culture of target and WBI-effector cells produced responses that were no different than untreated effector cells (lacking WBI pretreatment). In contrast to TT (which resulted in active suppression), there was no evidence that effector cells from mice subjected to 100 cGy were capable of active suppression of the PHA response of target cells when the spleens were removed on days 1 or 7 post-WBI.

D. MITOGENIC RESPONSES AFTER IRRADIATION AND TETANUS TOXOID

1. Effect on the PHA Response

Both irradiation and TT could act independently to depress the PHA response (Figure 5 and Figure 11 respectively), whereas only TT was capable of active suppression

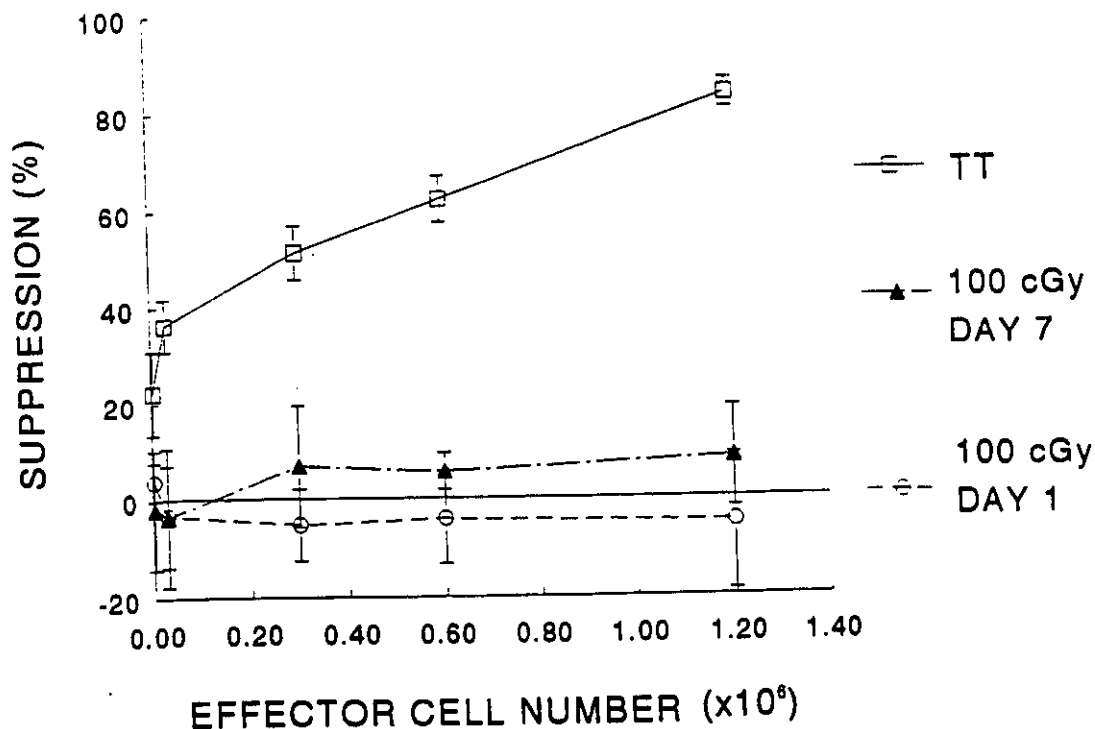


FIGURE 12. NO TRANSFER OF SUPPRESSIVE ACTIVITY WITH SMNC FROM WBI MICE

Mice were irradiated with 100 cGy and their SMNC were harvested on days 1 and 7 post-irradiation becoming effector cells. Varying concentrations (3×10^3 , 3×10^4 , 3×10^5 , 0.6×10^6 , and 1.2×10^6) of effector cells were co-cultured in triplicate with fresh autologous target cells (1.2×10^6) and stimulated with PHA for 3 days. Effector cells were irradiated with 30 Gy prior to co-culture so that proliferation was due only to the target cells. There was no significant suppression induced by irradiated effector cells as compared to non-irradiated effector cells. There also was no difference between SMNC harvested on days 1 and 7 post-irradiation. The data represents the mean and SEM of pooled data from 4 experiments.

(Figure 7). The resultant effect of irradiation and TT combined was a further dampening of proliferation in response to PHA (Figure 13). SMNC were removed 1, 4, and 7 days post-WBI and cultured for 48 hours with medium or 2.5 $\mu\text{g/ml}$ of TT. After the culture period the SMNC were washed and stimulated with the optimal PHA. As was seen previously, the PHA response was significantly suppressed by the prior presence of TT alone (no WBI) ($p < 0.01$). SMNC from mice exposed to WBI prior to culturing with TT showed a further reduction of the PHA response ($p < 0.05$). At doses of 400 and 700 cGy the PHA response was completely suppressed on a consistent basis.

2. Transfer of the Suppressive State with TT-treated SMNC from WBI Mice.

Transfer of suppression experiments were performed to determine if the increased suppression of the PHA response which had been observed with the combined effect of TT and irradiation represented simply an additive effect of TT-induced suppression and radiation-induced reproductive cell death, or whether radiation actually enhanced the number of suppressor cells or their activity in response to TT.

Experiments involved irradiating mice with 100 cGy and isolating SMNC on days 1 and 7 post-WBI. These SMNC were cultured in the presence or absence of TT (2.5 $\mu\text{g/ml}$) for 48 hours (effector cells), washed, and irradiated (30 Gy) prior to the addition of fresh autologous SMNC (after the 48-hour culture period of the effector cells). Varying doses of effector cells (3×10^3 to 1.2×10^6) were co-cultured with a constant number of target cells (1.2×10^6) in 24-well culture dishes and stimulated with PHA for 3 days. The activity of effector-induced suppressor cells was valuated by comparing the PHA

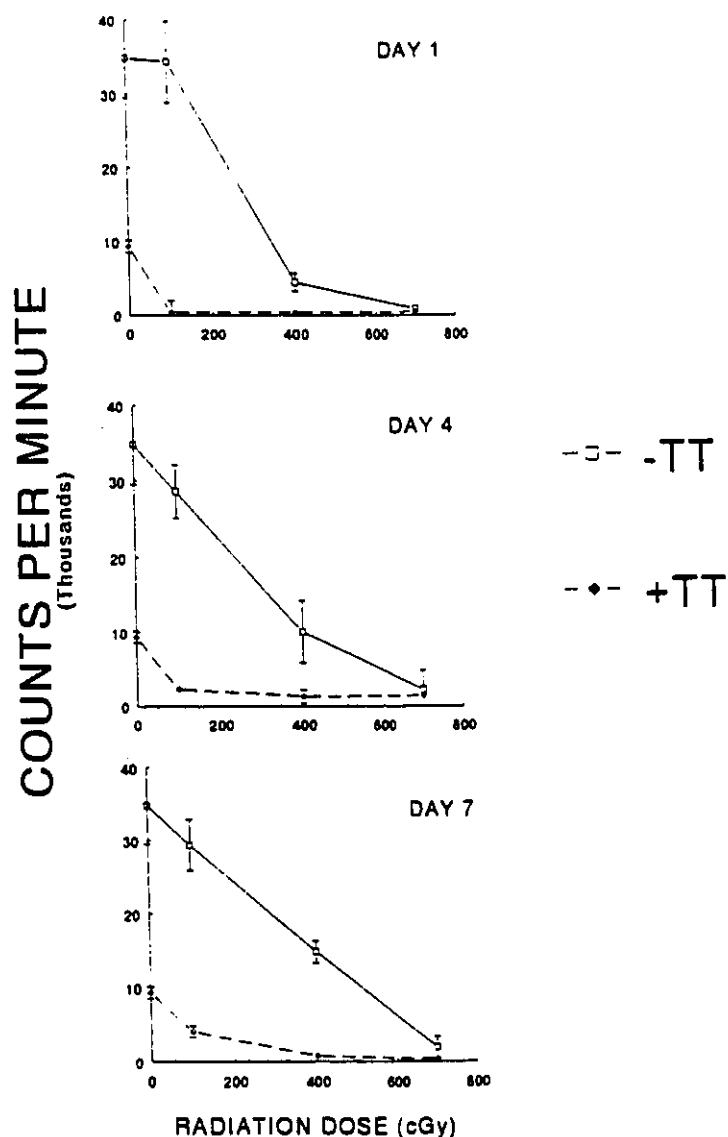


FIGURE 13. THE COMBINED EFFECT OF TT AND RADIATION ON THE PHA RESPONSE

Mice were irradiated (0-700 cGy) and their SMNC were harvested 1, 4, and 7 days post-irradiation. SMNC (2×10^6 cells/ml) were then cultured for 48 hours in complete medium with or without 2.5 $\mu\text{g/ml}$ TT. The SMNC were washed and stimulated with PHA (1:25) or given only medium for 54 hours (final cell volume of 0.2 ml). The data represent the mean ^3H -TdR incorporation and SEM for $n=4$. The PHA response was significantly suppressed by TT alone (0 cGy) ($p<0.01$). SMNC from mice exposed to WBI prior to culturing with TT showed a further reduction of the PHA response ($p<0.05$). At doses of 400 and 700 cGy the PHA response was completely suppressed on a consistent basis.

responses of co-cultures containing normal effector cells (no WBI, no TT) with responses of co-cultures containing irradiation/TT-treated effector cells. Results of the co-culture experiments are shown in Figure 14. The suppressive activity induced upon target cells was not different between that by TT-treated effector cells taken from unirradiated mice and that by effector cells harvested on days 1 and 7 following exposure to 100 cGy WBI.

D. FLOW CYTOMETRIC ANALYSIS OF MURINE SMNC

1. *Characterization of Fresh Splenic Mononuclear Cells*

Experiments were performed to characterize the major mononuclear cell populations of the spleen of C57BL/6 mice by flow cytometric analysis (FCA). The mean and standard deviation of each cell subset as a percentage of the total splenic mononuclear cell population (n=5) are shown in Table III. Any cell expressing a marker above non-binding isotype control levels was designated positive.

In single-labelling experiments B cells were labelled using an anti-CD45R antibody. The mean percentage of CD45⁺ cells among the total processed SMNC was 53.6% $\pm$ 9.2. T cells were labelled with anti-Thy1.2 mAb which made up 31.3% $\pm$ 3.3 of the total SMNC. Natural killer cells were labelled using anti-NK1.1 antibody which labelled 3.9% $\pm$ 1.6 of the total SMNC. Monocytes/macrophages were labelled using anti-F4/80 antibody which accounted for 9.1% $\pm$ 2.4 of SMNC. Class II MHC antigen expressing cells (including B cells, monocytes/macrophages, and other APC) were found to make up 55.3% $\pm$ 10.0 of SMNC. Cells expressing the CD25 or interleukin-2 receptor were not detectable. CD71, the transferrin receptor, was found on 3.4% $\pm$ 2.8

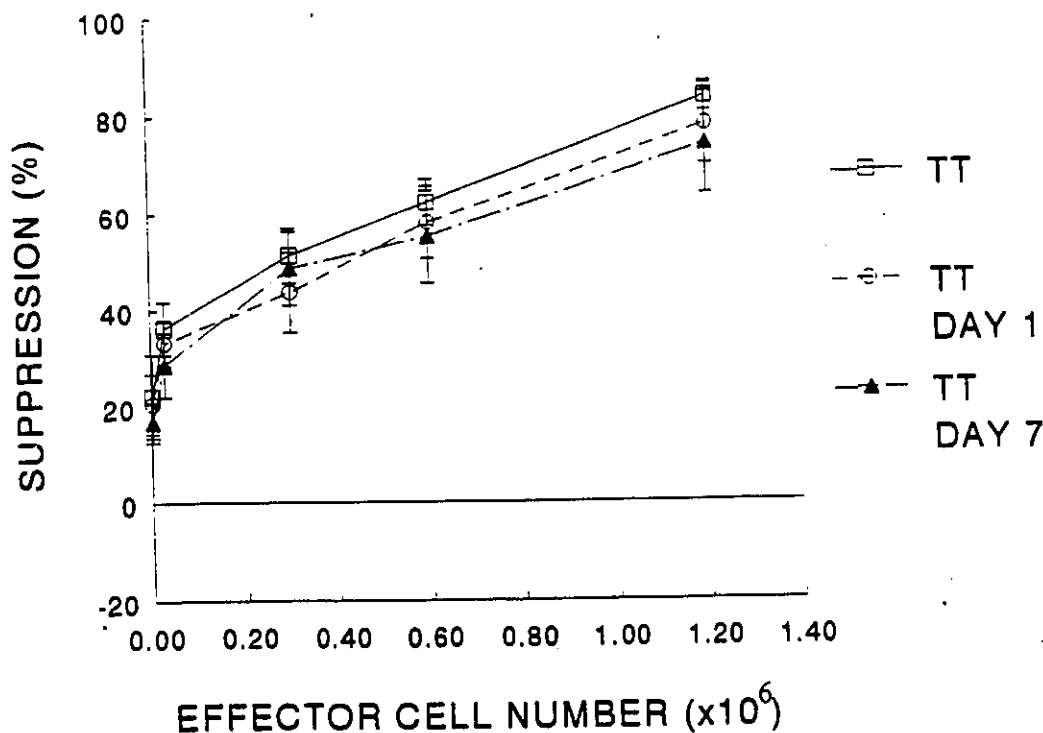


FIGURE 14. WBI DOES NOT ENHANCE THE SUPPRESSIVE ACTIVITY OF TT-TREATED EFFECTOR CELLS DURING CO-CULTURE.

Mice were irradiated with 100 cGy and their SMNC were harvested on days 1 and 7 post-irradiation. These SMNC (3×10^3 , 3×10^4 , 3×10^5 , 0.6×10^6 , and 1.2×10^6) were then incubated for 48 hours in the presence or absence of TT ($2.5 \mu\text{g/ml}$) becoming effector cells. The effector SMNC were washed and co-cultured with fresh autologous target cells (1.2×10^6) and stimulated with PHA for 3 days. Effector cells were irradiated with 30 Gy prior to co-culture to ensure that proliferation was due only to the target cells. There was no significant difference in the amount of suppression induced by TT-treated effector cells from WBI mice versus unirradiated mice. The percent suppression was performed as outlined in the Methods. Each point represents the mean and SEM of pooled data from 5 experiments.

of control SMNC. The percentage of cells positive for CD25, CD71, and class II MHC as well as the level of antigen expression were monitored as potential indicators of cell activation after exposure to irradiation and TT.

Table III. EXPRESSION OF SUBSET AND ACTIVATION MARKERS ON MURINE SPLENIC MONONUCLEAR CELLS

Cell Subset ^a	Mean Percentage (SD) ^b
Thy1.2 ⁺	31.3 (3.3)
Thy1.2 ⁺ CD4 ⁺	16.6 (3.6)
Thy1.2 ⁺ CD8 ⁺	9.6 (2.8)
CD45 ⁺	53.6 (9.2)
Ia ⁺	55.3 (10.0)
CD45 ⁺ Ia ⁺	56.6 (7.5)
NK1.1 ⁺	3.9 (1.6)
F4/80 ⁺	9.1 (2.4)
CD25 ⁺	ND ^c
CD71 ⁺	3.4 (2.8)
CD4 ⁺ Ia ⁺	3.7 (1.6)
CD8 ⁺ Ia ⁺	3.5 (2.0)

^aSubsets of SMNC identified by single and double-antibody labelling. Antibodies were FITC anti-CD4, anti-CD8, anti-CD45, and PE-mAb to other markers listed.

^bFrequency of total splenic mononuclear cells (n=5 where 2 spleens were used per experiment).

^cNot detectable.

In double-labelling experiments T lymphocyte subsets were characterized using anti-Thy1.2 mAb combined with anti-CD4 and anti-CD8 antibodies. Thy⁺CD4⁺ cells (including T helper/inducer) made up 16.6% $\pm$ 3.6 of the SMNC population, while Thy⁺CD8⁺ cells (including T cytotoxic/suppressor cells) comprised 9.6% $\pm$ 2.8. A representative double-labelling experiment for staining of the Thy⁺CD8⁺ subset is shown in Figure 15. The percentage of B cells expressing the class II MHC molecule was

56.6% $\pm$ 7.5 (Table III). The percentages of class II MHC cells expressing CD4 and CD8 were also examined since they are known to comprise a small percentage of the total population (Ko *et al.* 1979).

2. Effect Of Irradiation On Populations of SMNC

i. Absolute Numbers and Relative Proportions

Using flow cytometric analysis the effects of radiation on the major SMNC populations were examined. SMNC were detected by single or double-antibody labelling as outlined in the Methods section. Although radiation caused a large decline in the total number of spleen cells, as shown in Figure 6, the degree of cytoreduction varied among the different cell types. C57BL/6 mice were irradiated with 100, 400, and 700 cGy and SMNC were analyzed by flow cytometric analysis after 1, 4, and 7 days. As depicted in Figures 16-21, although the absolute number of all of the cell populations declined, the relative proportion, which is expressed as a percentage of the total number of SMNC, did vary among the cell types. Doses of 400 and 700 cGy resulted in significantly fewer SMNC as compared to control mice for each cell population ($p < 0.05$). The proportion of all T cells (relative to total SMNC) actually increased with radiation dose (Figure 16B). Doses of 400 and 700 cGy resulted in significant increases in the proportion of T lymphocytes on days 1 through 7 as compared to the control mice ($p < 0.05$). The CD4⁺ and CD8⁺ T lymphocyte subsets, however, demonstrated differences in their radiosensitivities; the proportion of CD4⁺ T cells increased with radiation doses of 400 and 700 cGy ($p < 0.05$) (Figure 17B), whereas the proportion of CD8⁺ T cells remained

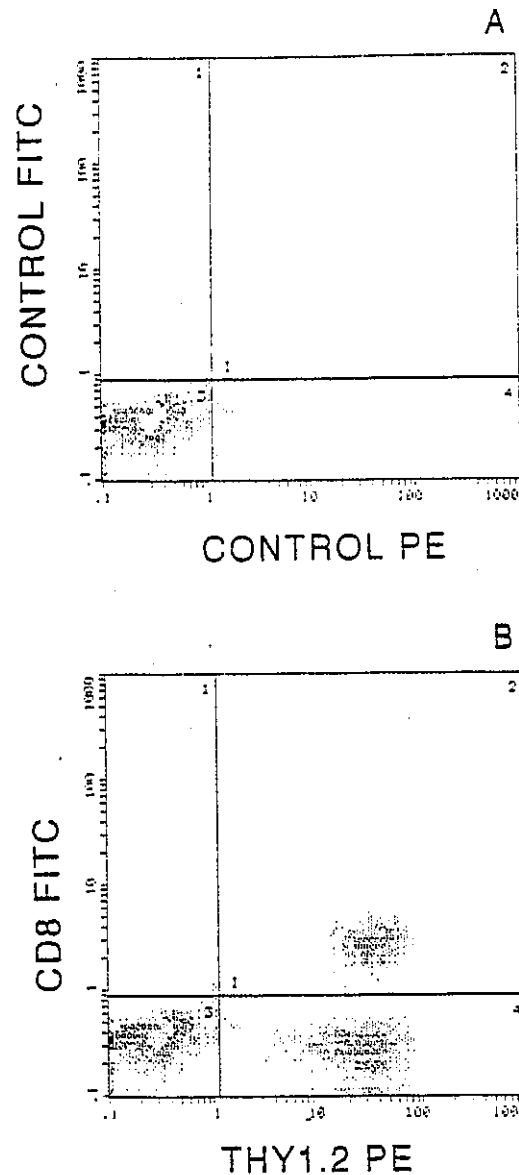


FIGURE 15. TWO COLOUR FCA OF SMNC USING ANTI-THY1.2 AND ANTI-CD8 ANTIBODIES.

The analysis is a dot-density plot with PE fluorescence on the horizontal axis and FITC fluorescence on the vertical axis. In (A) SMNC were labelled with PE IgG2a and FITC IgG2b isotype control antibodies. In (B) SMNC were labelled with PE anti-Thy1.2 and FITC anti-CD8 antibodies. Within quadrant 4 are cells which bound the anti-Thy1.2 antibody, but not the anti-CD8 antibody. These $\text{Thy1.2}^+\text{CD8}^-$ cells include the T helper cells. Quadrant 2 includes double-stained $\text{Thy1.2}^+\text{CD8}^+$ cells which include the T cytotoxic/suppressor cells. Quadrant 3 is the autofluorescence from cells which were negative for both antibodies.

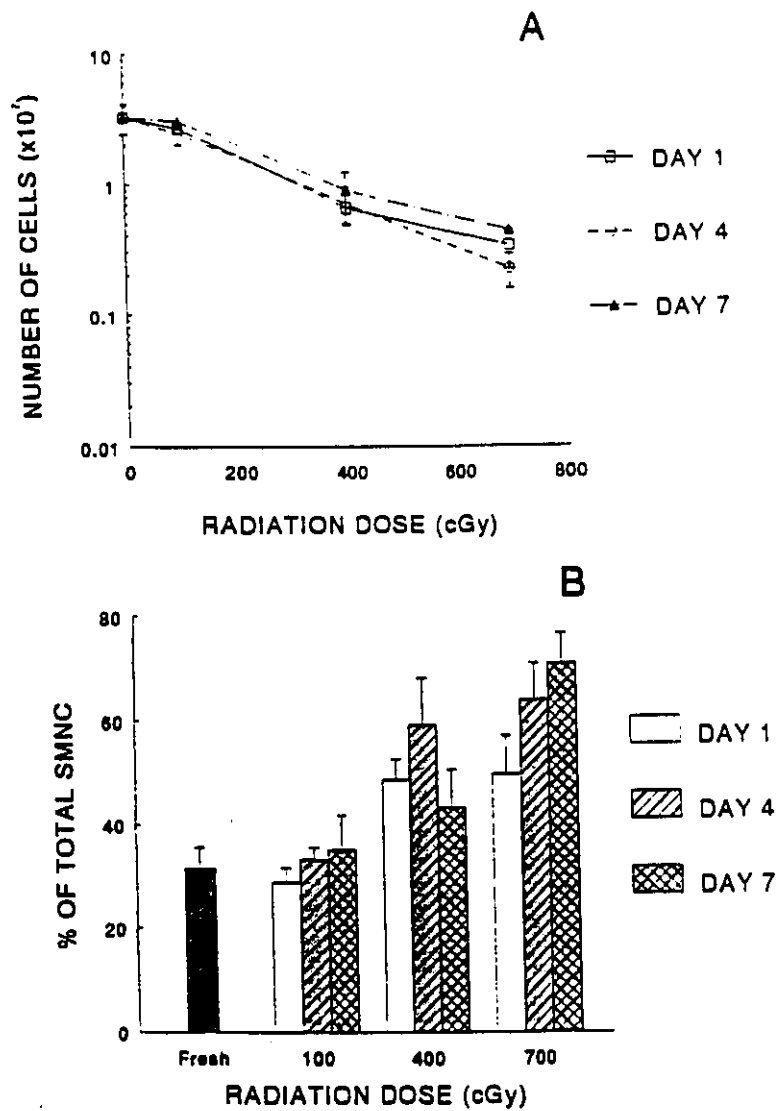


FIGURE 16. THY1.2⁺ SMNC (TOTAL T-CELLS): ABSOLUTE NUMBER AND RELATIVE PROPORTION AFTER IRRADIATION.

The absolute and relative number of Thy1.2⁺ cells (T lymphocytes) in C57BL/6 mouse spleens detected by single-antibody labelling is demonstrated. Mice were irradiated with 100, 400, and 700 cGy and SMNC were analyzed by flow cytometric analysis after 1, 4, and 7 days. The absolute number of positive cells (A) was determined by the following: total number of SMNC x percentage of positive cells. The relative proportion (B) is the number of positive cells as a percentage of the total number of SMNC. Each bar represents the mean of 3-10 animals (3 mice with control and 100 cGy, 8 mice with 400 cGy, and 10 mice with 700 cGy) in 4 separate experiments. The error bars indicate the SEM. A two-way ANOVA determined that doses of 400 and 700 cGy resulted in significant decreases in the absolute number of T lymphocytes and increases in the relative proportion of T lymphocytes versus fresh control cells ($p < 0.05$).

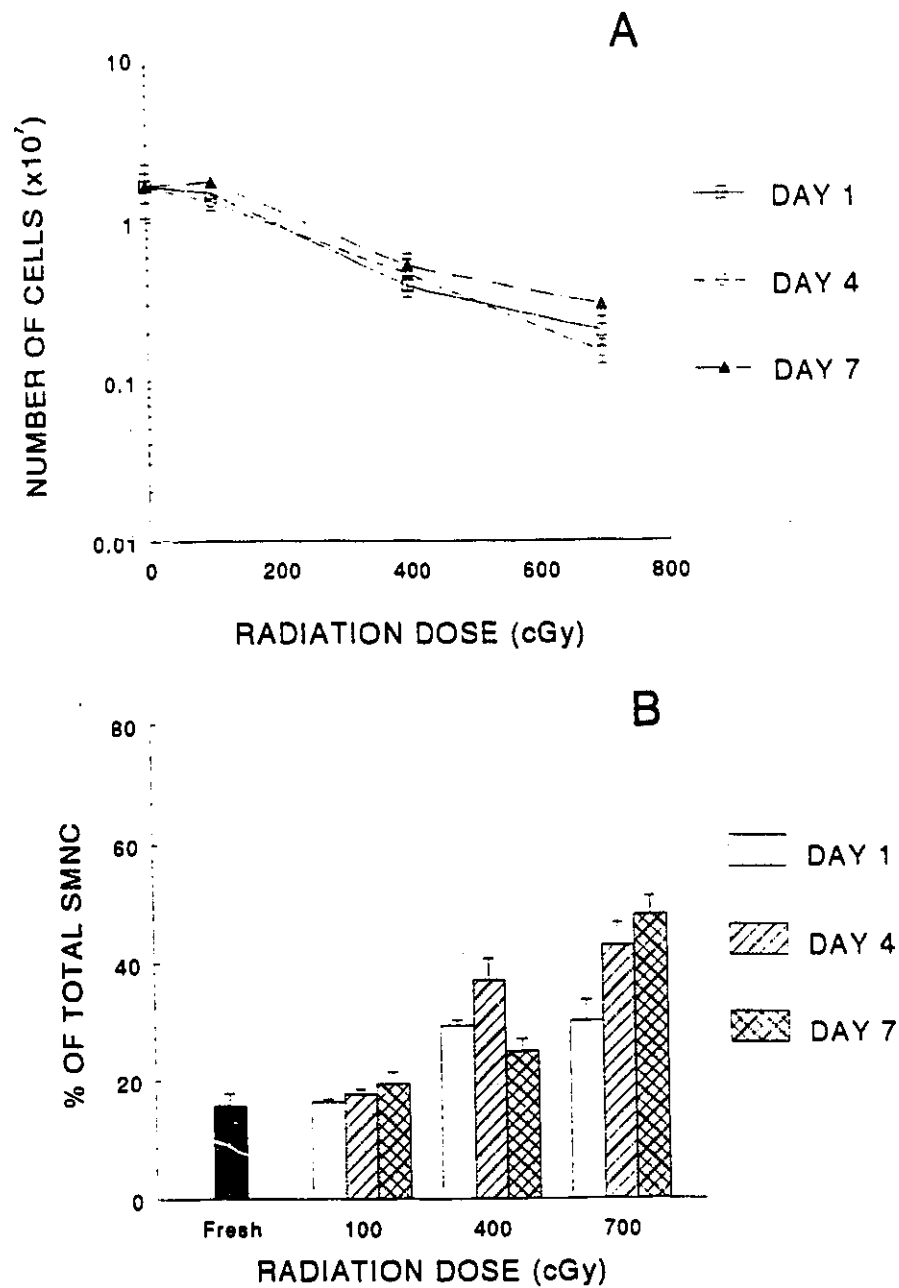


FIGURE 17. THY1.2⁺CD4⁺ (Th) SMNC: ABSOLUTE NUMBER AND RELATIVE PROPORTION AFTER IRRADIATION .

As in Figure 17 with (A) the absolute number and (B) the relative proportion of Thyl.2⁺CD4⁺ (T helper/inducer) cells in C57BL/6 mouse spleens detected by double-antibody labelling. A two-way ANOVA determined that doses of 400 and 700 cGy resulted in significant decreases in the absolute number and increases in the relative proportion of CD4⁺ T-cells versus fresh control cells ($p < 0.05$).

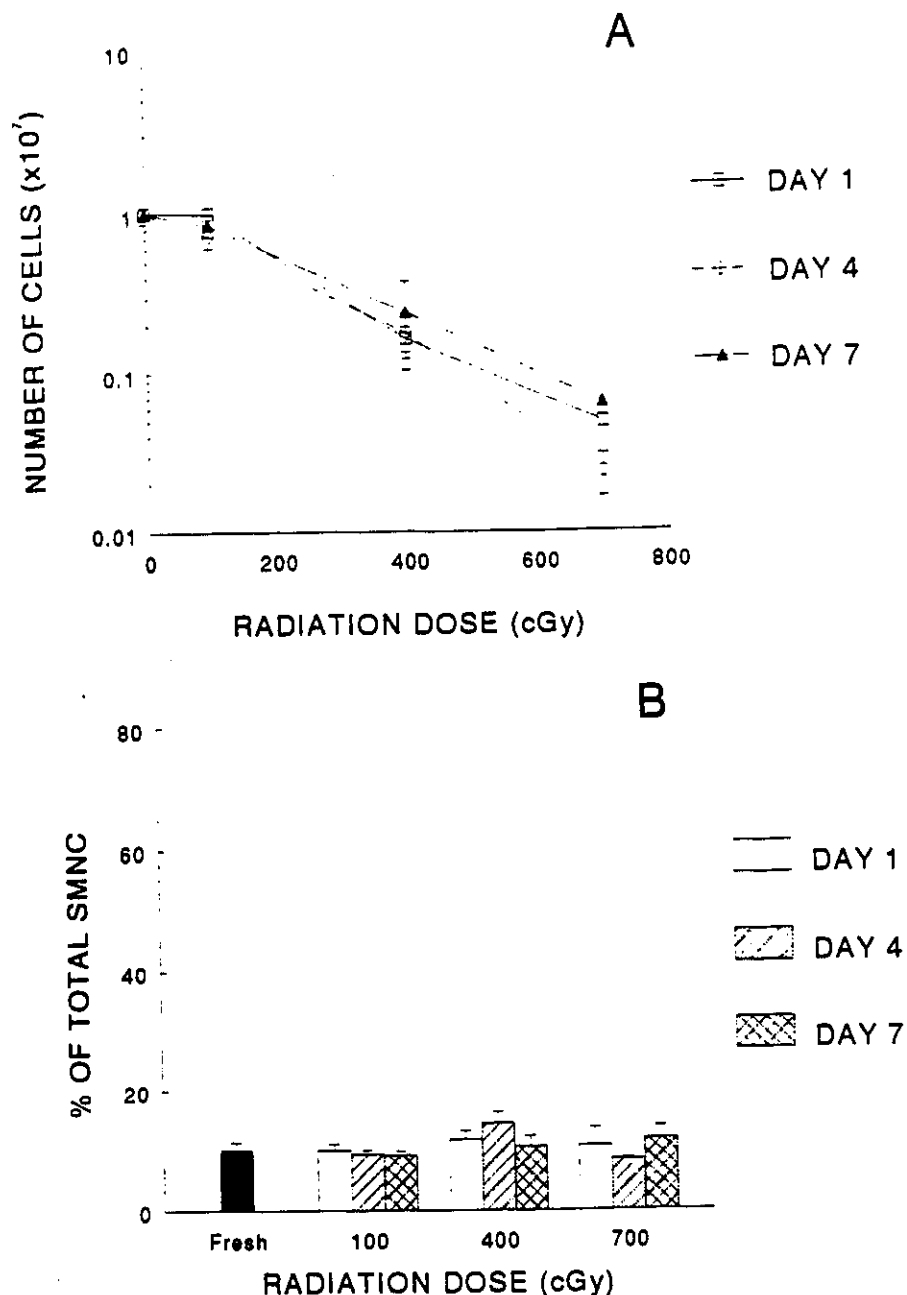


FIGURE 18. THY1.2⁺CD8⁺ (Tc/s) SMNC: ABSOLUTE NUMBER AND RELATIVE PROPORTION AFTER IRRADIATION.

As in Figure 17 with (A) the absolute number and (B) the relative proportion of Thy1.2⁺CD8⁺ cells (T cytotoxic/suppressor) in C57BL/6 mouse spleens detected by double-antibody labelling. A two-way ANOVA determined that doses of 400 and 700 cGy resulted in significant decreases in the absolute number of CD8⁺ T-cells versus fresh control cells ($p < 0.05$). In contrast, there was no significant change in the relative proportions.

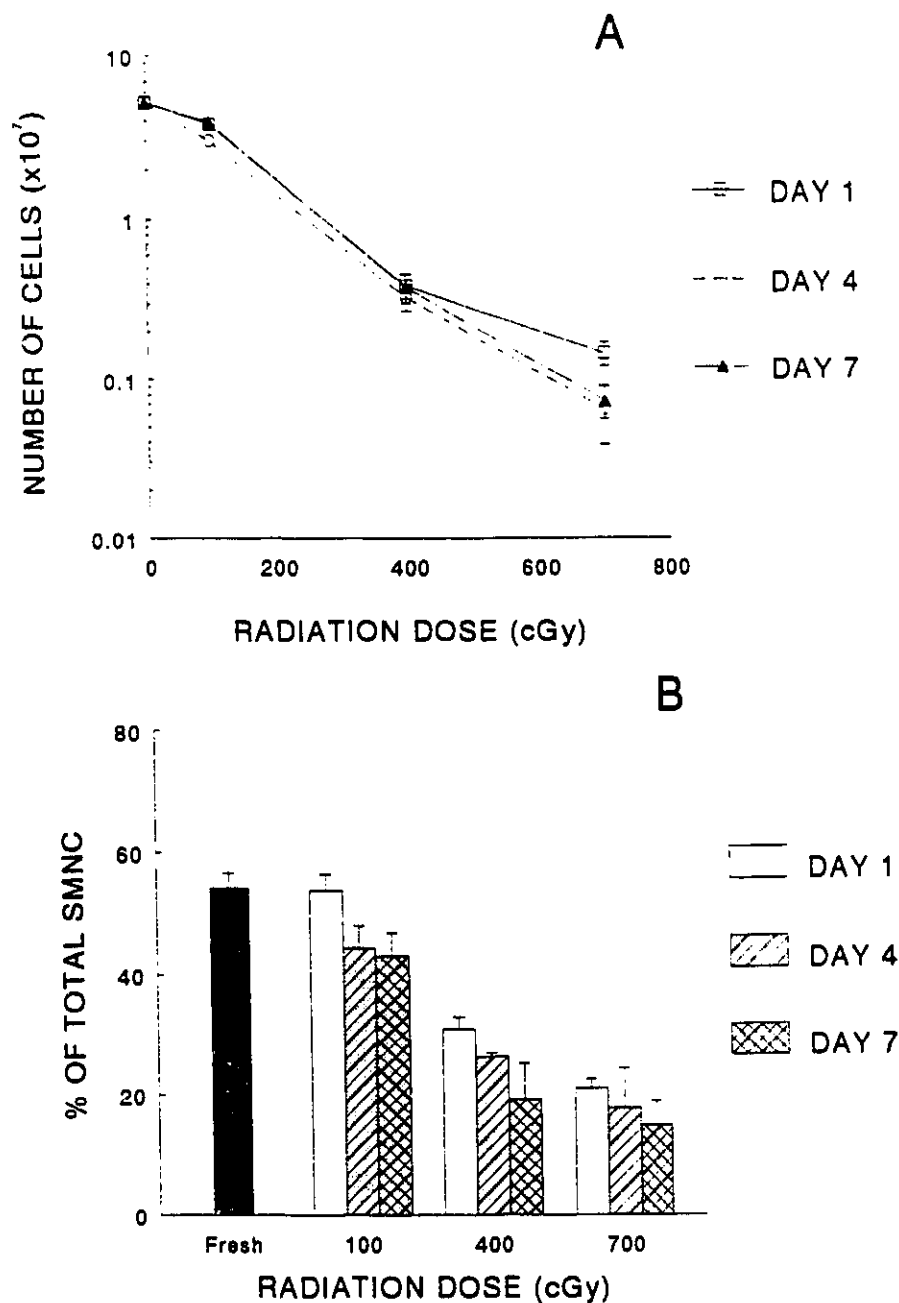


FIGURE 19. CD45⁺ (B-CELLS) SMNC: ABSOLUTE NUMBER AND RELATIVE PROPORTION AFTER IRRADIATION.

As in Figure 17 with (A) the absolute number and (B) the relative proportion of CD45⁺ cells (B lymphocytes) in C57BL/6 mouse spleens detected by single-antibody labelling. A two-way ANOVA determined that doses of 400 and 700 cGy resulted in significant decreases in both the absolute number and the relative proportion of B-cells versus fresh control cells ($p < 0.05$).

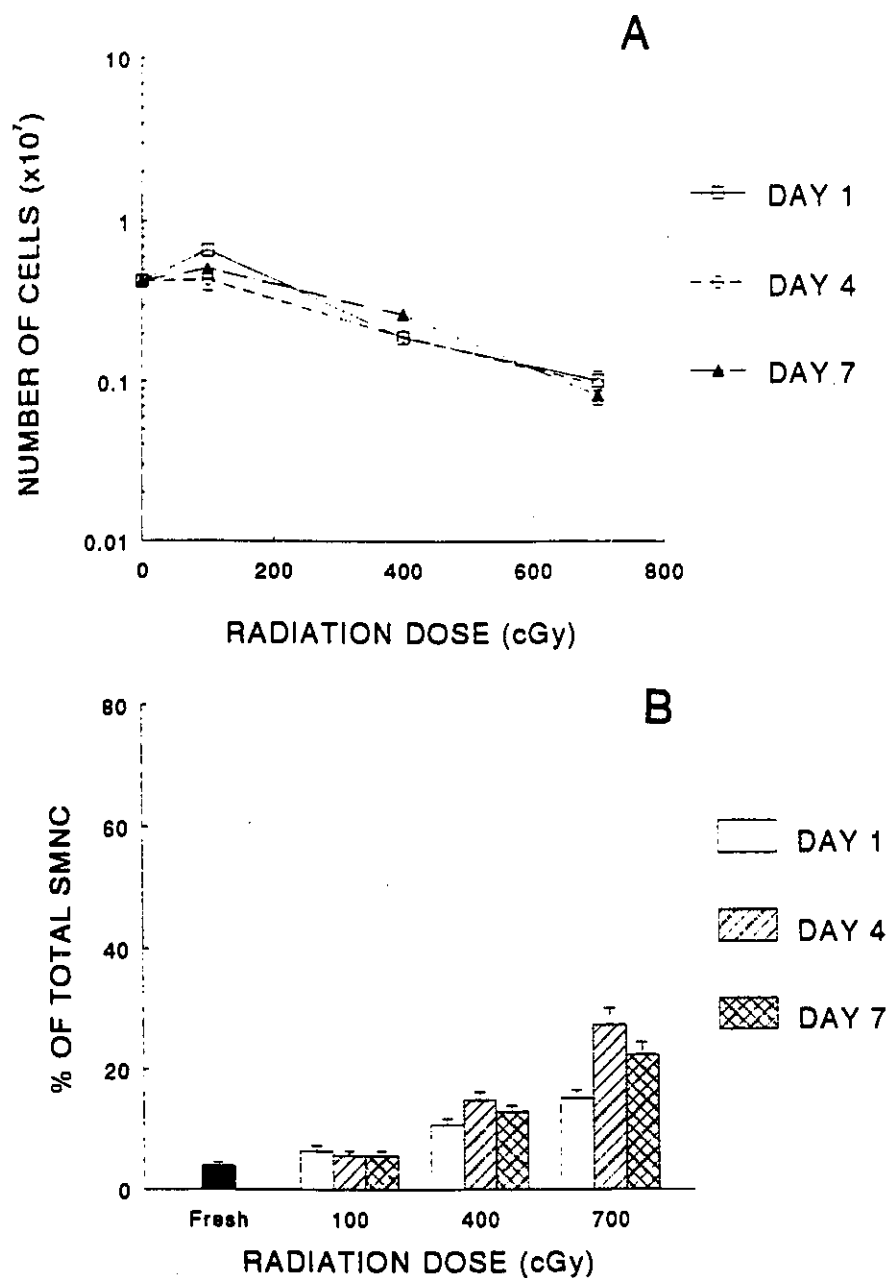


FIGURE 20. NK1.1⁺ (NATURAL KILLER CELLS) SMNC: ABSOLUTE NUMBER AND RELATIVE PROPORTION AFTER IRRADIATION.

As in Figure 17 with (A) the absolute number and (B) the relative proportion of NK1.1⁺ (Natural Killer) cells in C57BL/6 mouse spleens detected by single-antibody labelling. A 2-way ANOVA determined that doses of 400 and 700 cGy resulted in a significant decrease in the absolute number and an increase in the relative proportion of NK cells versus fresh control cells. Increasing the dose from 400 to 700 cGy resulted in an increase in the relative proportion with a peak by 4 days post-WBI ($p < 0.05$).

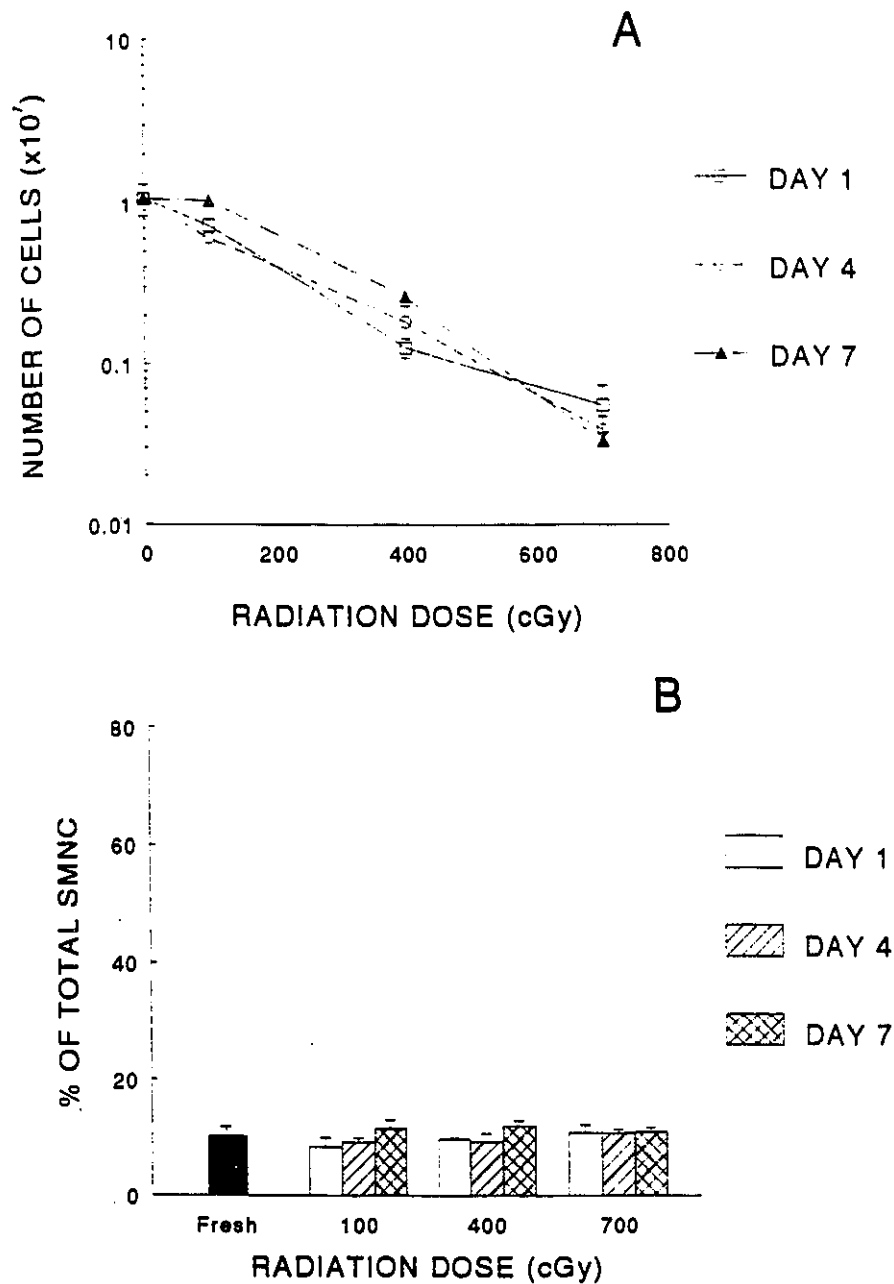


FIGURE 21. F4/80⁺ (MONOCYTES/MACROPHAGES) SMNC: ABSOLUTE NUMBER AND RELATIVE PROPORTION AFTER IRRADIATION.

As in Figure 17, with (A) the absolute number and (B) the relative proportion of F4/80⁺ cells (monocytes/macrophages) in C57BL/6 mouse spleens detected by single-antibody labelling. A two-way ANOVA determined that doses of 400 and 700 cGy resulted in significant decreases in the absolute number of F4/80⁺ SMNC versus fresh control cells ($p < 0.05$). In contrast, there was no significant change in the relative proportion.

unchanged (Figure 18B). The proportion of CD45⁺ B cells declined from 53.6% in control mice to 26.4% by day 4 post-WBI (700 cGy). Doses of 400 and 700 cGy resulted in significantly lower levels of B cells than in control mice at all time periods post-irradiation ($p>0.05$) (Figure 19B). In contrast, an increase in the proportion of natural killer cells (Figure 20) was observed as the radiation dose increased: from 3.9% in control mice to as high as 26% of the total SMNC (on day 4 after 700 cGy). Similar to CD8 T lymphocytes, F4/80⁺ macrophages declined in absolute numbers but their proportion of the total number of SMNC was not significantly different than that in the control mice (Figure 21).

For a more concise overall comparison of the differential effects of WBI on the MNC populations of the mouse spleen, Figure 22A shows the surviving fraction of each population with increasing dose on day 4 post-WBI. Although all cell populations decline, they do so at different rates. In ascending order of rate of decline are the NK cells, CD4⁺ T cells, CD8⁺ T cells, monocytes/macrophages, and B cells. Figure 22B shows the proportion of each population of splenic mononuclear cells expressed as a percentage of that cell type in unirradiated control mice. The proportion of natural killer cells increased the most dramatically to 7-fold control values, whereas the more radiosensitive B cells declined to 33% of control values. Macrophages and CD8⁺ T lymphocytes remained near control values, whereas the CD4⁺ T lymphocytes increased almost 3-fold. Such trends were found with all doses and time-durations post-WBI.

ii. Activation Markers

Monoclonal antibodies were also used to identify activated populations of cells.

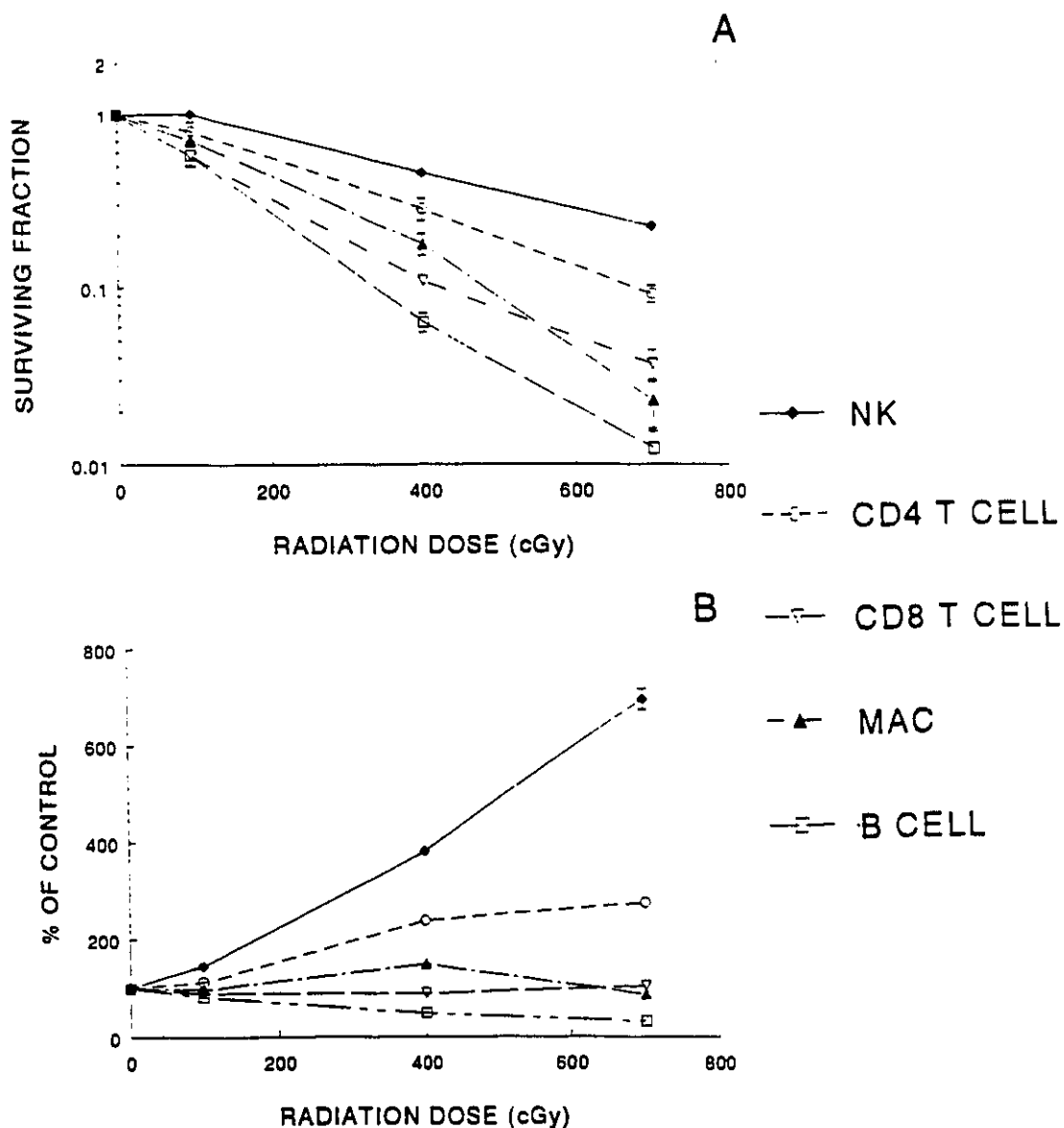


FIGURE 22. SUMMARY OF EFFECT OF IRRADIATION ON ABSOLUTE AND RELATIVE NUMBERS OF SMNC

A summary of the effect of WBI (0-700 cGy) on SMNC isolated 4 days post-irradiation. In (A) the effect on the absolute number of the major SMNC populations is shown. The surviving fraction is the ratio of the number of positive cells following a given radiation dose to the total number of positive cells in unirradiated control mice. In (B) the proportion of each SMNC population is expressed as a percentage of unirradiated control mice for those cells. The data represent the mean and SEM for four experiments with SMNC pooled from 8 mice for each experiment.

WBI produced no increase in the amount of CD25⁺ cells. The expression of CD71, the transferrin receptor, was found to be very low in fresh SMNC (controls) as can be seen in the one-parameter histogram in Figure 23. Only 3-4% of unirradiated spleen cells were found to stain positively for the marker (Table III). All doses of radiation produced no increase in the percentage of positive cells on day 1 post-WBI. However, in mice given 100 cGy, the percentage of transferrin expressing cells increased to 9% by day 4 and to 11% by day 7 post-irradiation ($p < 0.05$) (Figure 23). A higher dose of WBI, 400 cGy, did not produce an increase in CD71 expression until 7 days post-irradiation, but the increase was more dramatic, reaching of 21.3% of all SMNC ($p < 0.05$). With the very high dose of 700 cGy WBI, the expression of CD71 did not increase over the 7 day post-irradiation period. Therefore, a population of activated CD71 positive cells appeared at varying periods of time post-WBI depending on the dose given. In order to identify which SMNC population was expressing the CD71 molecule and hence was "activated", double-labelling experiments were carried out using anti-CD71 in combination with the T cell subset markers CD4 and CD8, and CD45R (a pan B cell marker). In each of these experiments there was no evidence of a double-stained population (Figure 24 A, B, and C). The population induced by irradiation to express CD71 was neither a T nor B lymphocyte.

iii. Intensity of Cell Surface Marker Expression

The level of expression of certain cell surface markers is also related to a cell's functional state of activation. The mean channel fluorescence (MCF) of the class II MHC was seen to decline with increasing radiation doses, whereas other cell surface markers

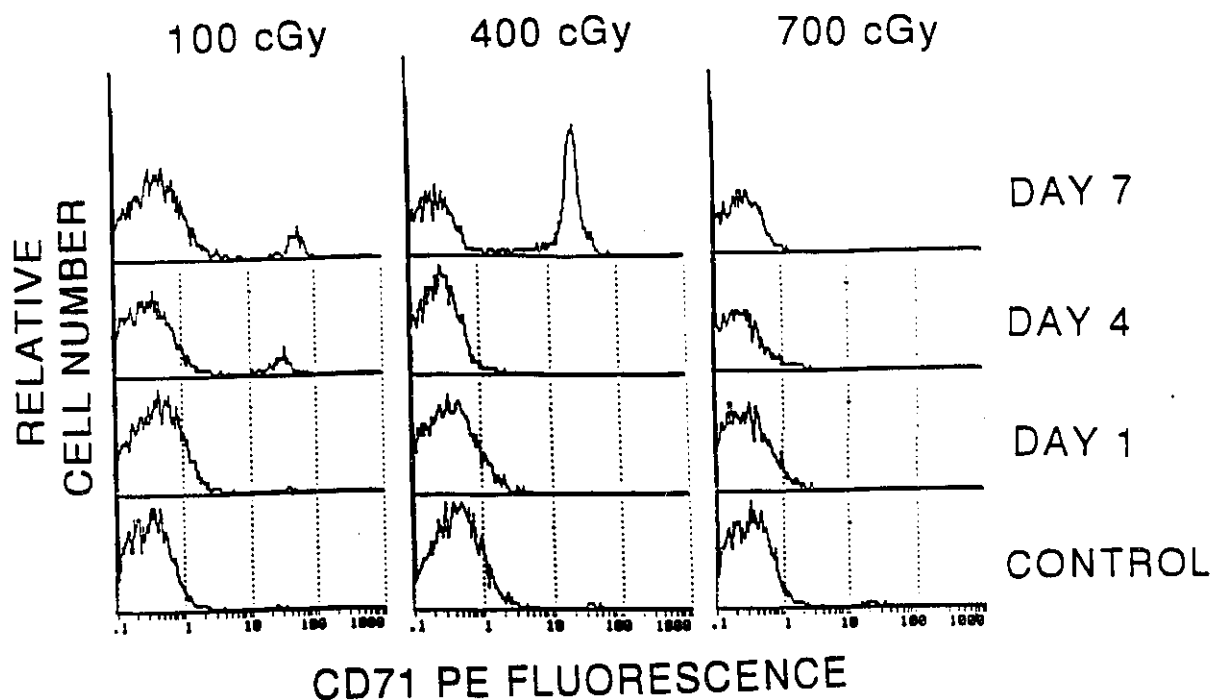


FIGURE 23. INDUCTION OF CD71 EXPRESSION AFTER WHOLE-BODY-IRRADIATION

Flow cytometric analysis of cell surface CD71 expression on SMNC. Mice were irradiated with 100, 400, and 700 cGy and after 1, 4, and 7 days their spleens were removed. The SMNC were then isolated and stained with anti-CD71 mAb. The percentage of CD71 positive SMNC from fresh untreated control mice was 3.4%. There was a significant increase in the percentage of CD71 positive cells with 100 cGy on days 4 and 7 post-WBI and with 400 cGy on day 7 post-WBI ($p < 0.05$).

had a stable expression. Figure 25 shows a representative of a total of 4 experiments comparing the level of class II MHC and of Thy1.2 expression on SMNC 4 days after exposure to 100, 400, or 700 cGy. A dose of 100 cGy did not alter the expression of class II MHC. Doses of 400 and 700 cGy, however, did result in significantly lower mean channel fluorescence (MCF) ($p < 0.05$). The MCF declined from 41.2 ± 5.3 in unirradiated mice to 11.5 ± 6.4 in mice exposed to 700 cGy. In contrast, the MCF for Thy1.2 was 48.0 ± 10.4 in unirradiated controls and 46.4 ± 9.4 in mice exposed to 700 cGy. Similarly, the MCF of other cell surface markers did not significantly change with any of the doses examined.

3. Effect of Culturing SMNC with Tetanus Toxoid.

i. Proportions of SMNC Populations

As was seen in the transfer of suppression experiments, radiation alone may not be enough to induce suppressor cell activity. The presence of an antigen may be needed in conjunction with radiation to induce a suppressive response. Flow cytometric analysis was performed on TT-treated splenic mononuclear cells to determine if TT alone caused any alteration in the cellular balance as was the case with irradiation, or whether TT caused an alteration in the expression of a surface marker which would be indicative of an altered cell function. SMNC were isolated and cultured for 48 hours in complete medium with or without TT (2.5 $\mu\text{g/ml}$). The SMNC were harvested and prepared for FCA as described in the Methods. As shown in Table IV, culturing in TT did not result in any significant differences in the proportion of the various SMNC populations.

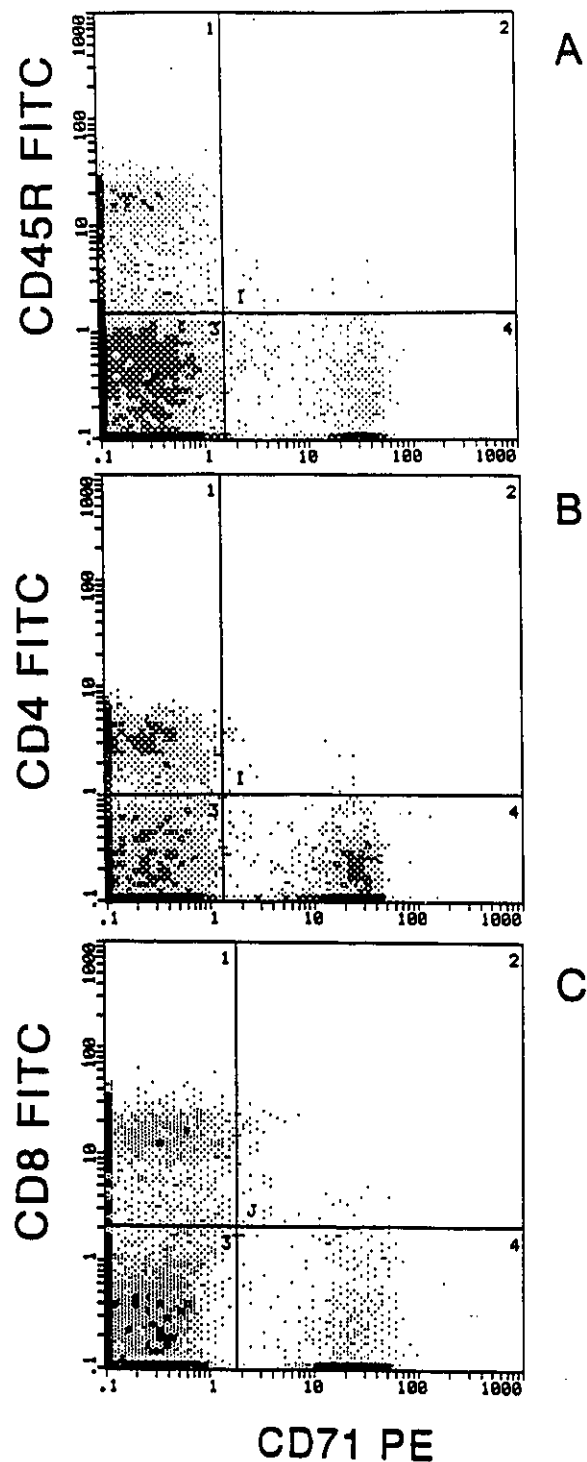


FIGURE 24. INCREASED EXPRESSION OF CD71 IS NOT FOUND ON EITHER T OR B LYMPHOCYTES.

Two-colour FCA of SMNC isolated 7 days post-irradiation (400 cGy) is demonstrated. The SMNC were labelled with PE anti-CD71 antibody (x-axis) and one of the following FITC-labelled mAbs (y-axis): anti-CD45R (A), anti-CD4 (B), and anti-CD8 (C).

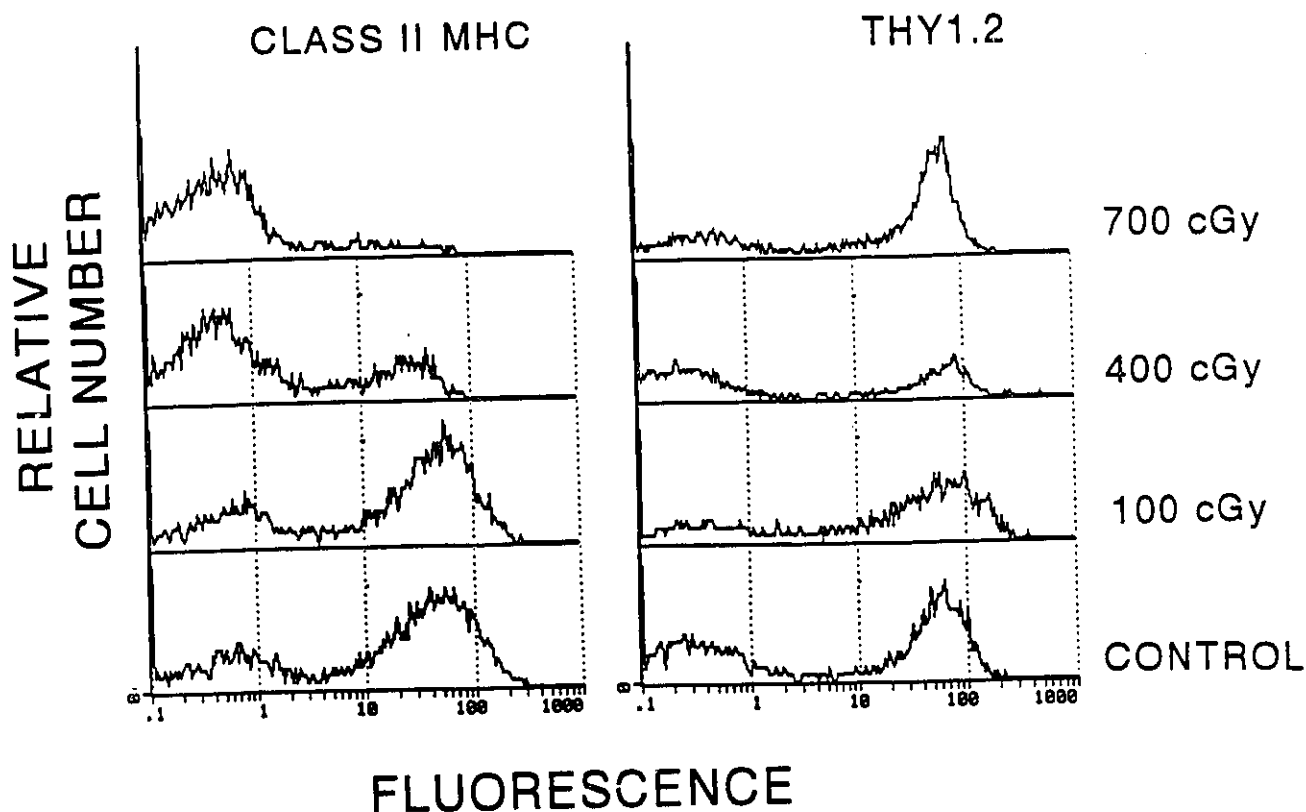


FIGURE 25. DECREASED EXPRESSION OF CLASS II MHC BY DAY 4 FOLLOWING WBL

A representative experiment (of a total of 4) in which SMNC were harvested 4 days post-irradiation and cell surface expression was examined by flow cytometry. The horizontal axis represents the log fluorescence intensity of (A) class II MHC and (B) Thy1.2 expression, and the vertical axis represents the relative number of cells. The mean channel fluorescence (MCF) for class II MHC decreased from 41.2 in non-irradiated controls to 11.5 following 700 cGy ($p < 0.05$). In contrast, other cell surface markers examined such as Thy1.2 (B) did not show such an alteration in expression. The MCF of Thy1.2 in unirradiated controls was 48.0 and following irradiation there was no significant change. No differences between non-irradiated and irradiated SMNC were observed using the appropriate negative isotype control antibodies.

TABLE IV. EFFECT OF CULTURING WITH TT (48 hr.) ON SURFACE EXPRESSION OF SMNC SUBSET ANTIGENS.

Cell Subset ^a	Mean Percentage (SEM) ^b		
	Fresh	48 Hour Culture	
		Medium	+ TT
Thy1.2 ⁺	31.3 (1.7)	25.3 (4.7)	25.9 (5.2)
Thy1.2 ⁺ CD4 ⁺	16.6 (1.8)	12.7 (3.6)	14.5 (2.7)
Thy1.2 ⁺ CD8 ⁺	9.6 (1.4)	9.2 (0.8)	10.4 (2.2)
CD45 ⁺	53.6 (4.6)	63.6 (5.8)	60.5 (3.7)
Ia ⁺	55.3 (5.0)	59.7 (3.7)	52.1 (3.9)
CD45 ⁺ Ia ⁺	56.6 (3.8)	59.9 (4.3)	59.2 (4.1)
NK1.1 ⁺	3.9 (0.8)	2.5 (1.0)	3.9 (2.2)
F4/80 ⁺	9.1 (1.2)	7.0 (2.3)	5.3 (2.7)
CD25 ⁺	ND ^c	ND ^c	ND ^c
CD71 ⁺	3.4 (1.4)	3.0 (2.1)	3.8 (2.8)
CD4 ⁺ Ia ⁺	3.7 (0.8)	4.4 (1.6)	3.7 (1.1)
CD8 ⁺ Ia ⁺	3.5 (1.0)	2.7 (0.7)	2.3 (1.4)

^aSubsets of SMNC identified by single and double-antibody labelling. Ant.bodies were FITC anti-CD4, anti-CD8, anti-CD45, and PE-mAb to other markers listed.

^bFrequency of total splenic mononuclear cells (n=5 where 2 spleens were used per experiment).

^cNot detectable.

Moreover, culturing in medium alone did not significantly change the SMNC proportions as compared to SMNC from fresh control mice. Culturing for periods longer than 48 hours, however, did alter the proportion of T and B cells as B cells die more quickly in culture than T cells (Freshney, 1983). In contrast to irradiation, TT did not cause an increase in the percentage of CD71⁺ cells (Table IV).

ii. Intensity of Cell Surface Marker Expression

To examine if the the inhibition of proliferation was the result of disrupted interactions between T cells and antigen-presenting cells, it was determined whether TT exerted any influence upon the level of expression of class II MHC molecules. The SMNC were cultured with medium in the presence or absence of TT (2.5 µg/ml) for 48 hours. SMNC were harvested and stained with anti-class II MHC or control antibody. The TT cultures showed decreased expression of MHC class II molecules after the culture period. Figure 26 shows a representative experiment comparing the level of MHC class II antigen on TT cultured control SMNC. In this figure, and in each of the other 4 pairs examined, TT-cultured SMNC expressed substantially lower levels of MHC than the controls ($P < 0.05$). Table V presents the results from 5 experiments in which the mean channel of fluorescence for the various cell surface molecules is shown. Class II MHC expression was significantly decreased for the TT-cultured SMNC from controls ($p < 0.001$). The expression of all the other surface molecules examined was not significantly altered after culturing with TT.

4. The Effect of Culturing with TT following WBI

i. Proportions of SMNC Populations

In order to correlate any effects observed in the transfer of suppression experiments when radiation and TT exposure were combined, FCA was also performed. Mice were irradiated with 100, 400, and 700 cGy and their spleens were removed 1, 4, and 7 days post-irradiation. MNC were isolated from spleens and ultured for 48 hours

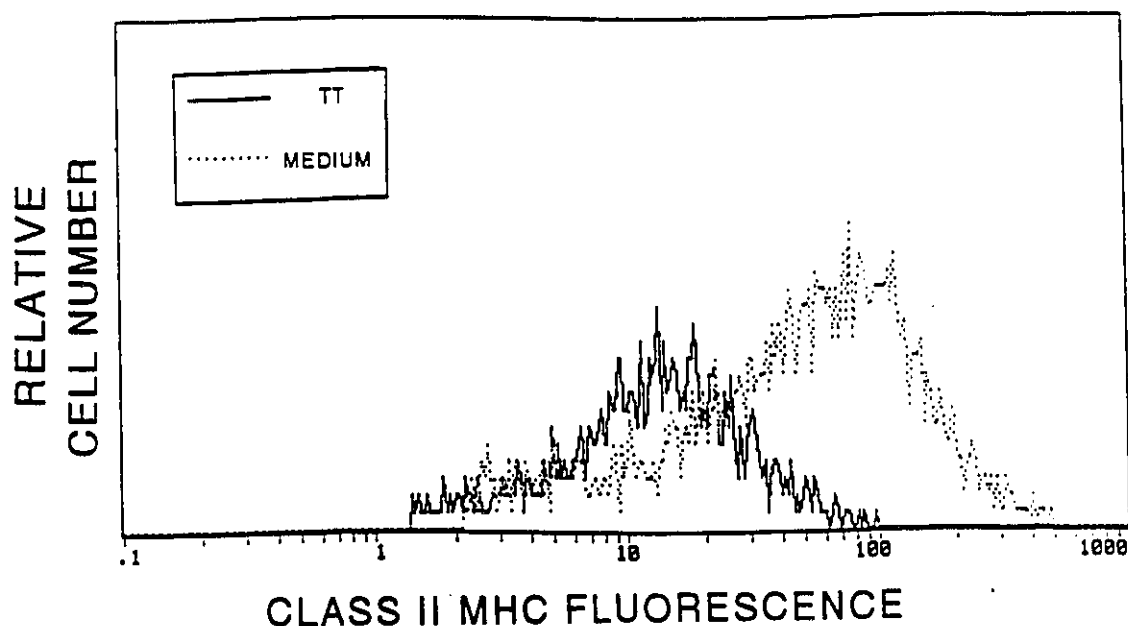


FIGURE 26. DECREASED EXPRESSION OF CLASS II MHC AFTER CULTURING WITH TT.

A representative experiment in which SMNC were cultured in medium only (stippled line) and TT (2.5 $\mu\text{g/ml}$) (solid line) for 48 hours and class II MHC expression was examined by flow cytometry. The horizontal axis represents the log fluorescence intensity of MHC expression and the vertical axis represents the number of cells. The mean channel fluorescence (MCF) values for control and TT-cultured SMNC were 44.9 and 12.1 respectively. No difference between medium and TT-cultured cells was observed using an IgG2a-PE negative control antibody (MCF 0.175 and 0.183, respectively).

in medium or TT (2.5 µg/ml). Following the culture period, SMNC were harvested from the culture flasks and prepared for FCA as described in the Methods.

TABLE V. CELL SURFACE EXPRESSION OF MOLECULES ON CONTROL AND TT-CULTURED SMNC^a

Molecule	Mean MCF (SEM) ^b	
	Control	TT
Thy1.2	20.7 (3.1)	15.1 (0.6)
CD45	4.8 (0.3)	3.8 (0.4)
Ia	47.4 (3.8)	^c 16.0 (3.1)
CD4	3.3 (0.4)	3.7 (0.2)
CD8	10.6 (1.9)	9.1 (1.8)
NK1.1	3.8 (0.2)	3.7 (0.2)
CD71	4.3 (0.2)	5.9 (1.9)

^aCell surface antigens analyzed by direct immunofluorescence and flow cytometry. The mean channel of fluorescence (MCF) was determined for each sample.

^bData are expressed as the mean of MCF±SEM of 5 experiments for the control and TT-cultured SMNC examined.

^c $p \leq 0.001$ using the Student's *t*-test.

Although TT alone was shown not to cause an alteration in the subsets of SMNC (Table IV), it was tested whether there would be any alteration in response to TT after mice were first exposed to WBI. As can be seen in Figures 27 through 32, culturing with TT did not result in any significant alterations in the proportions of populations (at the $p < 0.05$ significance level) other than the changes that were induced by WBI alone. In addition, culturing the SMNC from irradiated mice in medium for 48 hours did not significantly alter the proportion of SMNC compared to non-cultured cells.

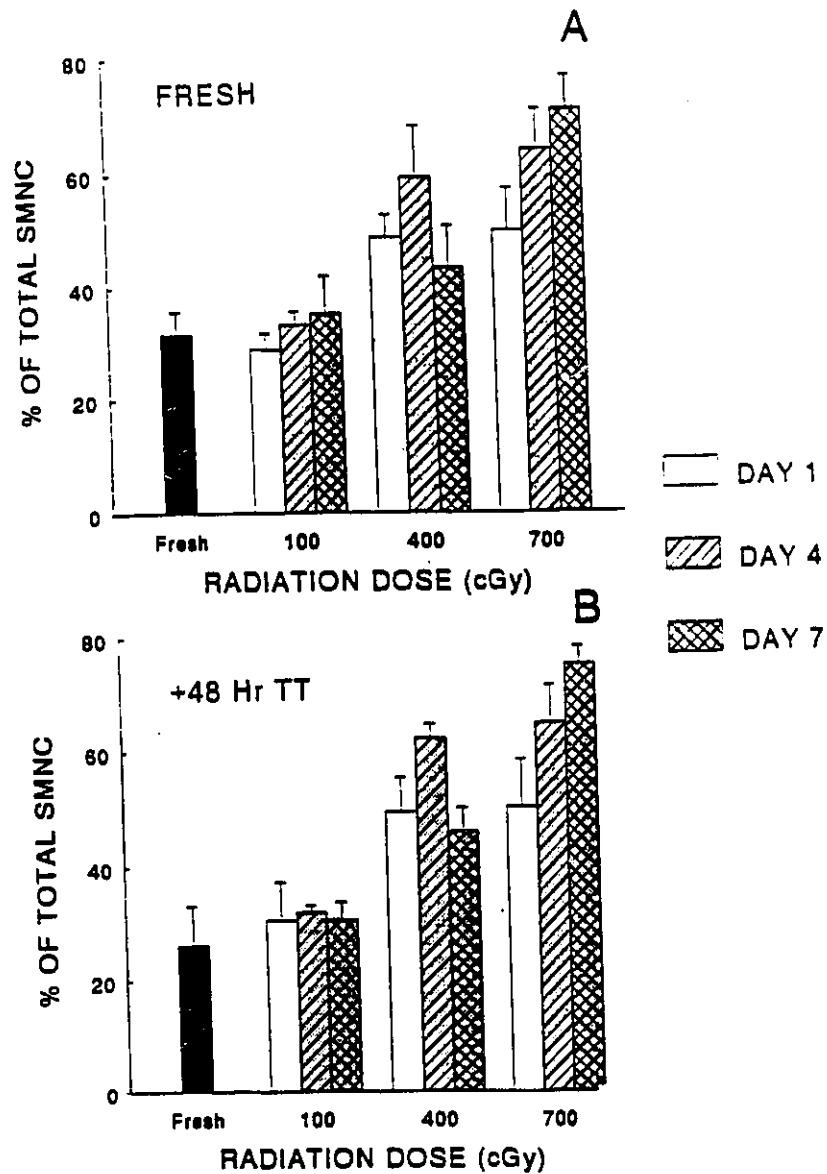


FIGURE 27. THY1.2⁺ SMNC: RELATIVE PROPORTION NOT ALTERED BY TT.

Mice were irradiated with 0-700 cGy and SMNC were analyzed by flow cytometric analysis after 1, 4, and 7 days (A). SMNC were also cultured post-WBI for 48 hours in complete medium containing 2.5 μ g/ml TT. Following the culture period, SMNC were harvested and analyzed by FCA (B). Culturing with TT had no significant effect on the relative proportion of Thy1.2⁺ SMNC. The relative proportion of SMNC cultured in complete medium alone was also not different (Table II in Appendix). Each bar represents the mean of 3-10 animals in 4 separate experiments. The error bars indicate SEM.

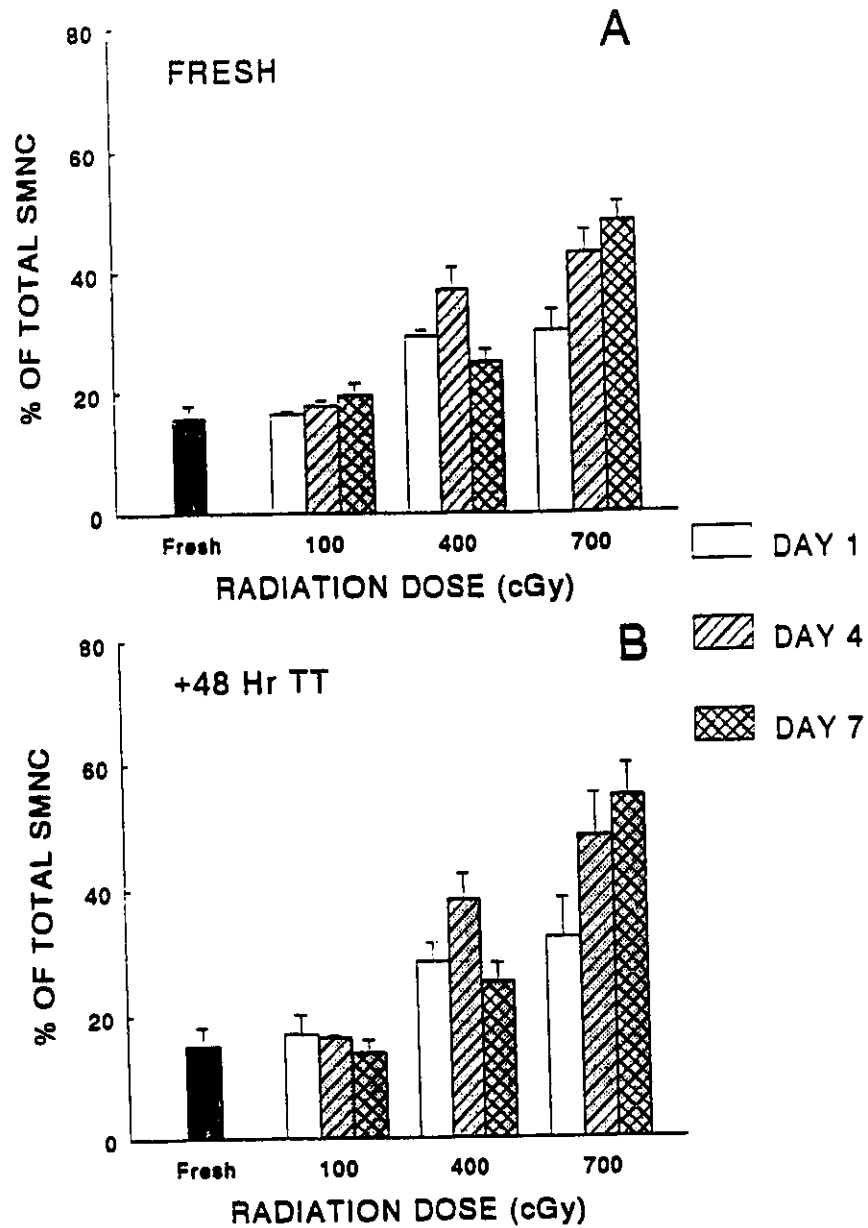


FIGURE 28. THY1.2⁺CD4⁺ SMNC: RELATIVE PROPORTION NOT ALTERED BY TT.

As in Figure 28 with the proportion of Thy1.2⁺CD4⁺ SMNC post-WBI (A), and following an additional 48 hour culture with TT (B).

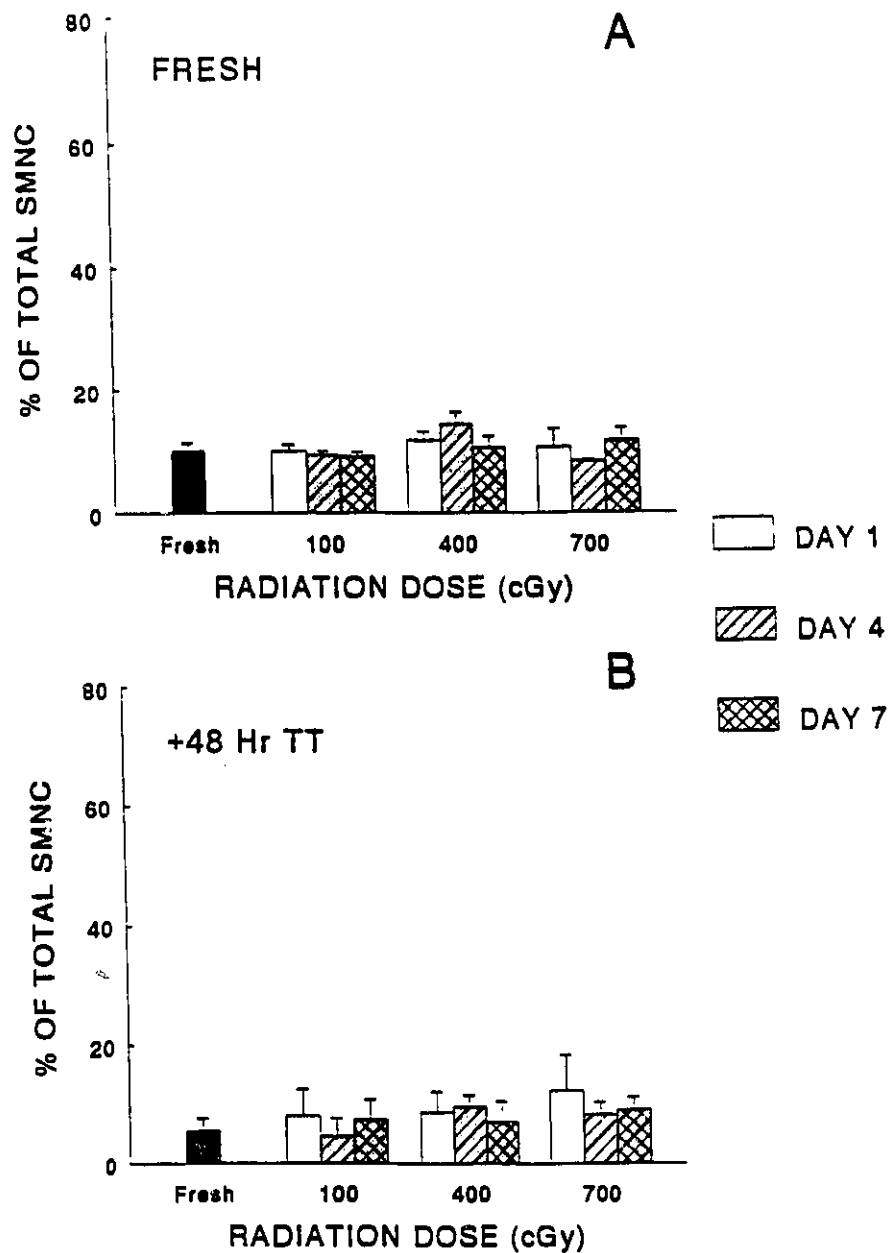


FIGURE 29. THY1.2⁺CD8⁺ SMNC: RELATIVE PROPORTION NOT ALTERED BY TT.

As in Figure 28 with the proportion of Thy1.2⁺CD8⁺ SMNC post-WBI (A), and following an additional 48 hour culture with TT (B).

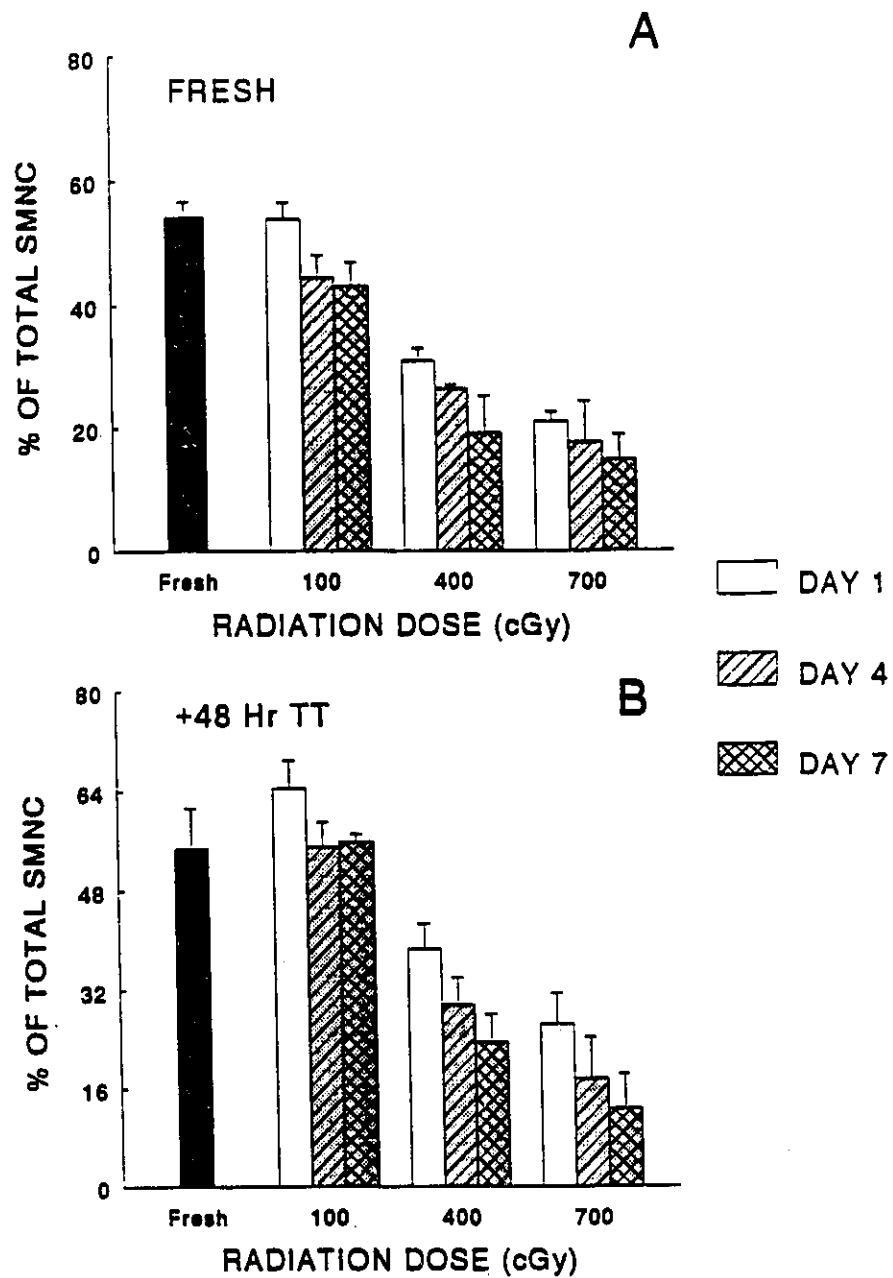


FIGURE 30. CD45⁺ SMNC: RELATIVE PROPORTION NOT ALTERED BY TT.

As in Figure 28 with the proportion of CD45⁺ SMNC post-WBI (A), and following an additional 48 hour culture with TT (B).

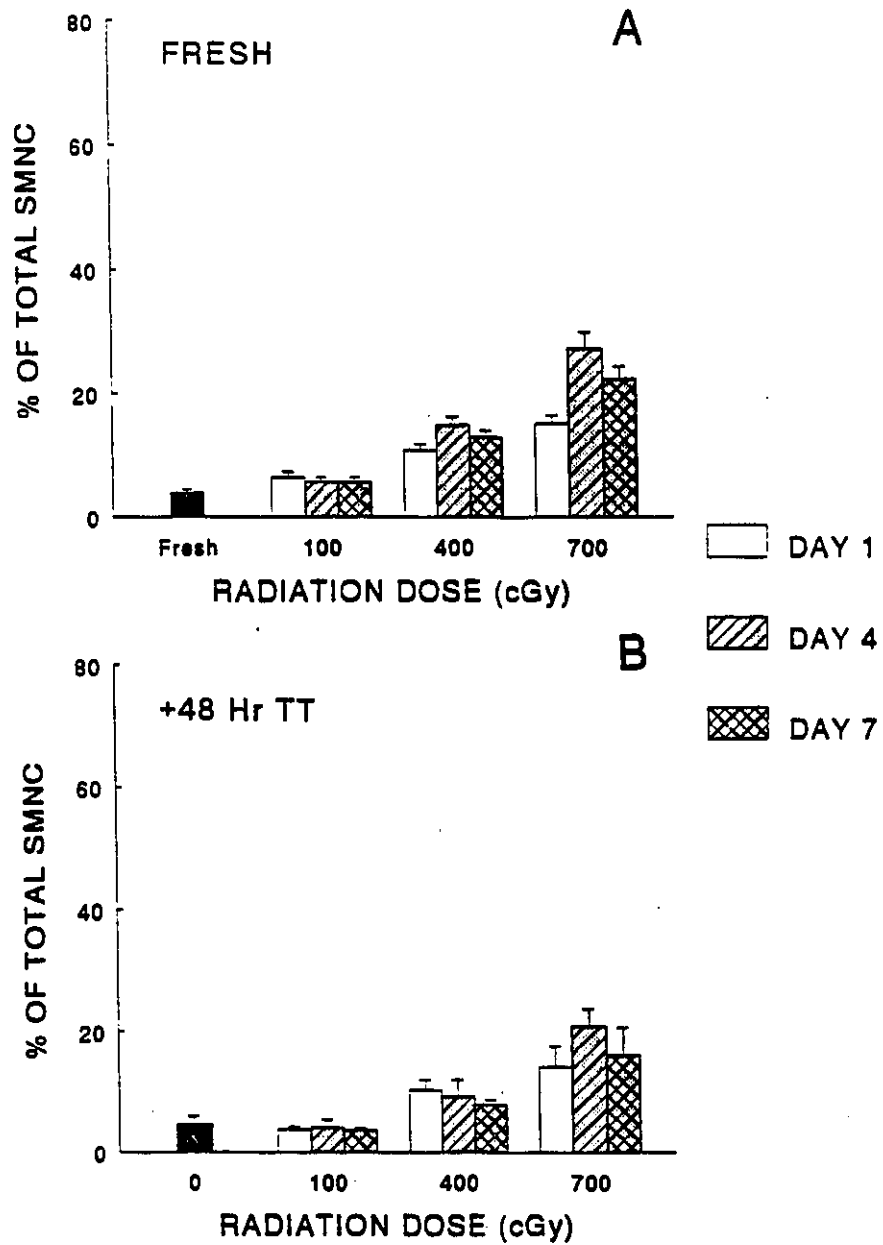


FIGURE 31. NK1.1⁺ SMNC: RELATIVE PROPORTION NOT ALTERED BY TT.

As in Figure 28 with the proportion of NK1.1⁺ SMNC post-WBI (A), and following an additional 48 hour culture with TT (B).

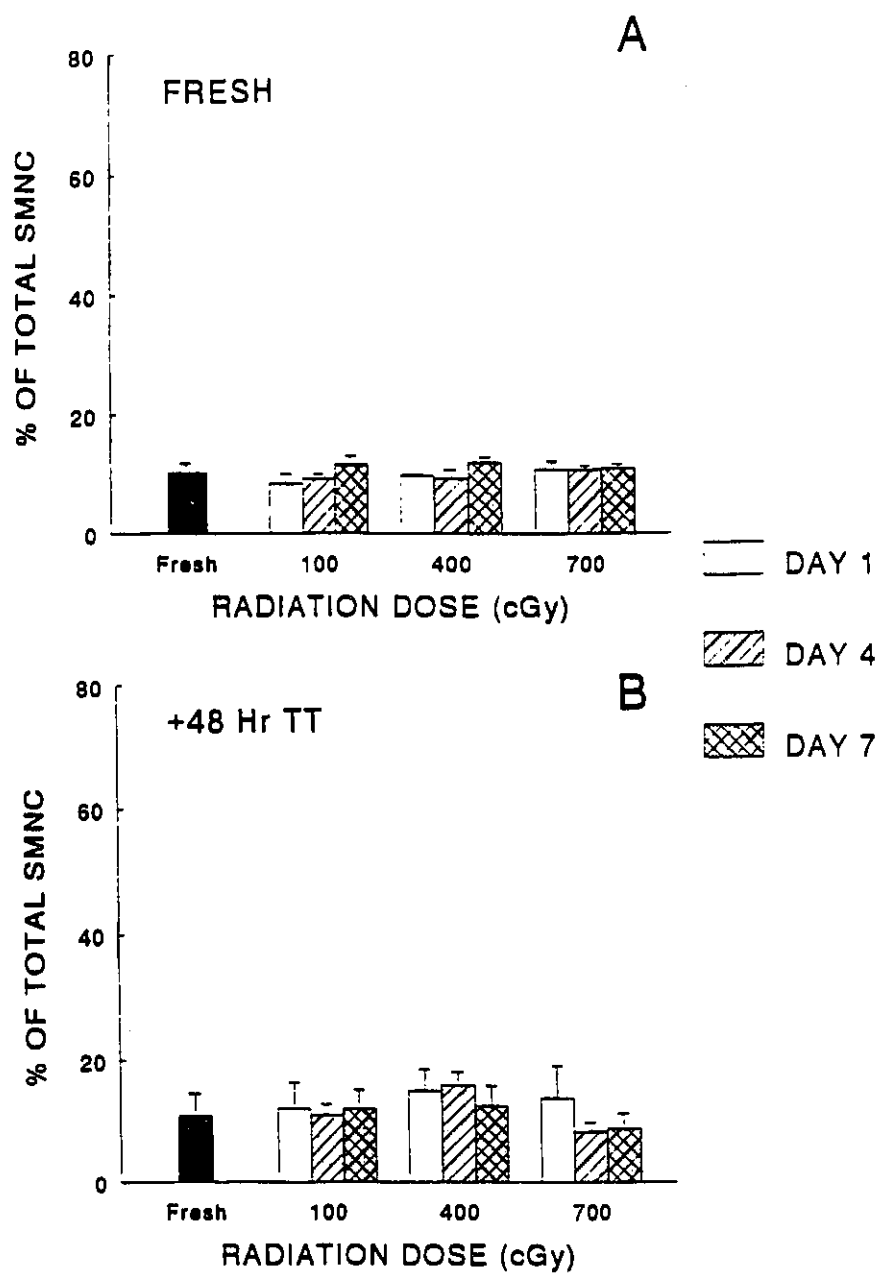


FIGURE 32. F4/80⁺ SMNC: RELATIVE PROPORTION NOT ALTERED BY TT.

As in Figure 28 with the proportion of F4/80⁺ SMNC post-WBI (A), and following an additional 48 hour culture with TT (B).

ii. Activation Markers

The effect of culturing with TT post-WBI on the expression of cell surface activation markers was also examined. As demonstrated previously, doses of 100 and 400 cGy induced the expression of CD71 by days 4 and 7 post-irradiation respectively. Table VI shows that culturing SMNC in the presence of TT had no effect on the time and radiation dose-dependent appearance of CD71 as compared to SMNC cultured in complete medium alone. Interestingly, being in culture for 48 hours post-WBI did result in an increase in the total percentage of CD71 positive cells, but this was not affected by TT.

TABLE VI. THE PERCENTAGE OF CD71⁺ SMNC FROM WHOLE-BODY IRRADIATED MICE AFTER CULTURING WITH TT.

Dose (cGy)	Control		48 Hr. Culture			
			Medium		+TT	
	Day 4	Day 7	Day 4	Day 7	Day 4	Day 7
100	9.0 (5.9)	9.7 (2.8)	12.3 (3.4)	8.7 (4.0)	10.4 (2.4)	7.7 (1.0)
400	3.0 (1.1)	21.3 (7.6)	4.0 (2.1)	34.9 (4.5)	2.7 (1.4)	35.7 (2.8)

Culturing SMNC in the presence of TT had no effect on the time and radiation dose-dependent appearance of CD71 as compared to SMNC cultured in complete medium. Having been in culture for 48 hours post-WBI did result in an increase in the total percentage of CD71 positive cells for 400 cGy.

*Frequency of total splenic mononuclear cells (mean percentage $\pm$ SEM, n=4).

Mice exposed to 400 cGy had 21.3% $\pm$ 7.6 CD71 positive cells whereas after culturing in medium, 34.9% $\pm$ 4.5 of SMNC were positive and after culture in TT 35.7% $\pm$ 2.8 were positive. This increase was not observed in cultures from mice exposed to 100 cGy WBI.

iii. Expression of Cell Surface Markers

The effect of WBI followed by culturing SMNC with TT on the expression of class II MHC expression was examined (Figure 33). As shown previously, TT was capable of downregulating the expression of MHC following 48 hours of culture. WBI of 400 and 700 cGy (but not 100 cGy) was also found to downregulate expression. Experiments were performed to determine if there was a relationship between the ability to transfer suppression to untreated syngeneic cells and alterations in the expression of class II MHC. SMNC from mice exposed to 100 cGy WBI did not have decreased MHC expression (Figure 25). When these SMNC were cultured with TT for 48 hours, however, the MHC expression decreased to the levels of unirradiated SMNC cultured with TT (Figure 33B). While the MHC expression of SMNC isolated after 400 cGy was decreased, culturing with TT further reduced expression to the levels of unirradiated SMNC cultured with TT (Figure 33C). The MHC expression of SMNC isolated after 700 cGy was reduced and no further decrease was observed after culturing with TT (Figure 33D). All other surface molecules remained stable after both WBI and culture with TT.

F. LEVELS OF IL-10 FOLLOWING IRRADIATION AND TT

It was investigated whether IL-10 may have a role to play in the immunosuppressive properties of TT/irradiation. Levels of IL-10 were measured by ELISA from culture supernatants of SMNC from various conditions after 24 and 48 hours and compared to an IL-10 standard curve (Figure 34). IL-10 was not found to increase as a result of culturing SMNC with TT or with TT and PHA. Similarly, SMNC from

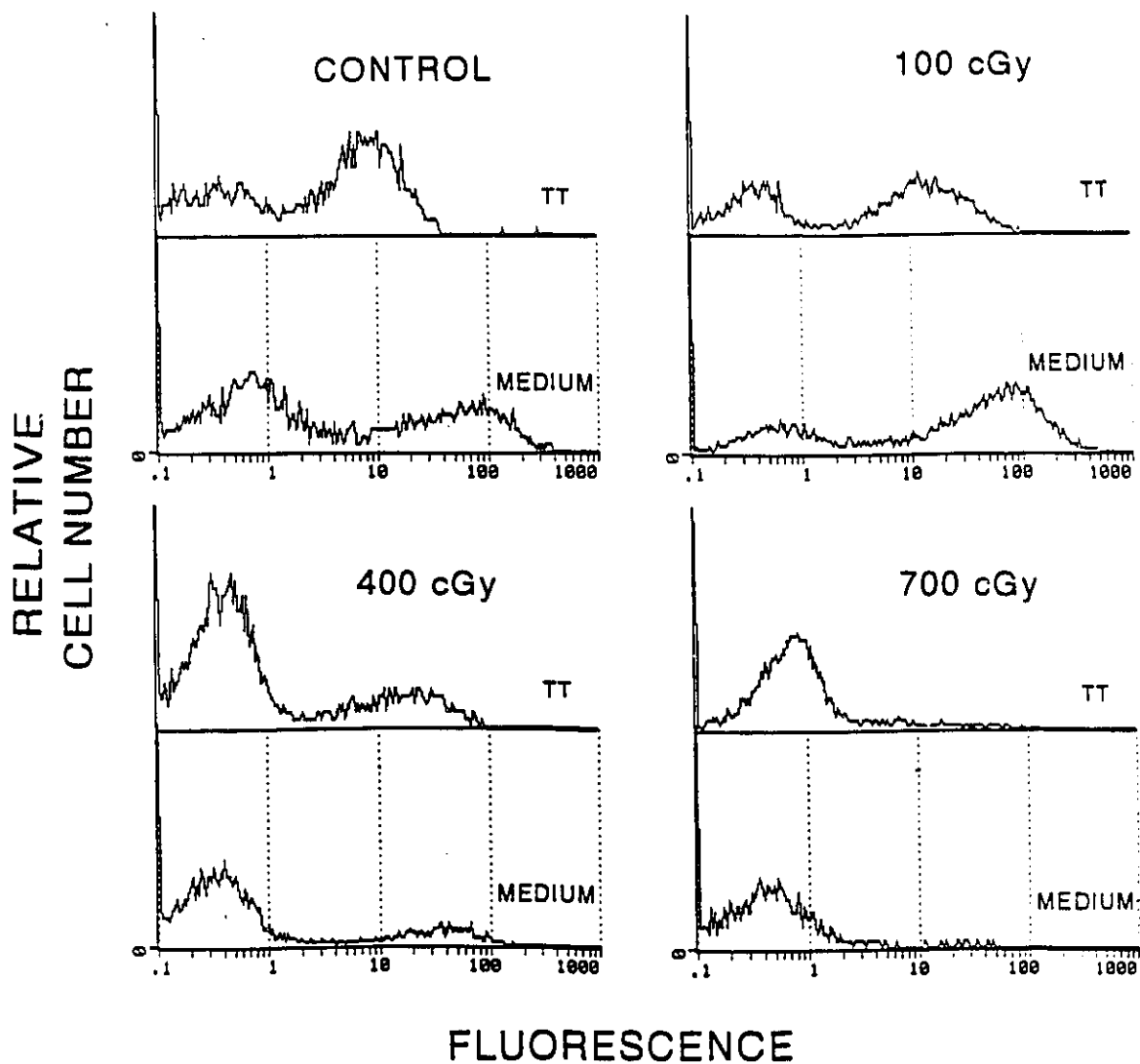


FIGURE 33. THE EFFECT OF WBI ON DAY 4 AND CULTURING WITH TT ON CLASS II MHC EXPRESSION.

SMNC were isolated from mice 4 days after WBI with 0 (A), 100 (B), 400 (C), and 700 (D) cGy. The SMNC were cultured in complete medium with or without TT (2.5 $\mu\text{g/ml}$) for 48 hours after which they were washed and prepared for flow cytometric analysis. A representative experiment (of a total of four) of class II MHC expression is shown. No difference between non-irradiated and irradiated SMNC was when observed using the appropriated negative control antibodies.

mice exposed to 100 cGy, harvested on days 1 or 7 post-irradiation did not have increased IL-10 levels. SMNC cultured with Con A was used as a positive control and had measurable levels of IL-10.

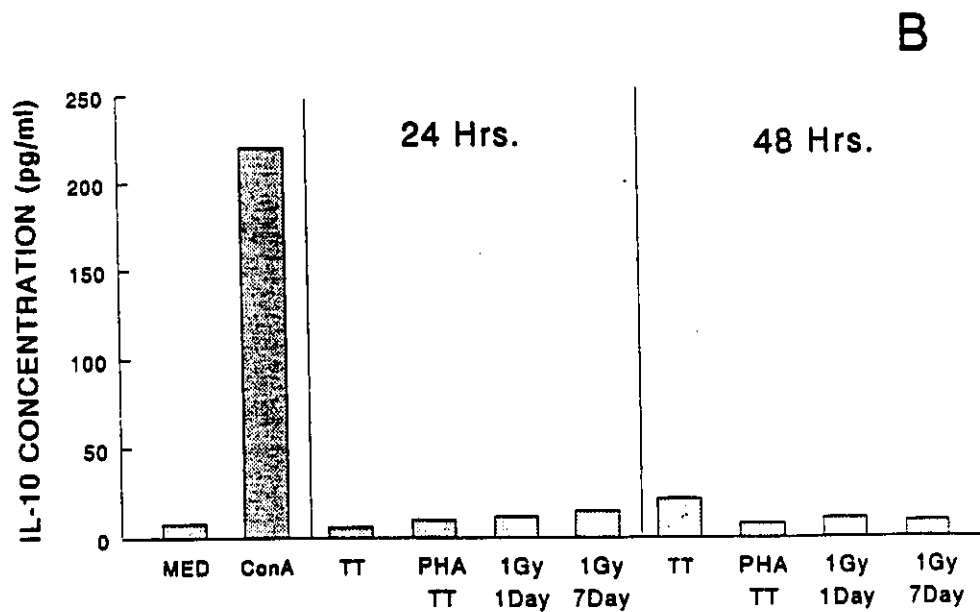
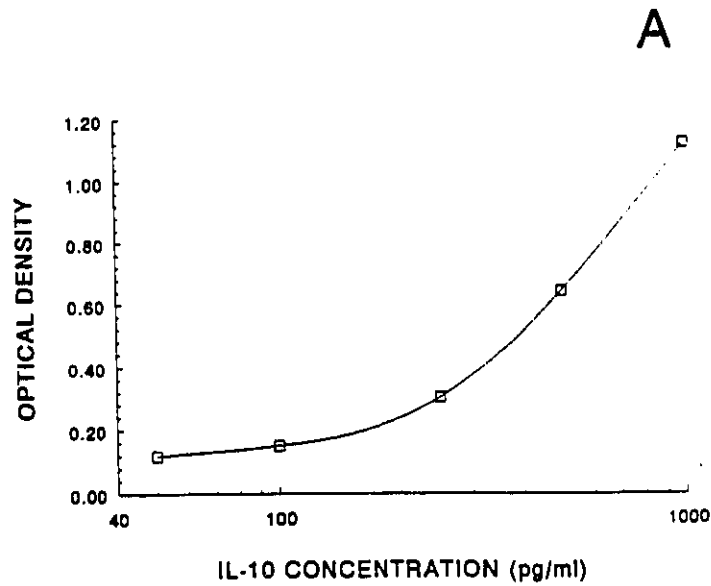


FIGURE 34. IL-10 LEVELS WITH CULTURED SMNC NOT INCREASED BY WBI AND TT.

An ELISA was performed to measure levels of IL-10 in culture supernatants from various conditions. In (A) the standard curve of mouse IL-10 concentrations is shown. In (B) SMNC were harvested on days 1 OR 7 post-WBI and put into culture. After 24 and 48 hours, culture supernatants were removed for ELISA. Similarly, SMNC from fresh control mice were cultured with TT and TT/PHA, and supernatants were removed after 24 and 48 hours. SMNC cultured with Con A was used as a positive control.

VL DISCUSSION

It has been demonstrated that Tetanus toxoid (TT) can be immunosuppressive to mouse cellular mitogenic responses *in vitro*. Many other bacterial, viral and fungal antigens and their toxins have been shown to have suppressive effects both *in vivo* and *in vitro* (Cuff *et al.* 1989; Rouse & Horohov, 1986; Taub *et al.* 1989; Wainberg & Mills, 1985). 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 would be necessary to eliminate pathogens (Asherson *et al.* 1986). The triggering of a suppressive pathway could reflect a shift in a group of cells which are reacting directly with antigen or indirectly as a result of factors produced by other cells encountering antigen. In addition, total-lymphoid irradiation (Oseroff *et al.* 1984; Strober *et al.* 1979), and in some cases whole-body irradiation, have been shown to produce active immune suppression (Waer *et al.* 1984). Radiation may directly activate suppressor cells, or their activity may be related to their relative resistance to radiation damage. The work described in this thesis examines the effects of toxin and radiation on lectin-induced proliferation and has attempted to delineate a mechanism by which TT can be immunosuppressive and how it may interact with irradiation to further suppress the immune response.

A. SUPPRESSION OF MITOGENIC RESPONSES BY TETANUS TOXOID

Experimental conditions were established such that normal mitogenic responses

of murine SMNC to PHA and Con A could be semi-quantified. TT clearly suppressed the blastogenic response to PHA (Figure 5). The suppression was dose-dependent and was complete at higher doses of antigen. TT was shown not to have any direct toxic effects on cells in culture since viability counts performed by Trypan Blue dye exclusion remained unchanged (Table II). Cell death due to toxicity thus could not explain the depressed blastogenic response. Moreover, if the TT were simply toxic, one would not expect the kinetics of induction of suppression to vary with the duration time of TT exposure as demonstrated in Figure 6.

It has been known for many years that compounds such as phytohemagglutinin and concanavalin A are strongly mitogenic for unprimed T cells (Ling & Holt, 1967; Simons *et al.* 1969). These compounds appear to bind directly to the TCR and TCR-associated molecules (CD3, CD4, CD8, etc.) on T cells and induce rapid DNA synthesis in a very high proportion of these cells. T cell responses to these artificial ligands generally require the presence of other cell types, termed "accessory" cells. As described in the Introduction, these cells seem to have two main roles. First, through non-specific binding, accessory cells can present the ligand to T cells in cross-linked form (cross-linking of TCR molecules appears to be a prerequisite for T cell triggering). Second, accessory cells synthesize certain soluble or cell-bound molecules which play a critical role in inducing or potentiating T cell activation.

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

Previously, HBsAg (Filion *et al.* 1989) and more recently live attenuated measles vaccine (Smedman *et al.* 1994) have been shown to be suppressive to PHA responses in both vaccinated and non-vaccinated individuals. This finding supports the view that antigen-related suppression may induce specific and non-specific phenomena, and explains why a broad range of antigens have been able to exert a negative modulation to a similar degree. For example, suppression has been demonstrated from culturing with PT, TA, gp 120, and soluble extracts from *Helicobacter pylori* and *Salmonella typhimurium*, (Arora *et al.* 1987; Luft *et al.* 1988; Shalaby *et al.* 1987; Knipp *et al.* 1994; Katsyhiko & Toshihiko, 1994). Antigen normally triggers antigen-specific suppressor cells by means of a feedback mechanism during infection following the induction of appropriate antibody responses. This process begins to peak at the time the pathogen is cleared (Roitt, 1989). This suppression is distinct from non-specific suppression which occurs prior to and irrespective of antibody production or DTH reactions.

Kinetic experiments were designed in order to determine the duration of exposure to TT which was necessary for suppression to be detected. Suppression of the PHA response began to occur between 1 hour (when no suppression was detected) and 24 hours (when suppression was pronounced, at 53%) (Figure 6). This time-dependent effect indicates that the suppressive action of TT was not simply the result of disrupted interactions between mitogen and the SMNC. Such an effect would have been observed almost immediately. Similar findings have been observed in Pertussis Toxin (PT)-induced suppression of CD3/TCR or PHA responses (MacIntyre *et al.* 1988); a 2-hour period was not sufficient to induce suppression by pertussis toxin. This is consistent with the

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 and washing out at different times to determine the extent of exposure to TT required.

1. *Suppression is an Active Phenomenon*

To determine if the nature of the TT-induced suppression was purely metabolic or the result of the induction of a tangible suppressor cell, TT-suppressed cells were combined with fresh autologous cells during PHA stimulation (Figure 7). Recent studies have demonstrated that some antigens can act as powerful and specific inhibitors of T cell activation by engagement of the TCR below a crucial affinity threshold necessary for full T cell activation (Sette *et al.* 1994). Transfer of a suppressive state by TT-treated cells to fresh autologous cells would eliminate such an explanation and support the idea of an active mechanism of suppression through the activation of a suppressor cell. Examination of the kinetics of the suppression and of the viability of the cell cultures ruled out "trivial" explanations of suppression, such as a shift in the kinetics of peak ^3H -TdR incorporation or suppression due to cell death. In addition, the induction of the suppression was found to be dose-dependent (Figure 8) and was not observed when cells were exposed to TT for 1 hour. The possibility of an antigen carry-over effect was thus eliminated (Figure 9). The actual transfer of suppression was not due to active replication of the suppressor cells, since irradiation with 30 Gy did not prevent the transfer of TT-induced suppression. It

remains possible, however, that the TT-treated cells were sucking up (adsorbing) all the IL-2, leaving little for the readout cells. Experiments in which IL-2 is added to cultures could be performed to see whether the suppression could be overcome.

2. Flow Cytometric Analysis and Culturing with TT

The phenotype of a possible suppressor cell was of interest. Previous studies in humans have shown that removal of CD4⁺ cells prior to induction of suppression by TT greatly diminished the suppression of the PHA response (Stefanovic *et al.* 1989). The CD4⁺ cell may be important in the induction of suppression, but the CD8⁺ cell may be the effector cell. The necessity for the interplay between both these cell types has been documented (Dorf & Benaceraf, 1984; Cantor *et al.* 1976; Germain & Benaceraf, 1981). Furthermore, the effector cell may be CD4⁺ or a monocyte, as "suppressor" CD4⁺ T cells and monocytes have been documented (Bottomly *et al.* 1983; Luft *et al.* 1988).

In order to determine if the cellular immune suppression induced by TT was the result of an alteration in cellular balance and/or the activation of particular subsets of splenic mononuclear cells, we turned to the technology of fluorescent cytometric analysis. One of the early objectives of this research was to establish a reproducible protocol for the FCA of murine splenic mononuclear cells. Although cell surface markers on human myeloid and lymphoid cells are well characterized, their murine equivalents are not. As a result antibodies obtained from manufacturers have not always been specific or standardized against mouse myeloid or lymphoid cells. Consequently the antibodies had to be titrated in order to determine optimal concentrations for the various SMNC

populations, as outlined in the Methods. Since total leukocyte counts were performed prior to FCA using a Coulter Counter, it was possible to determine the absolute number of each subpopulation of SMNC per spleen. Bearing in mind that enzymatic digestion and Lympholyte-M separation processing may select for some cell types (Garcia *et al.* 1987), FCA was performed on both unprocessed and processed SMNC in order to ensure that the cell population counts obtained reflected those in the native spleen. Similar results were obtained both for cell surface antigen expression and for the proportion of each subset (Table VII and VIII in Appendix), demonstrating there had been no selective elimination of a specific cell type.

Through single-labelling experiments, it was possible to characterize the major MNC populations of the murine spleen including T cells, B cells, NK cells, and monocytes/macrophages. The proportions of the various populations, given as a percentage of the total number of splenic mononuclear cells (shown in Table III), corresponded with values in the literature using classical immunofluorescent techniques (Johnstone *et al.* 1982). The T lymphocytes were subdivided into their respective subpopulations of CD4⁺ (T helper/inducer) and CD8⁺ (T cytotoxic/suppressor) in double-labelling experiments. The approximate 2:1 ratio of CD4⁺ to CD8⁺ T cells is consistent with values seen previously in both mice and humans (Roitt, 1989).

In addition to identifying the various mononuclear cell subsets present in the spleen, specific monoclonal antibodies were used to identify activated populations of cells. This is possible because certain cell surface molecules are expressed only when a cell becomes activated, such as the CD25 and CD71 molecules on lymphocytes (Waldmann,

1986; Reed *et al.* 1986). Other cell surface molecules are more intensely expressed when a cell becomes activated. Experiments using an anti-CD25 mAb, unfortunately, did not result in any detectable staining above isotype control levels. The CD25 molecule is the interleukin-2 receptor which is found on activated T cells and is required for T cell activation (Abbas *et al.* 1991). One of the initial aims of this thesis project was to examine the percentage of CD25⁺ and other activation markers following TT treatment. It was my hypothesis that a subpopulation of CD8⁺ cells was involved as an effector in the observed suppression. Recent work (Inoue *et al.* 1993) suggests CD8⁺ T cell clones can be subdivided into two subgroups based upon the pattern of cytokine production and upon CD45 isoforms which exactly correspond with the functionally assigned cytotoxic (CTL) and suppressor (Ts) subsets. This is analogous to the Th1 and Th2 subsets within the CD4⁺ T cell compartment. The Ts clones were characterized as the effector type Ts, which directly and non-specifically inhibited T cell dependent responses and produced IL-10 as a suppressive lymphokine. If this was indeed the case, the proportion of CD8⁺CD25⁺ cells in the experimental model presented here would be expected to increase if a CD8⁺ "suppressor" cell was activated by irradiation or TT. It is possible that the anti-CD25 mAb may not have been specific for the murine CD25 molecule or the sensitivity was not sufficient.

One of the major stumbling blocks of flow cytometric analysis is the procurement of suitable, specific monoclonal antibodies for various murine cell types. As the technology improves, examination of this hypothesis will become feasible. Other activation markers which were successfully characterized included the CD71 and class II

MHC molecules. The CD71 molecule, better known as the transferrin receptor, mediates cellular iron uptake via the internalization and recycling of transferrin. The expression of class II MHC molecules has been shown to be up-regulated in activated macrophages, T cells, and B cells (Adams & Hamilton, 1987; Ko *et al.* 1979).

In addition to determining the percentage of cells stained by a particular antibody (or antibodies), FCA provides a measure of the average fluorescent intensity of a labelled population of cells. Assuming a constant ratio of fluorochrome to protein for an antibody, the mean channel number is therefore a function of the number of receptors present on the surface to which the antibody molecules are able to bind. In order to ensure that mean channel number differences occurring between experiments were not a consequence of the FCA instrument itself, the machine was calibrated prior to each experiment as described in the Methods. After such calibration, differences in mean channel numbers between experiments involving the same antibodies can be attributed to changes in cell surface marker expression. Such changes may occur as a result of up- or down-regulation of a particular cell surface marker.

Flow cytometric analysis revealed that culturing with TT did not affect the proportion of any of the SMNC populations (Table IV). This indicated that the TT-induced suppression of the blastogenic response would appear to be dependent upon a change in T cell function rather than upon an alteration of the cellular balance. It was demonstrated, however, that the class II MHC expression on SMNC was down-regulated following the 48-hour culture period with TT (Figure 26). This observation was in contrast to the other cell surface molecules examined (Table V).

In the mouse spleen, class II MHC molecules are present primarily on monocytes/macrophages and B cells. Further double-labelling experiments could be performed in which the expression of MHC molecules on macrophages and B cells could be separately examined in order to determine if TT exerts its down-regulating effect on only one or both cell types.

Important accessory molecules on the surface of antigen-presenting cells include class II MHC molecules (Unanue & Allen, 1987) and the adhesion molecules ICAM-1 and LFA-1 (Springer, 1990; Dougherty *et al.* 1988). It has been shown that Ag-specific proliferation of Th0, Th1-like, and Th2-like CD4⁺ human T cell clones and the inhibition of proliferation are dependent upon the use of monocytes/macrophages as APCs and are associated with a down-regulation of class II MHC Ag on the surface of these cells (de Waal Malefyt *et al.* 1993). The presence of TT at a concentration of 2.5 µg/ml effectively inhibited lymphoproliferation and reduced the expression of MHC II on splenic mononuclear cells. The class II MHC molecule may thus be an accessory cell bound molecule which plays a critical role in the induction or potentiation of T cell activation to PHA. The observed down-regulation of class II MHC may represent an inappropriate costimulatory signal which, delivered by the monocyte, results in an inability of the T cell to respond to mitogen and/or to generate its own growth factors. At present it remains to be examined whether or not the reduction of class II MHC expression and the antiproliferative effect are closely connected events mediated by the same factor. The possible involvement of a suppressor cell in this phenomenon is supported by the identification of a splenic suppressor cell in neonatal mice which blocks the expression

of class II MHC molecules on macrophages (Snyder *et al.* 1982). The adhesion molecules ICAM-1 and LFA-1 have been shown to be involved in the key initial adhesive interaction between T cells and APCs (Ibrahim *et al.* 1994). The down-regulation of these adhesion molecules on accessory cells results in the suppression of T cell proliferation (Fedoseyeva *et al.* 1994). In order to determine if the effect of TT is restricted to class II MHC molecules, further FCA experiments could be performed to examine the expression of ICAM-1 and LFA-1 molecules.

3. *A Possible Cell Mediator of Suppression*

A good candidate for the mediation of responses in mitogenic reactions would be a phagocytic cell like the monocyte/macrophage which can take up antigen non-specifically, and in the course of processing it, become activated. Upon such activation, other cells could be triggered by the MΦ or factors produced by it (Unanue & Allen, 1987; Goldyne, 1988). The activation and regulation of the cellular immune system depends on cell-cell interactions. Monocyte/macrophages have an important role as accessory and regulatory cells in this system. As accessory cells, monocyte/macrophages are essential for the activation of T cells by antigens and mitogens (Unanue & Allen, 1987; Rosenstreich *et al.* 1976). MΦ have been shown to elaborate a wide variety of substances including leukotrienes, HETEs, and other metabolites of arachidonic acid, many of which have been shown to have immunomodulatory effects (Goodwin & Ceuppens, 1983; Goldings, 1986). PGE₂ is one of the end-products of the cyclooxygenase metabolic pathway and has long been associated with immunosuppression and

immunomodulation (Goodwin & Webb, 1980; Plescia & Racis, 1988). Previous work has found that TT-treated cultures treated with indomethacin showed a greatly diminished suppression, although a complete reversal was never seen (Stefanovic & Fillion, 1990). Indomethacin is a known inhibitor of the cyclooxygenase pathway and has often been effective in reversing suppressed mitogenic responses (Vercammen & Ceuppens, 1987). This pointed to the possible involvement of the cyclooxygenase pathway within the MΦ as being involved in the suppression. Because reversal of the suppression was never complete and varied according to the degree of suppression conferred by TT, it was speculated that additional mechanisms of suppression could be involved. While TT was found not to interfere with normal IL-1 or TNF- α production or release (Stefanovic, MSc Thesis, University of Ottawa, 1991), the observed down-regulation of class II MHC described in this thesis may represent just such an inappropriate costimulatory signal delivered by the monocyte/macrophage.

Further experiments could be performed to elucidate whether MΦ or activated lymphocytes are the target of the TT suppressive activity. For example, pretreatment of monocytes with TT would determine if this is sufficient to mediate inhibition of lymphoproliferation in the absence of TT after PHA-activation. Similarly, the effect of pretreating resting T cells with TT on the PHA-induced proliferative response could be examined. Most recently, immunosuppressive properties of mouse macrophages have also been found to be based on the generation and release of reactive nitrogen intermediates (RNI) (Stein & Strejan, 1993; Lejeune *et al.* 1994). Macrophages produce nitric oxide (NO) by the enzymatic oxidation of the guanido nitrogen of L-arginine. The highly

reactive NO is further oxidized to the more stable products, nitrite (NO_2^-) and nitrate (NO_3^-). The possibility of RNI involvement in the TT-induced suppression could be investigated by detecting nitrite levels and by adding a competitive inhibitor of nitric oxide synthetic pathway such as N^G -monomethyl-L-arginine (MMA) to cultures.

4. A Role for IL-10?

Among the regulatory factors limiting the macrophage response to infection and the expansion of Ag-specific T cells, IL-10 has received recent attention. Murine IL-10, initially identified as a product of Th2 CD4^+ T cell clones, is now known to be produced by a variety of hematopoietic cells including B cells (O'Garra *et al.* 1992) and macrophages (Fiorentino *et al.* 1991a). Murine IL-10 inhibits the IFN- γ production of Th1 cells by interfering with the accessory cell function of mouse macrophages (Fiorentino *et al.* 1991b). On the other hand, however, IL-10 does not impair the ability of macrophages to stimulate cytokine production by Th2 cells. It has been proposed that the inhibitory effects of murine IL-10 are mediated by the inhibition of a thus far ill defined costimulatory activity on the macrophage (Fiorentino *et al.* 1991b). Furthermore, IL-10 has been shown to inhibit the intracellular killing of bacteria (Bogdan *et al.* 1991) and the secretion of cytokines by monocytes/macrophages (Fiorentino *et al.* 1991a). IL-10 has also recently been reported to be an effector cytokine from Ts clones (Inoue *et al.* 1993; Hisatsune *et al.* 1994). Since it was found that TT down-regulated class II MHC and that suppression could be transferred to fresh autologous cells, it was worthwhile to investigate whether there was a role for IL-10 in our system.

Levels of IL-10, however, were not found to be increased in cultures of SMNC treated with TT as compared to control cultures either after 24 or 48 hours (Figure 34). Similarly, cultures treated with TT and PHA did not have increased IL-10 levels (to eliminate the possibility that IL-10 release required subsequent stimulation by mitogen). This would suggest that TT did not cause decreased class II MHC expression via IL-10. Recent studies suggest that while the inhibition of macrophage function by IL-10 in human cells is associated with down-regulation of class II MHC, the murine model involves the down-regulation of costimulatory activity from LFA-1 or ICAM-1 (Fiorentino *et al.* 1991b). It is possible that TT induced the production of some as yet unidentified factor to cause decreased MHC expression or perhaps TT bound directly to the MHC as has recently been described for the bacterial superantigen Staphylococcal enterotoxin-B (SEB) with x-ray crystallography (Jardetzky *et al.* 1994). This may result in internalization of MHC molecules or sterically interfere with its function. Similarly, it can be suggested that the ability to transfer the TT-induced suppressive state to fresh autologous cells was not due to the action of IL-10. This, however, does not rule out the possibility of an activated suppressor cell as other Ts cell soluble factors have been identified such as the soluble form of the $\alpha\beta$ heterodimer of the TCR (Chen *et al.* 1994) and RNI from macrophages (Stein & Strejan, 1993). The ELISA was sensitive to approximately 100 pg/ml (Figure 34A), whereas an ELISA for human IL-10 levels has been developed sensitive to 16 pg/ml (Kumar, personal communication) suggesting the lack of effect may be associated with sensitivity problems.

B. IMMUNE SUPPRESSION BY IRRADIATION

Whole-body irradiation of mice, especially at doses of 400 and 700 cGy, produced a dramatic decline in the number of nucleated cells per spleen presumably due directly to cell death and perhaps also indirectly to migration from the spleen into the circulation (Figure 10). WBI produced an exponential decline in cell survival of splenic cells as has been previously demonstrated both *in vitro* and *in vivo* (Coggle, 1973). With a dose of 700 cGy some animals may die within 30 days from hematopoietic death. Within a few hours of a whole-body dose of 2-10 Gy there is reduction in the number of nucleated bone marrow cells (Coggle, 1973). Severe damage to the bone marrow stem cell population may be lethal because it is the renewal system of the mature circulating blood cells. The latter are soon used up and have to be replaced. For example, the lifespan of the granulocytes in the mouse is characterized by a half-life of some 5-6 hours in blood.

Irradiation was also found to be capable of depressing the response of surviving SMNC to mitogen. Figure 11 demonstrates that there was a gradual decline in the proliferative ability of SMNC with dose. This supports the notion of a dose-dependent inhibition of ^3H -TdR uptake previously observed in human lymphocytes in response to PHA after exposure to a 2.5-25 Gy range of radiation and a wide range of PHA concentrations (Rickinson & Ilbery, 1971). Radiation has been demonstrated to retard and reduce DNA synthesis, and cause DNA strand breakage. This impediment to division results in the mitotic or reproductive cell death of functioning immune cells (Coggle, 1973) and delays specific points in the cycle of proliferating cells (Rickinson & Ilbery,

1971). Thus, following the doses of radiation examined in this study, animals were immuno-incompetent both as a result of a reduction in the number of viable SMNC and the decreased ability of the surviving SMNC to respond to mitogen. It was then examined if there was a role for active immune suppression by a suppressor cell population after exposure to irradiation and what effect TT would have on this system.

1. *A Role for Active Immune Suppression Following WBI?*

Although there is considerable evidence to indicate that total-lymphoid irradiation is capable of inducing active immune suppression, the evidence is not as clear for whole-body irradiation. It was found that SMNC from mice which were given 100 cGy WBI were not capable of suppressing the PHA response of fresh autologous cells on days 1 or 7 post-irradiation (Figure 12). It should be noted, however, that demonstrations of suppression have typically involved the mixed-leukocyte reaction (MLR) system rather than the PHA mitogenic response system (Oseroff *et al.* 1984; Strober *et al.* 1979). It is possible that any active suppression induced by irradiation, while having the ability to suppress the MLR, is not capable of suppressing the PHA response. It should be emphasized that the effects of mitogens such as PHA represent the summation of effects from the binding of these lectins to a large number of distinct molecules. Thus, mitogens activate T cells by pathways including and in addition to the TCR/CD3 complex (i.e., CD2, Thy1.2, Ly-6, CD28). Any suppressor activity which is stimulated by irradiation may not be able to suppress such potent stimuli. Further experiments could be performed using the MLR system, which is believed to involve T cell activation strictly through

TCR-MHC interaction, to determine if active immune suppression following irradiation is a real phenomenon or whether the immune system is strictly incompetent.

2. Flow Cytometric Analysis and WBI

FCA was performed following WBI to examine how the mononuclear cell populations were affected and if there was any evidence of active immune suppression. Oseroff *et al* (1984) found that the spleens of adult total-lymphoid irradiated (TLI) mice contain cells that can non-specifically suppress the mixed-leukocyte reaction, block the generation of cytotoxic cells, and allow the generation of antigen-specific suppressor cells. These cells were found to be phenotypically null, and did not readily fall within the T lymphocyte, B lymphocyte, or macrophage lineages. These null cells made up only a small proportion of spleen cells, but increased dramatically after TLI. The decline in suppression with time followed the kinetics (disappearance) of the null cells. The suppressor cells were only present when the spleens were predominantly hematopoietic organs and had few mature T and B lymphocytes (Oseroff *et al.* 1984). These regulatory null cells were termed natural suppressor (NS) cells since they were found for a 2 to 3-week period in both neonatal (naive) and TLI mice.

It is reported here that following WBI the spleen is similarly hypocellular (Figure 6), and the relative proportions of the mononuclear cell subsets are dramatically changed (Figures 16-21). Although all cell populations declined, they did so at different rates. In descending order of radiosensitivity were the NK cells, CD4⁺ T cells, CD8⁺ T cells, monocytes/macrophages, and B cells (Figure 22). The extreme radiosensitivity that we

report in the spleen has been known since 1973 when Nossal and Pike demonstrated that one day after WBI exposure to 8 Gy, the number of B cells fell by a factor of over 200 while the total cellularity decreased by only a factor of 10. A similar observation of a relatively greater radiosensitivity in the B-cell predominant areas was made on rabbit appendix (Blythman & Waksman, 1973). In this study, the natural killer cells were the most strikingly radioresistant cell population of SMNC (Figures 20 and 22). In control mice, NK cells made up only a small fraction on the total population (3%), whereas 7 days after 700 cGy NK cells comprised upwards of 25% of the total SMNC.

It is possible that the observed alterations in SMNC populations of the spleen were the result of a net difference in migration into and out of the spleen via the blood, but it has previously been shown that overall depletion of T and B cells occurs in the spleen, lymph nodes, thoracic duct lymph, and peripheral blood following irradiation (Anderson, 1976) and a comparable depletion of MNC to that in the spleen has been reported in the peripheral blood (Sprent & Miller, 1973). The alterations in MNC populations of the spleen likely represent differences in radiosensitivities and are not due to a net efflux/influx. Further experiments, however, could be carried out in which FCA is performed on the individual MNC populations of peripheral blood and other lymphoid organs to determine if the effects seen in the spleen are representative of the whole animal.

The identity of cells expressing NS activity is not clear. Strober (1984) could not determine whether the suppressor cells were monocyte/macrophage precursors, splenic stromal cells, or lymphocyte precursors. Their NS cells were found to have several

features in common with NK cells in that both are present before antigenic challenge, both show lack of antigen specificity, and both have the appearance of large granular lymphocytes. Much progress has been made since then in the characterization and identification of cells based upon the expression of phenotypic markers. This study has found that no "null" cell population which was transiently expressed. In fact, the results reported here suggest that the null cells are equivalent to the transiently increased NK cells; the introduction of more specific monoclonal antibodies has made such a distinction possible. Interestingly, it has recently been reported that lymphoid irradiation of Hodgkin's disease patients results in an expanded NK cell compartment which is thought to play a role in the long-term immunosuppression observed in these patients (Macklis *et al.* 1993).

It should be noted, however, that various experimental models have suggested that non-specific suppression may be mediated by T lymphocytes and cells of the monocytic lineage. Null cells from the neonatal spleen have been described which are able to suppress an *in vitro* antibody response and the expression of class II MHC antigens. These suppressor cells were found to differentiate into classic macrophages after 4 days in culture (Snyder *et al.* 1982).

It was demonstrated that culturing SMNC with TT resulted in decreased class II MHC expression and that these SMNC were capable of suppressing fresh autologous cells. It was thus suggested that the decrease in class II MHC was a possible mechanism by which responses to mitogens such as PHA were suppressed. In contrast, FCA

demonstrated that SMNC from mice exposed to radiation (100 cGy) did not have decreased MHC expression (Figure 26) and could not suppress fresh autologous cells (Figure 12). Decreased MHC expression, however, was seen with WBI of 400 and 700 cGy, while the expression of other surface molecules remained stable (Figure 25). If the MHC down-regulation was the result of an activated suppressor cell in both cases (TT and WBI), one would expect in further experiments to be able to transfer the suppression using SMNC from mice exposed to these higher doses. Interestingly, FCA has also revealed a reduction in the intensity of class II MHC expression on APCs after whole-body UVB (non-ionizing) exposures (Kitajima & Imamura, 1993). It should be emphasized, however, that while MHC marker expression was seen to decline with WBI, the proportion of class II MHC positive cells also declined with dose. This is demonstrated in Figure 25 by the decrease in the relative size of the positive peak as compared to the unstained cells with increasing radiation dose. Since both B lymphocytes and macrophages (and other APC) express class II MHC (and at different levels), the observed decline in MHC expression may reflect the differential decline in these SMNC populations with radiation dose. Further double-labelling experiments could be performed to examine MHC expression on B cells and monocytes/macrophages separately to determine if radiation can in fact cause decreased MHC expression on particular cell populations.

3. Flow Cytometric Analysis and Hematopoietic Recovery

Our findings, with respect to the effects of irradiation on SMNC, also have

particular relevance to the hematopoietic system. The survival of an irradiated animal depends in large part on the timely recovery of hematopoiesis. Following radiotherapy hematopoietic recovery will ensue from surviving endogenous hematopoietic stem and progenitor cells capable of giving rise to functional mature cells, or by the transplantation of healthy bone marrow cells from a suitable donor (Bond *et al.* 1965). One of the mechanisms supporting hematopoietic recovery appears to involve a unique class of T lymphocytes (helper anti-theta-sensitive regulatory cells (TSRC)) which have previously been demonstrated to be capable of stimulating hematopoietic recovery following bone marrow transplantation (Wiktor-Jedrzejczak *et al.* 1973). Such cells were found to exist in the murine thymus and spleen. TSRC were later found to actually consist of two distinct subpopulations of Thy1.2 cells: one subpopulation which can enhance hematopoietic recovery, and another which can suppress it (Sharkis *et al.* 1980). TSRC which enhance hematopoietic recovery have been found to be resistant to radiation whereas TSRC which suppress hematopoietic recovery are sensitive to radiation (Sharkis *et al.* 1981). The relationship of helper and suppressor TSRC to the immunoregulatory CD4⁺ helper and CD8⁺ suppressor T lymphocytes has not been determined. Williams *et al.* (1994) have recently found that CD4⁺ cells in the thymus and spleen of irradiated mice exhibited properties characteristic of the TSRC which stimulate hematopoietic recovery.

The results reported here are in agreement with Williams *et al.* (1994) in that the murine spleen contains a population of radioresistant CD4⁺ T cells. As a result of the radioresistance of these cells, the spleens of irradiated mice became proportionately "enriched" with CD4⁺ T cells. By seven days post-700 cGy WBI, CD4⁺ lymphocytes

made up almost 50% of all SMNC as compared to a proportion of 15% in control mice (Figure 20). The differential radiosensitivities between CD4⁺ and CD8⁺ T lymphocytes observed in the spleen (Figure 22) are comparable, respectively, to the radiosensitivities of helper and suppressor TSRC as previously reported (Sharkis *et al.* 1981).

The high sublethal doses of radiation used in these studies induce hematopoietic depletion which is followed by vigorous recovery initiated from endogenous stem and progenitor cells that survive the irradiation (Coggle, 1973). The proliferation and differentiation of such stem and progenitor cells are known to be regulated by a variety of cytokines produced by the cells that constitute the hematopoietic microenvironment. These cells include both the stromal elements, which make up the framework of the hematopoietic organs, and monocytoïd accessory cells from peripheral blood, such as T lymphocytes and macrophages (Torok-Storb, 1988). Immunological studies have shown that CD4⁺ cells produce extensive quantities of a wide range of cytokines (Kelso *et al.* 1991) such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), IL-3, and IL-6, all of which are known to be stimulators of hematopoiesis (Metcalf, 1989). The finding *in vivo* that antibody-mediated ablation of CD4⁺ cells prior to irradiation reduces hematopoietic recovery (Pantel & Nakeff, 1990), taken together with the finding of residual radioresistant CD4⁺ cells which become "enriched" following irradiation, is suggestive of the possible significance of CD4⁺ cells in hematopoietic recovery. Since these cells are readily identifiable after exposure to radiation, experiments could be performed to evaluate their ability to produce cytokines, either as a direct result of

radiation injury or because of subsequent antigenic stimulation. This would determine if they can contribute to hematopoietic recovery and if they are in fact identical to the proposed helper TSRC.

The enrichment of the spleen with NK cells post-irradiation may also be significant to the outcome of hematopoietic recovery. Activated NK cells have previously been shown to play a role in suppression of hematopoiesis after irradiation (Pantel *et al.* 1990). *In vitro* growth inhibition of hematopoietic cells has been consistently shown to emanate from NK and lymphokine-activated killer (LAK) cells with a similar range of activity directed against stem cells (multipotent colony-forming units [CFU-GEMM]) (Fujimori *et al.* 1987), progenitor cells committed to granulocyte-macrophage differentiation (granulocyte-macrophage colony-forming units [CFU-GM]) (Takahashi *et al.* 1988), and erythropoiesis (burst-forming units erythroid [BFU-E] and colony-forming units erythroid [CFU-E] (Mangan *et al.* 1984)). It has been shown that NK and LAK cells produce or release IFN- γ (Degliantoni *et al.* 1985; Takahashi *et al.* 1990), TNF- α and β (Gray *et al.* 1984), all of which are well known growth inhibitors (Dinarelli & Mier, 1987). In addition, the presence of an alternate pathway of inhibition by means of direct cell-cell contact between regulatory and target cells has been reported (Herrmann *et al.* 1987). Recent investigations suggest that the release of inhibiting cytokines might require a prior stimulation of NK cells, while resting NK cells release growth-stimulating factors (Pisotoia *et al.* 1989). Although the number of CD4⁺ T lymphocytes and NK cells was quite small when compared to that of control animals, due to an overall decreased cellularity, the selective enrichment persisted during the first 7 days post-WBI. The

balance between hematopoietic recovery-enhancing CD4⁺ T cells and recovery-inhibiting NK cells may be a key factor in the survival of irradiated animals.

4. Flow Cytometric Analysis and Activation Markers

Analysis of activation markers on SMNC following irradiation demonstrated a dose and time-dependent expression of CD71, the transferrin receptor. At a dose of 100 cGy, CD71 expression was seen as early as 4 days post-WBI (9%) whereas the higher dose of 400 cGy delayed the appearance to 7 days post-WBI at which time a greater proportion of SMNC were positive (22%) (Figure 25.) A whole-body dose of 700 cGy did not result in an increase in CD71 expression over the 7-day time period in our study. These findings have some interesting implications. What type of SMNC is being activated by irradiation? Is the CD71 expression an indicator of hematopoietic activity? Or is it indicative of an activated suppressor population? The first step was to try and identify which SMNC subset was expressing the activation marker post-irradiation. Double-labelling experiments were carried out, and it was concluded that neither the T nor B lymphocytes were the activated cell type. Due to limitations of appropriate antibodies at the time of this study, further FCA experiments were not possible for specific characterization. Thus, the CD71-expressing population belongs to either a monocyte/macrophage, NK, and/or progenitor cell lineage. As new monoclonal antibodies become available precise identification of this population should become possible.

It has been demonstrated over the range of doses used in this study that the cellularity of the spleen does not begin to recover until 10-14 days after irradiation, with

a peak value at day 17 (Williams *et al.* 1994). It is therefore unlikely that the 22% CD71⁺ SMNC observed 7 days post-WBI in this study are cells which have arisen from hematopoiesis. In addition, culturing the irradiated SMNC for 48 hours resulted in increased expression of CD71 to 35% of all SMNC (Table VI) further suggesting that the surviving SMNC were induced to express the transferrin receptor. Consequently, if CD71 expression were to be found on the NK cell lineage, it would correlate with the large increase in the proportion of NK cells seen in the spleen post-irradiation and would suggest their direct activation by irradiation. Alternatively, cells of the macrophage lineage are known to play a pivotal role in iron metabolism and have been estimated to be involved in 80% of normal iron turnover (Finch *et al.* 1970). Much of the acquired iron is recycled to hematopoietic cells for new erythrocyte synthesis. Macrophages have also been demonstrated to express high levels of CD71 receptors after activation (Taetle & Honeysett, 1988). Further studies investigating culture conditions to maintain CD71 positive cells, NK cells, and macrophages as stable, long-term cloned lines would resolve many of these issues and could determine if such cells are capable of immune suppression and/or hematopoietic recovery following WBI.

5. *Biological Dosimetry by FCA*

Can flow cytometry be used as a dosimeter or indicator of biological damage? The fact that FCA is a rapid technique and a simple blood sample would be the only requirement for analysis makes it an attractive possibility. The time- and dose-dependent appearance of CD71 reported here make it a potential dosimetry marker. A dose of 700

cGy resulted in no CD71 expression within 1 week post-irradiation, thereby making it possible to identify high exposures. Since doses of 100 and 400 cGy resulted in progressively greater levels of CD71 expression (11% and 22% respectively), it may also be possible to relate dose to level of expression. Further experiments would have to be performed over a wider range of doses and over a longer period of time to determine if flow cytometric analysis of CD71 expression could indicate the amount of radiation damage incurred, the range of doses over which it could be used, and the length of time following irradiation when such measurements could be made.

An ideal indicator of biological damage, however, would be one that could be measured immediately following a radiation injury in order to institute the proper treatment as early as possible. Further studies with an increasing available range of surface markers may make such assessments a possibility. The level or relative proportion of a particular cell sub-type, or the ratio of sub-types, may provide such an early indication of radiation damage. As reported here, the proportions of the various SMNC changed dramatically over the 0-700 cGy range. Flow cytometric analysis has recently been used to predict the effect of total body irradiation and to screen radioprotective agents by quantitating chromatin fragments (Shen *et al.* 1994) further suggesting the potential of FCA to serve as a biological indicator of radiation damage.

6. A Role for IL-10?

The role of cytokines in the response to ionizing irradiation is just beginning to be elucidated. IL-1 and TNF- α have been shown to be upregulated within hours by

irradiation (Sherman *et al.* 1991; Hallahan *et al.* 1989) and are known to contribute to natural resistance to lethal irradiation (Neta *et al.* 1992). Recent work has shown that IL-10 may have a role to play in the immunosuppressive effects of ionizing radiation (Rivas & Ullrich, 1992; Enk & Katz, 1992; Broski & Halloran, 1994). It is reported in this thesis that whole-body irradiation of 100 cGy did not result in increased levels of IL-10 within the first week post-irradiation (Figure 34). This would suggest that the lack of ability to transfer a suppressive state with SMNC from WBI mice (Figure 12) was not simply due to the inability of IL-10 to overcome a potent PHA response. It is possible that 100 cGy was simply not a sufficiently high dose to induce the production of IL-10. Further experiments using a wider range of doses could be performed to determine if there is a role for IL-10 in immunosuppression following WBI. It is also possible that the immunosuppression associated with irradiation may involve other as yet unidentified soluble mediators.

C. IMMUNE SUPPRESSION BY TETANUS TOXOID AND IRRADIATION

One of the best illustrations of the injurious effects of ionizing radiation on immunity is the decreased resistance of irradiated animals (usually in the 200-600 cGy exposure range) to specific pathogens. It appears that radiation-induced decreased resistance to infection primarily occurs not immediately but several days after exposure (Coggle, 1973). It was an initial hypothesis that there may be an integrative role for antigen and radiation in enhancing immune suppression via the activation of suppressor cells. Indeed, culturing SMNC from mice exposed to WBI with TT resulted in a weaker

blastogenic response to PHA than SMNC from non-irradiated animals (Figure 13). A whole-body dose of 100 cGy, however, did not result in any further increase in the ability to transfer suppression to fresh autologous cells within the first week following irradiation (Figure 14). Furthermore, SMNC from WBI mice and cultured with TT did not result in any alterations in the populations of SMNC other than that induced by WBI alone (Figures 27 to 33). Finally, there was no observable increase in the levels of the suppressive cytokine IL-10 from SMNC cultured with TT during the first week post-100 cGy (Figure 34). When taken together, these results suggest that irradiation of 100 cGy does not increase the number of suppressor cells or their activity in response to TT, rather, irradiation likely only prevents the division of lymphocytes directly via reproductive cell death.

The effect of WBI followed by culturing SMNC with TT on MHC expression was examined to determine if there was any relationship between radiation and TT-induced down-regulation of MHC. SMNC from mice exposed to 100 cGy had unaffected MHC levels, whereas mice irradiated with 400 and 700 cGy had reduced levels of MHC expression. Culturing these SMNC with TT resulted in a further reduction in all cases to levels observed with SMNC from unirradiated mice that were cultured with TT (Figure 33). There appears to be no interaction between TT and radiation at such doses (i.e., no synergistic reduction of class II MHC) or that MHC levels can only be reduced to a certain level. The fact that the MHC is expressed on several cell types and that there was a differential loss of cell types following irradiation, however, makes conclusions difficult. Transfer of suppression experiments using SMNC from mice exposed to 400 and 700 cGy

and double-labelling FCA experiments in which MHC expression is examined on various cell subtypes would have to be performed to correlate any change in MHC expression with suppression.

The serious effects of pathogens and endotoxins on the host following irradiation would seem to be due to the immune incompetence of the host rather than to the activation of suppressor cells. It is possible, however, as was mentioned previously, that immune suppression induced by irradiation cannot suppress mitogenic response but can suppress other systems of T cell activation such as the MLR. In addition, it is possible that such an interaction between irradiation and antigen may require doses higher and/or take a longer time to develop than the 100 cGy and 7 day post-irradiation period examined here.

VII. SUMMARY AND CONCLUSIONS

The current state of knowledge concerning the overall mechanism of suppression by TT can be summarized as follows: it is an early event which occurs within 24 hours of incubation with TT, possibly originating from the MΦ and involving PGE₂ and perhaps other mediators such as reactive nitrogen intermediates (RNI). In contrast, IL-10 does not seem to be involved in this suppressive pathway. Figure 35 represents a hypothetical model by which TT might suppress the blastogenic response of SMNC undergoing stimulation by mitogen. TT was found not to produce an alteration in the cellular balance, indicating that the suppression would appear to be dependent upon a change in T cell function. TT can trigger the production of PGE₂ (from the MΦ) which in turn could act on T cells to down-regulate IL-2 production but not receptor expression. The addition of IL-2 has previously been shown to diminish suppression by TT. A mechanism whereby IL-2 receptor expression occurs in the apparent absence of IL-2 production, and where suppression is alleviated by indomethacin, is highly consistent with PGE₂-induced suppression of mitogenesis. To confirm this, culture supernatants could be assayed for the presence and/or increase of PG. In addition TT, by some means, triggers the down-regulation of class II MHC expression. This may occur by TT directly binding to the MHC or by means of some intermediary mechanism. As mitogenic responses require the interaction of T cells and accessory cells, the down-regulation of class II MHC expression may represent an inappropriate costimulatory signal required for T cell activation.

TT is suppressive to PHA responses and has previously been shown to be

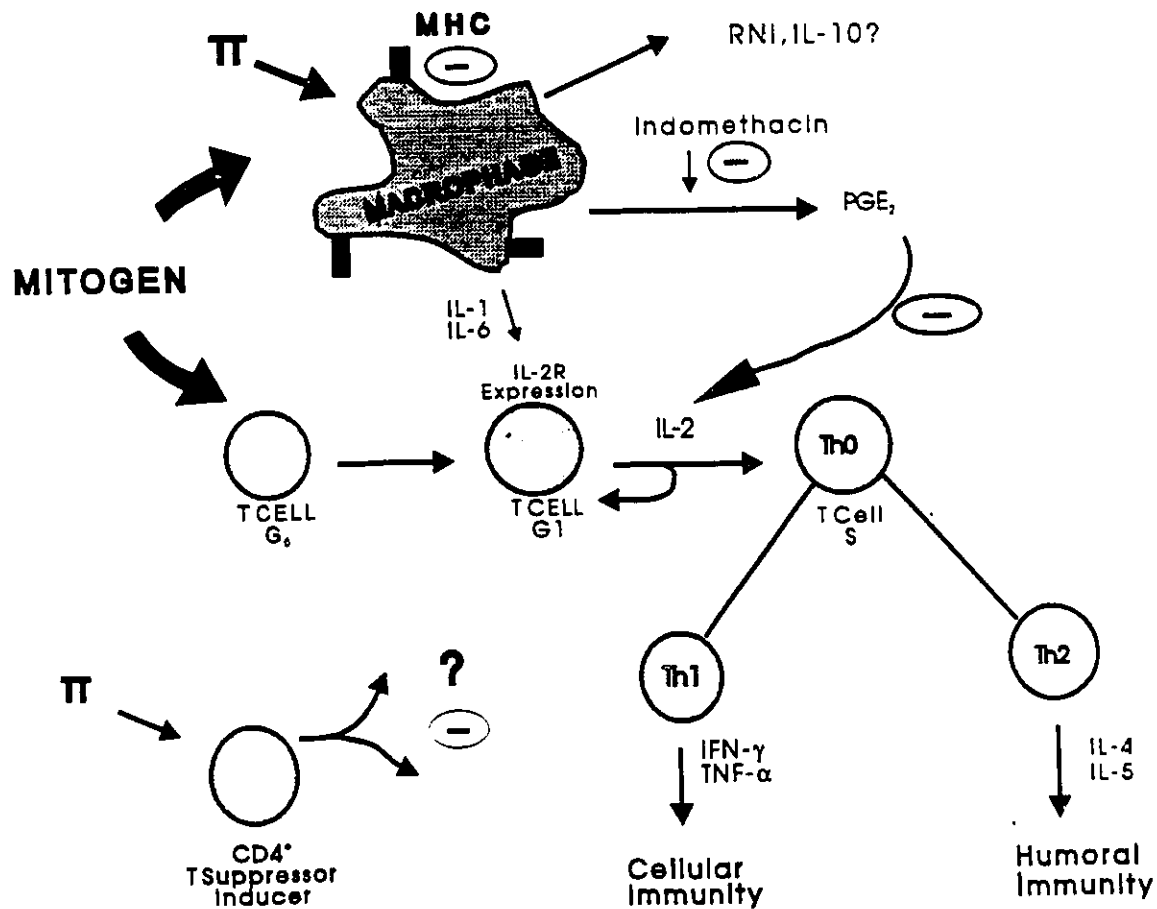


FIGURE 35. A MODEL FOR TT INDUCTION OF SUPPRESSION.

Mitogen stimulates monocytes/macrophages, T cells, and B cells. The resting T cell population progresses from G₀ to G₁ phase following mitogenic stimulation and MΦ signals in the form of IL-1/IL-6 and additional costimulatory molecules. The progression from G₁ to S phase is characterized by the production of IL-2 which acts on T cells in an autocrine and paracrine fashion. TT may trigger PGE₂ production in the MΦ, which prevents IL-2 production (indomethacin partially reverses suppression). In the absence of sufficient IL-2, T cells do not reach S phase, Th2 cells do not provide help to B cell in the form of IL-4 and IL-5, and Th1 cells do not provide IL-2 signals to B cells. TT causes the down-regulation of class II MHC expression which may represent an inappropriate costimulator signal between the MΦ and the T cell. In addition, TT may also induce a CD4⁺ suppressor inducer population which can induce a suppressor cell which can deliver negative signals at various stages in the system: Th1, Th2, monocyte/macrophage, and B cell.

suppressive to PWM responses, possibly at the same step since PWM-induced immunoglobulin production involves the cooperation of MΦ, T cells, and B cells. Normally Th1-type cells, once activated, send signals to Th2-type cells which require priming in order to secrete IL-4 and IL-5 which are 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 35).

A suppressor cell involvement is favoured because of the active transfer of suppression to fresh autologous cells. Previously, involvement of a cell bearing the CD4 marker has been implicated 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. The mechanism for the induction of a suppressive regulatory pathway is not known. Whether that cell is a T cell or a monocyte/macrophage and what the phenotype of the effector cell is may be determined by treating specific cell types isolated in cell-separation experiments with TT.

The suppression resulting from TT may be part of a more general mechanism of suppression that may be triggered by a variety of antigens. Which antigens can trigger suppressor pathways over helper ones, and the mechanism by which suppression is achieved, remain to be elucidated. TT could be studied along with a wide panel of antigens and compared for stimulatory and inhibitory potential. A common pathway of suppression might be revealed in related structures common to these antigens which might be triggering the suppressor mechanism. This knowledge could be useful in the treatment of many disease states, as well as in the elimination of detrimental effects encountered during vaccine production or administration.

Whole-body irradiation resulted in decreased cellularity of the spleen and decreased ability of surviving SMNC to respond to mitogen. There is no evidence from this study, however, to indicate that irradiation results in active suppression of the PHA blastogenic response via suppressor cells, as the suppression could not be transferred to fresh autologous cells. Similarly, irradiation of mice prior to culturing SMNC with TT did not affect the amount of suppression induced by TT. Experiments could be performed using higher doses (over 100 cGy) and over a longer time-period (greater than 7 days) to determine if WBI can cause activation of suppressor cells. In addition, the use of other systems of T cell activation such as the mixed-leukocyte reaction could be used to determine if suppressor cells are activated following WBI.

Whole-body irradiation produced dramatic alterations in the composition of the constituent splenic cell populations with selective enrichment of NK cells and CD4⁺ T lymphocytes. The observed increase in NK cells corresponds with the previously reported increase in natural suppressor (NS) cells following total-lymphoid irradiation. Further experiments to examine the ability of SMNC to suppress T cell responses when spleens are selectively enriched with these NK cells (i.e., 400-700 cGy) could be performed. In addition, the enrichment of NK cells may also be of importance in suppressing hematopoiesis following radiotherapy. The differential radiosensitivities between CD4⁺ and CD8⁺ T lymphocytes in the spleen observed in this project are comparable, respectively, to the previously reported radiosensitivities of helper and suppressor TSRC. This parallel may suggest a role for CD4⁺ T lymphocytes in hematopoietic recovery.

A dose- and time-dependent expression of CD71, the transferrin receptor, was

apparent on cells of the monocyte/macrophage, NK, and/or progenitor cell lineages following whole-body irradiation. This may indicate an activated population involved in immune suppression or conversely in hematopoietic recovery. The dose-dependent expression of cell surface molecules such as CD71, taken together with alterations in the relative proportions of cell subtypes, suggests the possible application of flow cytometric analysis as a biological indicator ("dosimeter") of radiation damage.

This work was undertaken to investigate the hypothesis that both TT and irradiation were capable of active suppression of the murine blastogenic responses *in vitro* and together are capable of enhancing immune suppression through an additive or synergistic effect. It was found that TT could produce active suppression of the PHA blastogenic response, whereas this was not the case for whole-body irradiation. Similarly, there appeared to be no interaction between TT and WBI to enhance suppression. It would appear that irradiation produces active immune suppression only in some circumstances such as in total-lymphoid irradiation, as has been previously observed. It cannot be excluded, however, that at some later time (beyond day 7) or at a higher dose, some suppression may be induced by irradiation.

TT did not produce an alteration in the cellular balance suggesting the suppression must have been due to a change in cell function, such as the observed down-regulation of class II MHC expression. In contrast, irradiation produced dramatic alterations in the proportion of mononuclear cell populations of the spleen. Whether this is related to reports of immune suppression following irradiation has yet to be determined.

The hypothesis that suppression was mediated through soluble factors was

investigated by examining a potential role for interleukin-10. This hypothesis was not supported since IL-10 levels remained unchanged after treatment with irradiation and/or TT. This does not rule out the involvement of IL-10 since there may have been problems with sensitivity. In addition, other factors could be involved such as reactive nitrogen intermediates and the newly described IL-13.

This project was successful in establishing a reproducible protocol for the flow cytometric analysis of murine splenic mononuclear cell populations. This has provided a better understanding of how these subtypes are differentially affected by irradiation with possible implications in immune suppression, hematopoietic recovery, and biological dosimetry. Similarly, flow cytometry has given further insight into the mechanism by which TT suppresses the blastogenic response which may be part of a more general mechanism of suppression that may be triggered by a variety of antigens or other factors.

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IX. APPENDIX

A. FLOW CYTOMETRIC ANALYSIS

In recent years, flow analysis and cell sorting have become standard research tools in immunology and in an expanding range of other disciplines. The power of flow analysis derives from its ability to make quantitative multiparameter measurements on statistically large numbers of cells. In flow cytometry a fluid flow system carries a single cell suspension through a sensing region which is usually illuminated by at least one focused laser beam. As the fluorescently labelled cells pass through the laser beam, light is scattered. As each cell passes through the sensing region, light scatter and fluorescence signals are collected and are translated into electronic signals by photomultiplier tube (PMT) detectors. The size of these signals is directly proportional to the light intensity. Forward light-scatter is a measure of cell size, whereas side light-scatter is indicative of the internal structure or granularity of a cell. Among equal sized cells those with more granularity scatter more light. The differences in light-scatter signals make it possible to define a region in a forward versus side scatter display that includes the cells of interest and excludes unwanted cells and debris.

As cells pass through the focused laser beam, the fluorochrome molecules on the antibodies which are bound to the cell surface antigen of interest are excited. Fluorescence is the process whereby a molecule absorbs light, moves into an excited state, and undergoes a transition back to the lower state with the emission of a photon of light. The emitted photon has less energy (i.e. a longer wavelength) than the original excitation

due to the loss of energy during internal transitions within states. This spectral shift makes it possible to measure very low levels of fluorescence, as the emitted light is measured at wavelengths where there is little or no background light. Under optimal conditions very small numbers, as low as a few thousand, of reagent molecules carrying fluorescent markers can be detected. Fluorochromes emit light with a spectrum determined by the nature of those molecules, and the emitted light is directed to separate detectors for each fluorescence signal to be measured. The electronic signals generated are digitized, stored and analyzed by computer.

There are several types of fluorescent reagents used in flow cytometry. Two that were used in this study were fluorescein isothiocyanate (FITC) and R-phycoerythrin (PE). Both chromophores are excited by the 488 nm line of the argon laser. FITC emits at 475-600 nm (green) and achieves peak fluorescence at 525 nm, whereas PE emits at 577-650 nm (yellow-red) and achieves peak fluorescence at 585 nm. These dyes can be detected in preference to one another by a photomultiplier detector with the appropriate filters. Since there is some overlap in the emission spectra, however, it is necessary, in the case of double-stained cells, to electronically subtract the amount of overlap in order to get "independent" measurements (Paul, 1989; Tsubono *et al.* 1990). This procedure is known as colour compensation and is provided for in most flow cytometers.

Monoclonal antibodies have been very important in expanding the use of flow cytometry, particularly multicolour analysis. Their specificity, availability, and reproducibility make them particularly suitable for quantitative measurements. Directly coupled monoclonal staining agents were used in the presented studies since they are

convenient, available, and provide adequate fluorescence signals for most purposes.

Once cells have been analyzed, the data generated can be displayed in several forms. These include: 1) single-parameter displays (e.g. histograms) in which frequencies are plotted as a function of the amount of signal collected by that sensor, 2) two-parameter plots which show cell frequency distributions as a function of paired measurements on two sensors, and 3) sample statistics, such as the percentage of cells falling within specified ranges and means or medians of signal levels.

B. THE EFFECT OF PROCESSING ON SMNC SUBSETS AND MARKER EXPRESSION

To ensure that the separation process for SMNC did not result in the selective elimination of a specific cell type, flow cytometric analysis of each subpopulation of SMNC was performed before and after processing (Lympholyte-M centrifugation). A two-tailed Student's *t*-test determined that there were no significant differences in the proportions of each cell subsets (Table VII) and cell surface antigen expression (Table VIII) between processed and unprocessed SMNC.

TABLE VII THE EFFECT OF PROCESSING ON THE PROPORTION OF SMNC SUBSETS

Cell Subset ^a	Mean Percentage (SD) ^b	
	Unprocessed	Processed
Thy1.2 ⁺	31.2 (4.5)	31.3 (3.3)
Thy1.2 ⁺ CD4 ⁺	17.2 (2.6)	16.6 (3.6)
Thy1.2 ⁺ CD8 ⁺	10.2 (0.7)	9.6 (2.8)
CD45 ⁺	55.1 (4.3)	53.6 (9.2)
Ia ⁺	55.2 (6.5)	55.3 (10.0)
CD45 ⁺ Ia ⁺	54.2 (6.7)	56.6 (7.5)
NK1.1 ⁺	3.8 (1.0)	3.9 (1.6)
F4/80 ⁺	8.5 (1.5)	9.1 (2.4)
CD25 ⁺	ND ^c	ND ^c
CD71 ⁺	3.1 (0.6)	3.4 (2.8)
CD4 ⁺ Ia ⁺	3.7 (0.4)	3.7 (1.6)
CD8 ⁺ Ia ⁺	3.9 (1.3)	3.5 (2.0)

^aSubsets of SMNC identified by single and double-antibody labelling. Antibodies were FITC anti-CD4, anti-CD8, anti-CD45, and PE-mAb to other markers listed.

^bFrequency of total splenic mononuclear cells (n=5 where 2 spleens were used per experiment).

^cNot detectable.

TABLE VIII **THE EFFECT OF PROCESSING ON
MARKER EXPRESSION OF SMNC**

Molecule	Mean MCF (SD) ^b	
	Unprocessed	Processed
Thy1.2	24.9 (7.2)	25.2 (5.2)
CD45	4.2 (0.6)	3.5 (1.7)
Ia	49.2 (6.6)	46.8 (5.5)
CD4	3.8 (0.7)	3.9 (0.5)
CD8	12.8 (3.0)	11.1 (2.2)
NK1.1	3.8 (0.6)	3.6 (0.4)
CD71	5.5 (4.3)	4.9 (3.5)

^aCell surface antigens analyzed by direct immunofluorescence and flow cytometry. The mean channel fluorescence (MCF) was determined for each sample.

^bData are expressed as the mean of MCF $\pm$ SD of 5 experiments for the unprocessed and processed SMNC examined.