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Immune Damage in Irradiated Mice: Contributions of Differential Radiosensitivity and Apoptosis in Mononuclear Cells, and Alterations in Natural Killer Cell Cytolytic Potential

By

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ABSTRACT

Damage to the immune system of exposed individuals renders the host susceptible to opportunistic infection and disease. The purpose of this investigation was to contribute to the understanding of mechanisms underlying depression of host immune responses following radiation exposure. Peripheral blood mononuclear cells (PBMC) of mice irradiated with 0-700 rad γ-whole body irradiation (WBI) were analyzed by flow cytometry (FCA). Natural killer (NK) cells and CD4+ T lymphocytes were selectively enhanced following radiation exposure, demonstrating radioresistance of these cell types over other PBMC, while B lymphocytes were dramatically radiosensitive. Dextran sulfate mobilization of mononuclear cells (MNC) from lymphoid tissues into the blood revealed that the same pattern of MNC loss had occurred throughout the lymphoid tissues. PBMC alterations reflected similar changes occurring in previous studies of splenic mononuclear cells {1}, and may promote immune dysregulation. The role of apoptosis in radiation-induced injury to the immune system in the low to intermediate dose range (0 to 400 rad) was investigated in PBMC. 25 rad induced apoptosis in PBMC above the unirradiated control within 2 hours post-irradiation; apoptosis induction increased with higher doses (100-400 rad). Additionally, the impact of ionizing radiation on NK cell function was assessed. 24 hours following radiation exposure, NK cytotoxicity against YAC-1 target cells was depressed by doses of 25 or 50 rad, with little change in the 100 to 400 rad range. By day 7, NK cytolytic potential was reduced or unaffected by doses lower than 200 rad, while a single exposure of 400 rad enhanced cytotoxicity. The results of this investigation have furthered our understanding of factors which may be important in the impairment of immune responses post-irradiation.

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LIST OF ABBREVIATIONS

ACK	Ammonium Chloride and Potassium
ACSS	Animal Care and Surgery Service
APC	Antigen Presenting Cell
ATCC	American Type Culture Collection
CD	Cluster of Differentiation
CO_2	Carbon dioxide
^{137}Cs	Cesium-137 (γ-emitter)
$DiOC_{18}(3)$	3,3'-dioctadecyloxacarbocyanine perchlorate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DS	Dextran sulfate
DTH	Delayed-type hypersensitivity
EDTA	Ethylenediaminetetra-acetic acid
FCA	Flow cytometric analysis
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
g	Gravity
GlyCAM	Glycosylation-dependent cell adhesion molecule
GM-CSF	Granulocyte-macrophage colony stimulating factor
gp	Glycoprotein

HEV	High endothelial venule
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen-DR
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
KIR	Killer Inhibitory Receptor
LFA	Leukocyte function-associated molecule
LFL	Log fluorescence
mAb	Monoclonal antibody
MHC	Major histocompatibility
MLR	Mixed leukocyte reaction
MNC	Mononuclear cells
mPEG	Monomethoxypolyethylene glycol
NK	Natural killer
NZB/NZW	New Zealand Black/New Zealand White Mice
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PI	Propidium iodide
SCID	Severe combined immunodeficiency

SDS	Sodium dodecyl sulfate
SD	Standard deviation
SMNC	Splenic mononuclear cells
TCR	T cell receptor
Th	T helper
TLI	Total lymphoid irradiation
TNF	Tumor necrosis factor
UV	Ultraviolet irradiation
WBI	Whole-body irradiation

I. INTRODUCTION

Through atomic weapons testing and medical and industrial applications of radioactivity, human exposure to ionizing radiation has increased, bringing new risks to human health. These health risks have been studied for decades, and are the basis for this investigation, a new approach to determining the causes and degree of risk associated with exposure to ionizing radiation.

Exposure to ionizing radiation has detrimental consequences, particularly on host immunity. Individuals exposed to radiation become susceptible to opportunistic pathogens, which would be eliminated by hosts with otherwise healthy immune systems. Investigation into mechanisms responsible for depressed immune responses post-irradiation are warranted and necessary, and may suggest avenues for intervention to counteract depressed immunity.

The observed depression in host immunity post-irradiation is certainly largely due to an overall reduction in the number of immune competent cells available to respond to antigenic challenge. Cell death (apoptosis or necrosis), inhibition of proliferation ("mitotic death"), and altered lymphocyte homing and migration may all contribute to reduction in immune competent cells available to respond to antigenic challenge. However, evidence suggests that radiation may depress immunity beyond the scope of reduced cell numbers alone, indicating that surviving immune cells are further downregulated post-irradiation. Mononuclear cells interact and communicate with one another in complex and intricate pathways, and they do so either by direct cell-cell contact (eg. antigen presentation by an antigen presenting cell (APC) to a T lymphocyte)

or by the use of biochemical mediators such as cytokines and interferons {2}. These mechanisms are responsible for the tight regulation of the immune response, such that an imbalance among the mononuclear cell populations or in the relative amounts of chemical mediators could have a severe, detrimental impact on the viability and functional competence of the specific immune response.

This investigation was undertaken to contribute to the understanding of potential mechanisms responsible for depressed immune responses in mice following exposure to ionizing radiation. The differential composition of peripheral blood mononuclear cells of mice was measured post-irradiation to identify alterations in cellular balance which may occur, and which could contribute to immune dysregulation. Dextran sulfate was employed to mobilize mononuclear cells from other lymphoid and peripheral tissues to determine whether cell loss was occurring preferentially in some tissues, or uniformly throughout the body. The role of apoptosis in radiation, induced cell destruction at low and intermediate doses was investigated by flow cytometric analysis of PBMC expressing phosphatidyl serine. In addition to investigation of potential mechanisms underlying radiation, induced immune suppression, a brief study of the impact of radiation exposure on functional immunity, specifically cytotoxicity by natural killer (NK) cells, was also conducted in hopes of further characterizing the scope of radiation, induced immune suppression. Previous studies had demonstrated the radiosensitivity of NK cells in mice post, irradiation {1}, although preservation of their cytolytic function has not yet been ascertained.

A. RADIATION INJURY

Ionizing radiation has a profound impact on biological material, beginning at the level of ions and molecules, and producing effects that reach as far as critical organ systems and the organism as a whole. Radiation induces immediate chemical changes by ionization of water and the formation of hydrogen and hydroxy radicals that react with proteins and nucleic acids. Additionally, radiation may act directly on these critical molecules without the free radical intermediate {3}.

1. Biological Effects of Ionizing Radiation

At the protein level, ionizing radiation may fragment polypeptide chains, alter protein solubility, disrupt secondary and tertiary organization, cross link and aggregate proteins, and destroy amino acids {3}. Any of these effects may inactivate or destroy enzymes or structural proteins essential for cell function and survival, which in turn, will impact tissue or organ function. In nucleic acids, radiation induces single or double-strand breaks, destruction of nitrogenous bases, mutations in DNA sequence, alterations in gene expression, and inhibition of DNA synthesis and cell division {3}. The susceptibility of nucleic acids to radiation injury renders mitosis and cell division among the most radiosensitive cell functions. This in conjunction with radiation damage to plasma membranes, endoplasmic reticulum, lysosomes, and mitochondria, severely compromises cell function and survival post-irradiation {3}. Sensitive cells are rapidly eliminated, while cells resistant to radiation-induced destruction may suffer delayed effects such as inhibition of effector function or carcinogenesis {4}, and these effects

may further translate into organ system injury.

2. Acute Radiation Syndrome

Radiation exposure results in characteristic injuries to human hematopoietic, gastrointestinal, and neurovascular systems, which comprise the life-threatening acute radiation syndrome, the progression through which is dose-dependent.

Following a prodromal phase (occurring within 1 to 2 hours post-irradiation) characterized by nausea, fatigue, apathy, and listlessness, the hematopoietic syndrome is the first to manifest, and occurs in the low to midlethal dose range (400-1000 rad) {5}. Injury occurs primarily to the bone marrow and cells of the immune system, resulting in bone marrow depression and cessation of blood cell production. The onset of this syndrome in humans occurs 8 to 10 days following exposures below 700 rad {5}. Alterations in the peripheral blood mononuclear cell profile can be observed 24 hours post-exposure, accompanied by severe hemorrhaging and anemia, as well as diminished resistance to infection in accordance with the cytoreduction of the immune system in following weeks. Unless a minimum of 10% of hematopoietic stem cells remain uninjured and competent to repopulate the immune system, death is expected within 4 to 6 weeks of the radiation exposure {5}. Whether injury of this nature occurs to the immune and hematopoietic systems at very low doses is not yet clear.

The gastrointestinal and neurovascular syndromes prevail at doses in the 700 to 3000 rad range (in humans) over the hematopoietic syndrome, due to the earlier onset and more serious clinical manifestation. Victims suffer severe electrolyte imbalance, hemorrhage, diarrhea, hypotension, deteriorating consciousness, coma and eventual

death, the onset and course more rapid with higher doses {5}. The prognosis for victims with gastrointestinal or neurovascular injury is bleak: death is predicted within 2 weeks for the gastrointestinal syndrome, and within 2 days for those suffering the neurovascular syndrome {5}. The hematopoietic syndrome can, however, be treated depending on the degree of damage sustained. Radiation-induced immune suppression can be counteracted somewhat with the use of antimicrobials and immunomodulators to reduce or prevent infection and stimulate hematopoiesis and repopulation of the immune system in patients with moderate injury, but in those with more severe immunologic injury, bone marrow transplantation is indicated {6}. Application of specific cytokines (IL-3, IL-8, GM-CSF) can enhance recovery with or without transplantation, and afford some additional protection against infection in the early stages of recovery {7, 8}.

B. IMMUNOSUPPRESSION

Exposure to ionizing radiation is not always accidental. The range of therapeutic applications for ionizing radiation spans cancer treatment, enhancement of graft survival following organ transplantation, and the suppression of autoimmune diseases. The use of radiation localized to the area of a malignant tumor is commonly used to destroy tumor cells, since rapidly dividing cells are highly susceptible to death by radiation. A patient that has received a tissue or organ transplant may be treated with radiation in order to suppress the hosts immune response against the foreign donor tissue to prevent transplant rejection. Sensitization of UV-irradiated mice with alloantigen results in antigen presenting cell (APC)-induced generation of alloantigen-specific suppressor cells that

suppress graft-versus host disease against an allograft in a recipient mouse {9}. A study in which NZB/NZW mice with lupus-like renal disease were treated with total lymphoid irradiation (TLI), demonstrated another therapeutic use of radiation, and resulted in the prolonged remission of the autoimmune disease without suppression of the general immune system {10}. TLI may suppress autoimmune disease by eliminating certain specific subsets of T or B lymphocytes that mediate autoimmune disease {10}. This leads to the possibility that alterations among the proportions of various mononuclear cells following irradiation could lead to immunosuppression due to the removal of a controlling mechanism. Several mechanisms, previously described or yet to be discovered, may act independently or in concert to produce the profound immune suppression observed as a consequence of radiation exposure. Examples of underlying mechanisms may include cytoreduction in immune, competent cells, leukocyte homing or migration defects, alteration in the relative proportions or effector functions in particular mononuclear cell populations, or disturbances in the cytokine makeup of the microenvironment.

Several diverse agents, including drugs and pathogens (or products thereof), may suppress the immune system, and examination of the various mechanisms of action may lend some insight into immune suppression by ionizing radiation. *Helicobacter pylori*, a gram negative bacterium, is able to protect itself from the human immune system by suppressing the proliferative response of human peripheral blood mononuclear cells (PBMC) to antigens and mitogens without affecting cell viability, and the suppression of lymphocytes has been linked to a reduction in IL-2 receptor (CD25) expression {11}. A soluble extract of *Candida albicans* induces antigen-nonspecific suppressor cells that

inhibit the antibody response of normal B cells in vitro {12}. Tetanus toxoid induces nonspecific suppressor cells that can dampen mitogenic responses {13}. Antigen-specific immune suppression has also been shown in severe combined immunodeficient mice (SCID) treated with a covalent conjugate of mPEG (monomethoxypolyethylene glycol) which tolerizes mice to human peripheral blood leukocytes by generating CD8+ T cells to down- regulate CD4+ T helper cells in an antigen, specific manner {14}. In addition, the protozoan *Leishmania donovani* impairs host immunity by decreasing the proportions and possibly the activity of CD4+ T cells, but CD8+ T cells are not involved {15}.

Viruses represent another class of agents that are capable of inducing immune suppression by a variety of mechanisms in the absence of radiation. Infection of mice by immunosuppressive retroviruses causes derangement of T cell cytokines to induce immune suppression, coinciding with suppression of the primary antibody response {16}. Other mechanisms of virus, induced immune suppression include lymphocytopenia, impairment of proliferation in response to mitogens, and in some instances, suppressor cells (not necessarily antigen-specific) that have been generated in response to a viral particle {17}. Viruses can elicit their suppressive effects in many ways, including direct effects of viral replication on lymphocyte functions, activity of soluble factors of viral or host origin from infected cells, thereby affecting their function in natural and specific immunity, and finally, viral triggering of an imbalance in immune regulation {18}. Measles virus represents a virus with multiple, sustained effects on the immune system, generating immune suppression. Following clearance of the measles virus from the host, long-term immunosuppression heightens susceptibility to secondary infections. This

immunosuppression is attributed to defects in monocytes (which are infected by measles virus and often lysed), and a decrease in total T lymphocyte number, accompanied by reduced IL-2 production and decreased cell-mediated immune response {19}. Clearly, from these varied examples of antigenic or viral mechanisms of immunosuppression, we see that immune suppression can occur by disturbing any one of several levels of interaction of the components of the immune response, whether by disrupting the function or viability of one or more subtypes, or by altering levels of stimulatory or inhibitory chemical mediators.

C. RADIATION-INDUCED IMMUNOSUPPRESSION

1. Cytoreduction of the Immune and Hematopoietic Systems

Exposure to ionizing radiation results in depression of host immune defences, and the severity of this depression is dose-dependent {20}. Much of the depression can be attributed to cell destruction, considering that cells of the immune system are among the most radiosensitive cells in the body {21-25}. Radiation can cause the cessation of cellular metabolism and disintegration of a cell, or at the very least, the inhibition of proliferation, which is extremely detrimental, since the ability to rapidly undergo unlimited proliferation is vital for protective immunity. As well as death of leukocytes, the young, rapidly proliferating and undifferentiated stem cells of the bone marrow are highly susceptible to radiation injury. Stem cell damage and depletion hinder the regeneration of mature, functional circulating leukocytes and repopulation of the immune system to counteract the cytoreduction caused by the initial exposure. Cells exposed to

ionizing radiation may be destroyed by implementation of one of two death mechanisms, apoptosis or necrosis.

2. Apoptosis

Apoptosis is a fundamental cell death process in all multicellular organisms, and is comprised of a series of rapid, distinct, ordered biochemical events culminating in characteristic morphological changes {25, 26}. Condensation of chromatin into large masses associating along the nuclear envelope and nuclear shrinking {23} are quickly followed by the loss of plasma membrane phospholipid asymmetry and cell shrinkage. There is an early loss of plasma membrane phospholipid asymmetry, specifically the redistribution of phosphatidyl serine (PS) from the cytoplasmic to the exterior surface of the lipid bilayer; this is the basis for a flow cytometric technique for identification of apoptotic cells (by staining phosphatidyl serine with Annexin V-FITC). Nuclear DNA is fragmented by DNA endonuclease activity into characteristic oligosomal DNA fragments of 180 to 200 base pairs in length or multiples thereof, which produce a characteristic ladder pattern upon electrophoresis. Blebbing of the cell surface and sequestration of cellular contents into membrane-bound apoptotic bodies is a later stage in the apoptotic cascade, preceding their engulfment by neighboring phagocytes {23, 25, 26}. The engulfment of apoptotic cells may be mediated by the appearance of phosphatidyl serine on the outer surface and subsequent recognition by vitronectin receptors on macrophages {27}.

Apoptosis, often termed "programmed cell death", results in cell death in the absence of any notable inflammation {25, 26, 28, 29}, and is considered to reflect

"physiologic rather than pathologic cell death" {26}. Necrosis, or "pathologic cell death", is characterized by cell swelling and lysis {26, 28}, and may also produce DNA fragments {26}. Necrotic death is associated with degradation of the cell membrane prior to DNA fragmentation, with a local inflammatory response, whereas apoptosis is not accompanied by inflammation, and the cell membrane integrity remains intact during the fragmentation of nuclear DNA {26, 29}. Apoptosis, aside from being an important process in development and differentiation of different organ systems, may be triggered by injury imparted by the same stimuli as those which evoke necrosis, but at lower doses. At higher doses, the noxious agents or physical injury supersede the biochemical pathways which elicit the cell suicide program and produce cell lysis and nonspecific nuclear degradation of DNA {22, 25, 26, 29}. Due to the regulated death process which is a hallmark of apoptosis, and the vague morphology and inflammation that occurs with necrosis, apoptosis is often contrasted from necrosis as a deliberate event, whereas necrosis is considered to be "accidental" {26}.

i. Biological Significance of Apoptosis

The occurrence of apoptosis as a regulatory process is essential for a wide range of biological processes and systems {26}, often aiding some aspect of development, differentiation, or elimination of dangerous cells. In the immune system, apoptosis is crucial for the process of thymocyte maturation by inducing the deletion of 80 to 85% of thymocytes, composed of non-reactive or autoreactive clones {25, 30, 31}. Elimination of virus-infected or transformed cells by cytotoxic T lymphocytes (CTL) or natural killer (NK) cells may involve apoptosis induction in target cells {25, 32}. Both CTL and NK

cells can mediate cytotoxicity by the perforin-dependent pathway involving secretion of cytotoxic factors {32}, or may also elicit cell killing by inducing apoptosis in susceptible targets through Fas surface signaling, using Fas ligand present on effector cells {25, 32}. The choice of the death pathway employed may depend on the level of surface expression of Fas on target cells: NK targets with low Fas levels undergo perforin-mediated lysis quickly, whereas in targets with high Fas levels, necrosis is delayed, and apoptosis occurs 15 to 20 minutes after contact with effector cells {32}.

One of the hallmarks of the immune system is the rapid expansion of clones in response to specific antigenic challenge. Clonal expansion is maintained by the persistence of antigen, and requires IL-2 {22}. Following elimination of the antigen, the cellularity of clones declines to resting levels, and this may occur by apoptosis {25}. As the challenging antigen is reduced, cells are no longer activated, and therefore, no longer produce certain survival factors, including IL-2, and cells starved of these factors may be eliminated by apoptosis {22}. As well, surface expression of Fas on T lymphocytes is upregulated as T cells age, providing a mechanism for control of lymphocyte lifespan.

Apoptosis is essential for eliminating lymphocytes activated inappropriately, providing a critical "safety-net" for the prevention of propagation or continuation of deleterious immune responses {22}. Lymphocytes activated in the absence of signalling through all essential and appropriate co-receptors (aberrant activation signalling) will be eliminated by apoptosis, such as in the instance of CD4 ligation prior to TCR/CD3 signalling in T lymphocytes {22}.

ii. Apoptosis as a Response to Cell Injury

Elimination of injured cells by apoptosis is a crucial safety mechanism for the protection of the host. Many deleterious stimuli are capable of eliciting apoptosis, falling into the categories of physiological stress, chemical insult, membrane signalling, and physical injury {29}. Physiological stress stimuli, including glucocorticoids, removal of key survival cytokines, or factor starvation are potent inducers of apoptosis {22, 29}. Chemical insults which trigger apoptosis include cytotoxic drugs such as etoposide and mafosfamide {33}, sodium azide, and reactive oxygen species {23}. Examples of membrane signals which initiate the cell suicide program include Fas/Fas Ligand interaction, and tumor necrosis factor receptor signalling. Both signalling mechanisms involve hydrolysis of sphingomyelin in the plasma membrane to generate the second messenger ceramide, which influences nuclear transcription factors, leading to apoptosis {34}. Finally, physical injury to a cell, in low or intermediate intensity will elicit apoptosis, and such stimuli would include heat shock or ionizing radiation {29}. Again, high intensity physical injury to the cell will supersede the death program, and induce necrosis, likely due to the extent of injury overwhelming the cell's ability to produce or activate the necessary enzymes to carry out the series of events occurring during apoptosis {25, 26, 29}. This "altruistic suicide" (apoptosis) allows for the elimination of dangerous cells which may perpetuate detrimental immune responses or mutations {22, 25}.

There is much debate in the literature as to whether the cell membrane or the nucleus is the primary target of radiation induction of apoptosis {22}. Some evidence suggests that radiation damage to cell membranes contributes to the Ca^{2+} influx which is

characteristic of the apoptotic pathway, and this in addition to mobilization of Ca^{2+} from intracellular binding, activates DNA endonuclease {29}. Additionally, activation of the inositol triphosphate/diacylglycerol system at the membrane of irradiated cells activates protein kinase C and a nuclear transcription factor producing apoptosis in some cell types {29}. Radiation exposure hydrolyses sphingomyelin and generates ceramide as a second messenger which in turn activates DNA endonuclease, a membrane-associated apoptosis signal shared with the Fas and TNF receptor signalling pathways {29, 34, 35}.

Ionizing radiation-induced physical damage to DNA is associated with upregulation of the p53 tumor suppressor gene {29, 35}, the expression of which varies from one cell type to another, contributing to the differential susceptibility of various cells to radiation-induced apoptosis {24, 26}. The p53 gene and gene product are thus far exclusively associated with cellular damage, while other physiological processes regulated by apoptosis appear to occur independently of p53 {23, 35}. The mode of action of p53 protein is unclear, although evidence suggests that p53 acts as a transcription factor, which upregulates transcription of the apoptosis accelerator *bax*, and induces Fas expression {23, 35}.

3. Radiation-Induced Interference with Lymphocyte Homing

Aside from the contribution of cell death (apoptosis or necrosis) to cytoreduction of immune-competent cells, radiation-induced impairment of leukocyte migration and homing may play a role in immune suppression post-irradiation {36}. Maintenance of the integrity of the membrane and of the association of the membrane with surface glycoproteins and proteins involved in signal transduction is essential for proper

lymphocyte migration {20, 37}. Damage to the plasma membrane may promote cell disintegration upon the "attachment and rolling" step in lymphocyte migration through high endothelial venules (HEVs) in passage between blood and lymph, during which cells are subjected to a strong shearing force that healthy cells would otherwise be capable of withstanding {37}. During the migration process, surface attachment and recognition is absolutely required, and is initiated through recognition of CD34 and GlyCAM-1 molecules on HEVs by L-selectin on lymphocytes {37}. The firm "sticking and arrest" stage which precedes transendothelial migration is also mediated by specific recognition by surface adhesion molecules: leukocyte function-associated molecule 1 (LFA-1) on lymphocytes recognizes and interacts with intercellular adhesion molecule 1 (ICAM-1) and ICAM-2 on the HEV surface {37}. A G-protein-mediated activation event is absolutely required for lymphocyte migration in lymph nodes and Peyer's patches {37}, again demonstrating the importance of maintenance of membrane integrity and its association with critical recognition and signal transduction molecules. Irradiated lymphocytes demonstrate a loss of normal homing ability, probably due to damage to the cell membrane {36} and/or alteration in the expression of adhesion molecules {20}, such as ICAM-1. Moreover, total body irradiation imparts damage to high endothelial venules of rat cervical and mesenteric lymph nodes, interfering with normal passage of lymphocytes from the blood into lymph nodes {38}, indicating that damage to key structures within the internal environment may play a role in impaired circulation of lymphocytes.

The ability of lymphocytes to rapidly expand and eliminate foreign antigen in an efficient, effective, and intricately coordinated manner is vital to specific immunity {36,

37, 39, 40}. For such protection to occur, lymphocytes must encounter antigen as rapidly as possible, reducing the opportunity for spread of infection and expediting its elimination. Much of the efficacy of specific immunity is attributable to the dissemination of protective immunity throughout the entire body, by the recirculation of lymphocytes from the blood, through the lymphatic system and throughout non-lymphoid peripheral tissues, and back to the blood {36, 37, 40}. This recirculation allows the distribution of the protective immunity throughout the body, providing effective immune surveillance against invading pathogens or challenges and the guarantee of perfusion of all tissues, enabling local immune responses throughout {36, 40}. Antigen-primed lymphocytes mainly recirculate to and within the tissue where the initial antigen stimulation took place, which is adaptive in providing sustained protection at a site where a specific antigenic encounter is more likely {36}. Large numbers of naive lymphocytes circulate through lymphoid organs, increasing the probability of encountering their specific antigen {40}. This system is maintained in part by differential expression of L-selectin between naive and antigen-activated lymphocytes, and the fact that epithelial surfaces are not designed for massive lymphocyte circulation, promoting surveillance by effector and memory cells at the site of initial infection {40}.

Lymphocyte migration and homing is specifically regulated to enhance the efficiency of the adaptive immune response, directing naive or primed lymphocytes via surface recognition and adhesion molecules, and thereby controlling their differential but necessary distribution throughout the host's lymphoid and non-lymphoid tissues.

i. Dextran Sulfate

Dextran sulfate is a non-toxic, heparinoid polyanion {41-43} capable of mobilizing stores of mononuclear cells, particularly lymphocytes, from reserves in the spleen and lymph nodes (>30% of entire lymphocyte distribution) {36} into circulation {44}. This compound and other polyanionic mobilizing agents promote movement of lymphocytes from the cortex of lymph nodes and from the white pulp of the spleen, to the peripheral blood via the efferent lymphatics {44}. The mechanism of this mobilization is not well understood, but it has been suggested that the negative charge of dextran sulfate is of utmost importance in leukocyte mobilizing potential, by altering the positive charge of the membrane {41}. This may influence cell adhesion and surface rigidity in such a manner as to alter adherence and promote movement from lymphoid tissues into the periphery. This mobilization increases mononuclear cell number in the peripheral blood 3 to 5-fold, with cell numbers returning to normal levels within 5 hours {41}.

Dextran sulfate has been employed as an *in vivo* method for determining immune competence post-irradiation. MNC mobilization into the peripheral blood was found to be indicative of the MNC distribution in the spleen, lymph nodes, and other peripheral tissues, available to respond to invading pathogens {43}. Here, DS treatment and immunophenotyping of PBMC by flow cytometry was employed to determine the distribution of MNC throughout lymphoid and non-lymphoid tissues, beyond the peripheral blood, to indicate the overall survival of particular MNC subsets post-irradiation.

Through the reduction of surviving numbers of immune-competent cells, and interference with their migratory capabilities, radiation severely impacts host defence,

rendering the host ill-equipped to eliminate threats. However, evidence suggests that after radiation exposure, the immune system is further down-regulated such that surviving immune-competent cells are less responsive to mitogens, and therefore, the immunosuppression observed following radiation exposure cannot be attributed solely to cell loss or inappropriate distribution {20, 45}.

Alterations in cell-cell interactions of critical lymphoid regulatory or effector cells, shifts in the cytokine microenvironment {21}, or altered expression of surface molecules essential to cell-cell or cell-environment interactions, may lead to immune dysregulation and contribute to impaired host defence. Suppressed immunity may be the result of generation of conditions unfavorable to adaptive immune responses, or favorable to the development of active suppression.

4. Immune Dysregulation

i. Alterations in Proportions of Mononuclear Cell Populations

Functions of the immune system, such as antigen presentation by accessory cells, T lymphocyte activation, antibody production, and clonal expansion, are all dependent on interaction with other cells of the immune system. Imbalance in the proportions of interacting populations, or loss of effector function by a critical cell may down-regulate these functions. Two distinct subsets of CD4+ T helper cell have been identified, and may be distinguished on the basis of their cytokine profile and the immune responses they support. T helper 1 (Th1) cells secrete IL-2 and IFN-γ, and promote cell mediated immune responses necessary for the elimination of many infectious agents. T helper 2 (Th2) cells secrete IL-4 and IL-10, and inhibit Th1 cytokine secretion and cell-mediated

immunity {46}. As well, functionally distinct subsets of CD8+ T cells produce different combinations of cytokines, and appear to play an important part in determining patterns of cytokines produced by CD4+ T cells as well as the isotype of immunoglobulins expressed by B cells {47}. Loss of antigen-presenting cells, or impaired antigen-presentation function would severely impair host immune response to antigenic challenge, providing less than adequate presentation of antigen to activate T lymphocytes {48, 49}. Therefore, it is conceivable that alterations in CD8+ or CD4+ T lymphocytes relative to the remaining mononuclear cell population, or significant loss of APC available to present antigen to T lymphocytes, could result in suppression of certain immune functions {21}, given the apparent importance of these cell types in immune regulation.

Lymphoid populations are differentially susceptible to radiation damage or death {10, 20, 21}, and so immune dysregulation on the basis of alterations among interacting subpopulations is a viable possibility. The role of trafficking of particular MNC types to or from the spleen compartment may contribute to these alterations, and this mechanism cannot yet be ruled out without investigation into the kinetics of alterations in peripheral blood mononuclear cells post-irradiation.

ii. Alteration of Cytokines in the Microenvironment

Ionizing radiation may alter cytokines of the microenvironment indirectly by altering the proportions of cells that produce particular cytokines, as outlined above. Additionally, radiation exposure, in many instances, induces alterations in the cytokine profile by activating or deactivating cytokine genes {50}. DNA may be altered directly

by the radiation hit, or indirectly by reaction with oxygen radicals produced by the radiation exposure {51}. Radiation damage to the membrane may also influence intracellular signalling pathways, transducing a change from the membrane to the level of cytokine genes {51}. For example, TLI inhibits IL-2 transcription {52}, and diminishes IL-12 production by monocytes, favoring a Th2-type cytokine profile which is suppressive to cell mediated immune responses {53, 54}. A key cytokine implicated in radiation-induced immune suppression is IL-10. Keratinocytes exposed to UV radiation (another form of electromagnetic radiation imparts damage to DNA) demonstrate an upregulation in the production and secretion of IL-10 {55}, which interferes with macrophage antigen-presentation function {46}, inhibits IL-2 production by Th1 cells {56}, reduces T cell proliferative capacity {57}, and downregulates cytotoxicity by CD8+ T and NK cells {46}. In addition, IL-10 release following ultraviolet radiation exposure reduces delayed-type hypersensitivity reactions by impairing the ability of APC to stimulate cytokine production by Th1 cells {56}, and may play a role in the growth of cutaneous malignancies {58}. The changes in cytokine production and release outlined seem to promote a Th2-type cytokine profile, which is generally suppressive, as exemplified by the case of HIV infection. Progression of HIV infection and disease is marked by a shift in the cytokine profile elicited by Th cells from Th1 to Th2, and is considered to be, in part, responsible for the profound and prolonged immune suppression associated with the disease {46}. Patients treated with total lymphoid irradiation for Hodgkin's disease demonstrate prolonged partial immunologic tolerance, due in part to suppression of cytokine production by Th1 cells {59}.

iii. Active Suppression

The detrimental effects of radiation injury may be enhanced synergistically when combined with antigenic challenge. The use of total-lymphoid irradiation along with sensitization with a specified antigen to induce antigen-specific non-responsiveness, is common in therapy for enhancement of allograft survival {9, 60}, although the mechanisms of this suppression are poorly understood. It is therefore not surprising that radiation exposure in conjunction with challenge from a pathogen, such as *Borrelia burgdorferi* down-regulate any immune response to that organism in an antigen-specific manner {61}, whether by generation of a cytokine environment suppressive for the cell-mediated response {51, 62}, or by the generation of antigen-specific suppressor cells {63, 64}.

Following TLI, approximately 80% of splenic mononuclear cells (SMNC) express a null surface phenotype which are referred to as natural suppressor (NS) cells, and maintain suppressive activity of the mixed leukocyte reaction (MLR) for 6 to 8 weeks *in vitro* {65}. In this same study, NS cells blocked generation of cytolytic cells, but allowed the generation of alloantigen-specific suppressor cells *in vitro*. The existence of antigen-specific T suppressor cells is controversial, although evidence of antigen-specific immune suppression is abundant and widely accepted. Through recent investigations of the suppressive cytokine IL-10, its upregulation following ultraviolet radiation, and its proposed role in inhibition of the antigen-specific delayed-type hypersensitivity (DTH) response, it may be that suppressor T cells actually represent cells rendered unresponsive (ie. tolerant) when challenged with a specific antigen in an environment providing inadequate stimulatory signals, or an altered cytokine profile. This would further support

a role for radiation-induced immune dysregulation in the promotion of immune suppression, involving not only T lymphocytes, but also APC as potent producers of key cytokines involved in immune regulation.

D. FUNCTIONAL IMMUNITY POST-IRRADIATION

1. Natural Killer Cell Cytotoxicity

Given the lack of investigation into the *in vivo* effects of γ-radiation on NK cell function, and the reported resistance of NK cells to radiation-induced cell destruction {1, 20, 67}, investigating the preservation or impairment of cytolytic potential of murine NK cells post-irradiation was of relevance.

i.Natural Killer Cells

Natural killer (NK) cells are large granular lymphocytes with cytotoxic activity similar to that exhibited by cytotoxic T lymphocytes {68}. NK cells contain numerous, prominent granules containing perforin which integrates into the target cell membrane forming pores, and granzymes which exhibit cytotoxicity {68, 69}. As well as the perforin-dependent cytotoxicity pathway, NK cells can induce perforin-independent apoptosis in susceptible targets, mediated by Fas {32}, although other Fas-independent pathways may exist.

Although NK cells resemble lymphocytes in their morphology, phenotypically they are quite distinct {68, 70, 71}. NK cells lack surface expression of the CD3 antigen as well as the known T cell receptor (TCR) chains α, β, γ, and δ, nor do they express the

surface immunoglobulins characteristic of B lymphocytes {68, 71}. Natural killer cells are identified by expression of CD16 (FcγRIII) and CD56 surface antigens in humans, and expression of the NK-1.1 surface antigen is definitive of murine NK cells. The cell-mediated cytotoxicity conducted by these cells is in an MHC-unrestricted fashion, and NK cells do not require prior sensitization to combat foreign antigens {69, 71}, although their activity is dramatically enhanced by interferon-γ (IFN-γ). This cytokine may increase NK activity by inducing NK proliferation, by promoting differentiation from a precursor to an effector cell, by increasing synthesis and release of cytotoxic granules or factors, or by enhancing target cell binding {69}. The role of NK cells is suggested to be that of a surveillance cell, providing an early line of defence against cells with virus or tumor antigens {69, 72}.

Given the lack of MHC restriction in NK-mediated cytotoxicity, the mechanisms by which an NK cell distinguishes between "self" and "non-self" is elusive. Much evidence in the literature suggests that recognition of MHC class I antigens, particularly the α-1 domain, on "self" is inhibitory of the binding and subsequent lysis by NK cells {69, 73, 74}. The level of expression of class I MHC molecules on target cells is inversely proportional to the sensitivity of target cell lysis by NK cells {75}. By the "missing self hypothesis", since all nucleated cells express MHC class I, cells lacking this antigen are therefore foreign {75}. An alternative, yet not mutually exclusive theory, suggests that recognition of class I MHC molecules by the NK cell may deliver negative signals back to the NK cell, inhibiting further progress in the cytotoxicity pathway {75}. However, MHC class I on non-foreign cells is not the only molecule responsible for inhibition of NK cytotoxicity against self, because K562 cells, a human tumor cell line

susceptible to human NK lysis, transfected with MHC class I are not protected {75}. Recent studies have revealed a novel antigen present on murine NK-sensitive targets (YAC-1), and binding this target antigen with the IgM monoclonal antibody 18C2 prevents conjugate formation with NK cells, and inhibits cytolytic activity {75}. This and other target antigens may contribute to NK recognition of transformed or virus-infected cells, tagging them for destruction.

Although we are beginning to learn about target-cell antigens which may promote recognition and subsequent lysis by NK cells, little is known about NK cell receptors for those recognition or inhibitory target antigens. A type II transmembrane glycoprotein expressed on the surface of murine NK cells has demonstrated inhibitory function when engaged by carbohydrate determinants of class I molecules {74}. Recently, another murine inhibitory receptor has been identified; gp49, a type I transmembrane receptor of the immunoglobulin superfamily, has been demonstrated to be a homologue of human killer inhibitory receptors (KIR) {76, 77}. How these inhibitory molecules, Ly-49, gp49, and KIR, transduce inhibitory signals upon receptor engagement is not known, although all three receptors share a tyrosine-based signalling motif in their cytoplasmic tails {76}.

ii. Natural Killer Cell Cytotoxicity and Radiation

Much of the research regarding the impact of radiation exposure on the cytolytic function of natural killer cells is conflicting and therefore, controversial. *In vitro* exposure to γ-radiation at low doses stimulates human NK cytolytic activity against K562 cells, while high doses inhibit cytotoxicity {78}. Patients receiving total lymphoid irradiation (TLI) demonstrate a short-term deficit in NK activity, but demonstrate that

after the initial decrease, NK function is maintained or even enhanced in TLI patients {72, 79}. On the other hand, *in vitro* exposure of PBMC to ultraviolet B (UVB) radiation show a dose-dependent decline in cytotoxicity against K562 targets {80, 81}.

Many argue that the conflicting reports may be explained by considering that different types and qualities of radiation (ie. gamma versus ultraviolet), different dose rates, and fractionation of exposures may all impact the response of NK cells to the exposure {78, 82}. Also, the enhancement in NK activity may be attributed to the selective enrichment of radioresistant, highly cytotoxic subpopulations post-irradiation, arguing in favor of heterogeneity in response to radiation within the NK cell surviving fractions {72, 82}. It is therefore imperative that cytotoxicity be corrected for the surviving NK fraction, to accurately reflect the cytotoxicity by NK cells on a per cell basis {78}. The disparity in the literature may also reflect the fact that much of the research regarding NK cytotoxicity post-irradiation has been conducted under pathological conditions such as systemic lupus erythematosus {83} or Hodgkin's disease {59}, and therefore, the results are confounded by the diseases themselves {84}. As well, most clinical studies involved a limited number of patients, and lack of adequate controls {84}.

II. RATIONALE

Given the potentially serious consequences of radiation exposure on the immune system, investigating mechanisms underlying the crippling immune suppression may reveal opportunities for intervention. Previous investigations conducted in this laboratory

revealed differential radiosensitivity of various splenic mononuclear cell (SMNC) populations in mice: specifically, a selective enrichment in the surviving fraction of natural killer cells within the splenic MNC pool, and the dramatic radiosensitivity of B lymphocytes {1}. This current study has focused on investigation of alterations among key mononuclear cell populations as a potential component of immune dysregulation, which may contribute to immune suppression. In addition to flow cytometric analysis and monoclonal antibody labeling of murine PBMC post-irradiation for comparison with prior SMNC investigations {1}, dextran sulfate was employed to further investigate the loss of MNC throughout lymphoid and non-lymphoid tissues post-irradiation.

III. PURPOSE

To detect and quantify radiation injury to cells of the immune system of C57BL/6 mice, to lend further insight into mechanisms of radiation-induced decrease in immune competence.

IV. HYPOTHESES

Many mechanisms may contribute to immune depression observed following exposure to ionizing radiation. The working hypotheses are: 1) alterations in splenic mononuclear cells previously observed {1} will be mimicked in peripheral blood MNC, revealing the potential importance of certain cell types in generation of immune suppression; 2) dextran sulfate administration post-irradiation will reveal uniformity of

cytoreduction in immune-competent cells throughout lymphoid and non-lymphoid tissues; 3) apoptosis is a key player in cytoreduction of the immune system post-irradiation; 4) radiation exposure results in impairment of function of particular cell types, specifically NK cells.

V. OBJECTIVES

Addressing the following objectives may uncover and elucidate some of the mechanisms underlying immune depression following radiation exposure.

1) characterization of alterations in peripheral blood mononuclear cell subsets post-irradiation, to determine whether these alterations are a reflection of changes observed in splenic mononuclear cell populations;

2) to investigate the capacity of dextran sulfate, a potent mobilizing agent, to mobilize reserves of lymphocytes from the spleen, lymph nodes, and peripheral tissues into the circulation post-irradiation;

3) to investigate the role of apoptosis in radiation injury to mononuclear cells in the peripheral blood;

4) to determine the effects of radiation on the function of natural killer cells.

VI. MATERIALS & METHODS

A. ANIMALS

Animals used in all studies were C57BL/6 female mice, 18-20 g in weight and 7 to 8 weeks in age. Mice were supplied by Charles River (Montreal) and housed in cages of five at the University of Ottawa Animal Care Facility (ACSS). Animals were fed Purina Mouse Chow pellets and acidified tap water (pH=2.7) *ad libitum*. All experimental protocols were approved by the University of Ottawa Animal Care Committee prior to commencement, and were in accordance with the Guidelines of the Canadian Council of Animal Care.

B. IRRADIATION

Whole-body irradiation of animals was performed using a ^{137}Cs GammaCell-40 shielded drawer radiation source (Nordion, Kanata, Ontario) located at the University of Ottawa Health Sciences Centre, at a dose rate of 110 rad/minute (1 rad = 1 cGy). Single exposures in the range of 0 to 700 rad were administered in a ventilated chamber.

C. ASEPTIC HARVEST OF MURINE SPLENIC MONONUCLEAR CELLS

Prior to spleen harvest, each mouse was anaesthetized with methoxyflurane (provided by the ACSS) and euthanized by cervical dislocation. The left flank was thoroughly bathed in 70% ethanol and with sterile scissors and forceps, a circular incision was made, exposing the spleen. The spleen was removed, taking care to remove connective and adipose tissue, and placed in a sterile glass petri dish with approximately

1 mL of sterile phosphate buffered saline (PBS) (Gibco, Burlington, ON). The spleen was cut open at both ends and massaged with two sterile 18½ gauge needles bent at a 90° angle to gently push out the cells. The contents of the petri dish were aspirated and dispensed two or three times with a 10 cc syringe and a 23½ gauge needle, to form a single cell suspension. The suspension was added to a 15 mL polystyrene test tube while the petri dish was rinsed with an additional 2 mL PBS to flush any remaining cells. In an accuvette (Coulter Electronics, Burlington, ON), 40 µL of the cell suspension was combined with 20 mL Isoton II balanced electrolyte solution (Coulter) and counted on a Coulter-ZM Counter (Coulter Electronics). The splenic mononuclear cell (SMNC) suspension was diluted to a final concentration of 10^7 cells/mL, followed by overlaying on Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario) at room temperature, in a 1:1 ratio in a fresh polypropylene 15 mL test tube. This suspension was centrifuged for 20 minutes at 1300 x *g* at room temperature, and the resulting buffy coat collected, transferred to a sterile 15mL test tube and washed with sterile PBS. After a 10 minute room temperature spin at 300 x *g*, the pellet was resuspended in PBS or culture medium, cells were recounted, and the cell concentration was adjusted as specified in each particular assay.

D. CELL VIABILITY ASSESSMENT

Determination of splenic mononuclear cell viability was determined by trypan blue dye exclusion. Fifty microlitres of a cell suspension was combined with 50 µL of trypan blue, and allowed to incubate at room temperature for 2 minutes. Ten microlitres of stained cells were loaded onto a Bright Line hemocytometer (Hausser Scientific,

Horsham, PA); the number of viable cells (those that exclude trypan blue) counted within the defined 1 mm^2 ruling was multiplied by 2 x 10^4 to yield a count of viable cells per mL.

E. ANALYSIS OF PERIPHERAL BLOOD MONONUCLEAR CELL SUBSETS

1. Peripheral Blood Mononuclear Cell Counts

The number of PBMC/mL of murine whole blood was determined by analysis on a Coulter-ZM Counter (Coulter Electronics). Briefly, 20 µL whole blood was diluted in 10 mL Isoton II, a balanced electrolyte solution (Coulter Electronics) and mixed with 6 drops of Zap-oglobin II (Coulter Electronics) for erythrocyte lysis prior to analysis on the Coulter Counter. Each sample was counted three times, and the mean of the counts was recorded.

2. Preparation of Murine PBMC for Flow Cytometric Analysis (FCA)

Blood harvested from C57BL/6 mice by orbital bleeding using an 80 µL heparinized microcapillary tube (Fisher Scientific, Ottawa, ON) was placed in a 12x75 mm test tube (Sarstedt Canada Inc., St. Laurent, QUE) with 20 µL Hepaleane (1000 units/mL) (Organon Teknika, Toronto, ON) to prevent blood coagulation. Eighty µL of normal rat serum (10 mg/mL, Cedarlane) were added for Fc receptor blocking, and samples were incubated on ice for 30 minutes prior to addition of fluorescent monoclonal antibodies (mAbs) or isotype controls (Pharmingen, Mississauga, ON), followed by incubation on ice for 20 minutes. Each sample was washed in 2 mL of PBS + 0.1%

sodium azide (NaN_3) (Sigma, St. Louis, MO), centrifuged at 300 x *g* for 5 minutes, and the supernatant aspirated. Isolation of PBMC from whole blood was accomplished by lysis of erythrocytes with 1 mL ammonium chloride and potassium (ACK) lysing buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH=7.2), incubation at room temperature and mild shaking for 5 minutes. Samples were washed in PBS + 0.1% NaN_3 with 5 minutes of centrifugation prior to resuspension in PBS + 0.1% NaN_3 to a final volume of 1 mL and analyzed on a Coulter Epics XL flow cytometer (Coulter Electronics). The proportions of T lymphocytes, B lymphocytes, natural killer (NK) cells, CD4+ T cells, and CD8+ T cells relative to the entire PBMC population were measured by applying the appropriate monoclonal antibodies as indicated in Table 1. Prior to each experiment, the flow cytometer was calibrated using Standard Brite Beads, lot # 7211 (Coulter Electronics).

3. Viability Assessment by Flow Cytometry

Viability of cells detected by FCA was determined by a Live/Dead Viability/Cytotoxicity Assay (Molecular Probes Inc., Eugene, OR). Calcein-AM, a substrate for intracellular esterase activity which emits fluorescence in the 530 nm range (LFL1) when cleaved, was used at a concentration of 0.1 μM to detect live cells. Ethidium homodimer emits fluorescence in the 645 nm range (LFL2) upon binding nucleic acid in dead cells, and was used at a concentration of 4 μM. Following ACK lysis of murine whole blood, 100 μL each of calcein, AM and ethidium homodimer were added to each sample and allowed to incubate at room temperature for 45 minutes prior to FCA.

TABLE #1: PANEL OF MONOCLONAL ANTIBODIES EMPLOYED FOR PERIPHERAL BLOOD MONONUCLEAR CELL SUBSET ANALYSIS

All monoclonal antibodies (mAbs) were supplied by Pharmingen (Mississauga, ON). Each mAb was titrated for optimal concentration prior to use in immunophenotyping of murine peripheral blood mononuclear cells in irradiation studies, with or without dextran sulfate administration. FITC: fluorescein isothiocyanate (LFL 1; $\lambda = 530$ nm); PE: phycoerythrin (LFL 2; $\lambda = 645$ nm).

Sample	Antibody	Isotype	Concentration µg Ab/10E5 cells	Cell/ Marker
1	Autofluorescence			
2	Isotype Controls	PE-rat IgG2a FITC-rat IgG2b	2 mg 0.4 mg	
3	Thy 1.2-PE	IgG2b	0.4 mg	T cells
4	CD45/B220 FITC	IgG2b	0.5 mg	B cells
5	Thy 1.2-PE CD4-FITC	IgG2b IgG2a	0.4 mg 0.5 mg	T cells CD4+ cells
6	Thy 1.2-PE CD8-FITC	IgG2b IgG2a	0.4 mg 4 mg	T cells CD8+ cells
7	F4/80-FITC	IgG2a	2.5 mg	Mac/Mono
8	NK 1.1-PE	IgG2a	0.2 mg	NK cells

F. DEXTRAN SULFATE MOBILIZATION

1. Kinetics of MNC Mobilization into Periphery

C57BL/6 mice were given subcutaneous injections of 1 mg dextran sulfate (DS) (Dextran Products Ltd., Scarborough, ON) in 0.2 mL of sterile PBS, for a final dose of 50

mg/kg body weight. Blood samples (10 μL) were harvested by bleeding from the retro-orbital sinus with heparinized microcapillary tubes, and counted using a Coulter-ZM Counter as previously described, and the number of PBMC per mL was recorded. Blood samples were drawn from DS-treated mice at times 0, 1, 2, 3, and 4 hours post-injection, in parallel with samples drawn from DS-untreated mice.

2. Analysis of PBMC Subsets Mobilized by Dextran Sulfate Treatment After Radiation

C57BL/6 mice were injected subcutaneously with DS at 50 mg/kg body weight, immediately following orbital bleeding of 40 μL whole blood sample from each mouse. Half of each blood sample was counted on the Coulter-ZM Counter to obtain a measure of PBMC per mL whole blood prior to dextran sulfate treatment. The other half of each sample was set aside for monoclonal antibody staining and flow cytometric analysis to determine the differential PBMC composition of the blood prior to dextran sulfate treatment. After 2 hours, a second 40 μL blood sample was harvested from the same mouse, half of which was counted on the Coulter-ZM Counter in the manner described above, and the other half of each sample was held for flow cytometric analysis of the PBMC composition after DS treatment, for comparison with the pre-DS levels.

Blood for later processing and analysis by flow cytometry was deposited into 12 x 75 mm round, bottom polystyrene test tubes, and stored on ice. Staining and processing of whole blood for flow cytometric analysis of DS treated or untreated mice was conducted as previously described.

G. APOPTOSIS DETECTION AND QUANTIFICATION

1. Confirmation of Recombinant Human Annexin V-FITC Interaction with Murine MNC

Splenic mononuclear cells (SMNC) harvested from a C57BL/6 mouse were cultured in RPMI-1640 + 10% heat-inactivated fetal calf serum (FCS) in the presence or absence of 2 ng/mL TNF-α (R&D Systems, Minneapolis, MN) at 37 °C, in a total volume of 10 mL. After three hours, cells were harvested from the flask, incubated at room temperature with 0.5 μg Annexin V-FITC (BioWhittaker, Walkersville, MD) and 5 μg propidium iodide (PI) (Sigma) per 10^5 cells, for 15 minutes prior to flow cytometric analysis. Induction of apoptosis by exposure to whole-body γ-irradiation (WBI) was also assessed. Splenic mononuclear cells (SMNC) were harvested from a C57BL/6 mouse 4 hours after a single dose exposure of 400 rad whole body irradiation (WBI). Cells were diluted to a final concentration of 10^6 cells/mL, and divided into 100 μL samples (ie. 10^5 cells per sample). Samples were stained with Annexin V-FITC and/or PI as above, and analyzed by flow cytometry. Cells which are in the early stages of apoptosis are identified as cells positive for Annexin V-FITC (LFL1) but negative for PI (LFL2), which would be indicative of compromise of plasma membrane integrity.

2. DNA Extraction and Laddering

C57BL/6 mice treated with 0, 100, or 400 rad WBI were sacrificed 4 hours post-exposure, and their spleens harvested.

i. Extraction

Four aliquots of 5 x 10^6 SMNC from each mouse were resuspended in 100 μL

PBS and 1.25 mL lysis buffer (10 mM Tris, HCl, 10 mM EDTA, 75 mM NaCl, 0.5% SDS, pH 8.0) in 1.5 mL Eppendorf tubes. Samples were mixed gently without vortexing at room temperature for 15 minutes, followed by microcentrifugation at room temperature for 15 minutes at 13,000 x *g*. The pellets (intact DNA) were discarded, and the supernatants (fragmented DNA) were retained. The samples were incubated with RNase (100 μg/mL) (Sigma) in a 37 °C water bath for 30 minutes, and Proteinase K (100 μg/mL) (Boehringer Mannheim, Laval, QUE) in a 50 °C water bath for an additional 30 minutes.

ii. Precipitation of Fragmented DNA

Each of the four aliquots of fragmented DNA were halved (1 mL into two aliquots of 500 μL), and to each sample 100 μL of 6N NaCl and 800 μL of 100% ethanol were added. The fragmented DNA was allowed to precipitate overnight at 20 °C. Samples were microcentrifuged at 4 °C at 20,000 x *g* for 15 minutes, the ethanol was discarded and tubes were allowed to dry for one hour. All pellets derived from the same stock of SMNC were combined and resuspended in a final volume of 50 μL TE buffer (10 mM Tris, HCl, 1 mM EDTA, pH 8.0). Absorbance at 260 nm in an Ultrospec II Spectrophotometer (Fisher Scientific, Ottawa, ON) was used to determine the DNA concentration of each sample, and DNA purity was determined by absorbance at 280 nm. The DNA purity index was calculated as absorbance at 260 nm divided by absorbance at 280 nm, and samples were determined to have a purity index in the range of 1.75 to 2.00 {85}. Three μg DNA from each sample plus 1 μL loading buffer (in a total volume of 25 μL) were loaded onto a 1.5% agarose (Gibco) gel. Electrophoresis was conducted in a

Horizon 58 Gel Electrophoresis apparatus (Gibco/Life Technologies), at 100 mV for 45 minutes, and upon completion of the run, the gel was stained in 0.5 μg/mL ethidium bromide in H_2O for 30 minutes at room temperature. The gel was rinsed with water and visualized under UV light using a 2000 i Gel Print gel imager (BioPhotonics, Ann Arbor, MI).

3. Detection and Quantification of Apoptotic Events in Murine PBMC

Whole blood samples (20 μL) were harvested from the retro-orbital sinus of a C57BL/6 mouse and dispensed into a 12 x 75 mm round-bottom polystyrene test tube (Sarstedt). Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by lysis of contaminating erythrocytes with 1 mL ammonium chloride and potassium (ACK) lysis buffer per sample, incubated at room temperature for five minutes with mild shaking. Samples were washed by filling tubes to maximum capacity with PBS + 0.1 g/L $CaCl_2$ and centrifugation at 300 x *g* for five minutes at room temperature. The pellet is resuspended in PBS + 0.1 g/L $CaCl_2$ to a final volume of 500 μL, and incubated with 0.5 μg Annexin V-FITC and 5 μg PI for 15 minutes at room temperature prior to flow cytometric analysis. As with detection of apoptotic events in murine SMNC induced by TNF-α or *in vivo* radiation, apoptotic events in PBMC are indicated by cellular events staining positively for Annexin V-FITC (LFL 1) but not for PI (LFL 2).

H. NATURAL KILLER CELL CYTOTOXICITY

1. Target Cells

YAC-1, a murine lymphoma cell line susceptible to murine natural killer cell cytotoxic activity, but not sensitive to cytotoxic T lymphocyte activity, was obtained from the American Type Culture Collection (ATCC, Rockville, Maryland), and used as target cells in the NK cytotoxicity assays. This cell line is propagated in RPMI-1640 medium with 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 100 units/mL penicillin, 100 mg/mL streptomycin, 50 mg/mL gentamicin (Gibco), and 5 mg/mL amphoterocin B (Sigma).

P815, a murine mastocytoma cell line resistant to murine NK cell lysis, was also obtained from the ATCC (Rockville, Maryland), for use as a negative control in NK cytotoxicity experiments. These cells are propagated in Dulbecco's Modified Eagle Medium (DMEM) with 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 100 units/mL penicillin, 100 mg/mL streptomycin, and 50 mg/mL gentamicin (Gibco).

Cell lines stored in liquid nitrogen were rapidly thawed by rubbing between palms until all ice crystals disappeared. As soon as cells thawed, they were added to a 15 mL polypropylene test tube, and dropwise, 10 mL of their respective culture media were added. Cells were centrifuged at 300 x *g*, room temperature for five minutes, culture media was replaced, and cells were transferred to 75 cm^2 flasks (Costar Corporation, Cambridge MA). Cultures were maintained at 37 °C, and culture media was replenished 2 to 3 times per week.

2. Target Cell Preparation for Use in Cytotoxicity Assays

Target cells used were maintained in log phase by subculturing 24 hours prior to each experiment as follows. Cells were harvested from their flasks and placed in 50 mL conical polypropylene test tubes, and centrifuged at 300 x *g* for 5 minutes at room temperature. Supernatant was aspirated and the pellet was resuspended in 5 mL culture media. Cell viability was assessed by trypan blue dye exclusion, and cells were counted on a hemocytometer as described above. 2×10^6 cells were placed into a fresh 75 cm^2 vented tissue culture treated flask with 35 mL of the appropriate culture media, and returned to the incubator (37 °C, 5% CO_2).

For use as targets, cells were stained with 1 μg/mL $DiOC_{18}(3)$ in culture media at a concentration of 2×10^5 cells/mL, and incubated in a 25 cm^2 vented flask (Costar) for 20 minutes at 37 °C. Following staining, cells were washed twice in Ca^{2+}/Mg^{2+}-free PBS, and resuspended to a final concentration of 2×10^4 cells/mL in RPMI-1640 + 10% FCS without phenol red.

3. Flow Cytometric Assay for NK Cytotoxicity Determination

SMNC were harvested from mice given 0, 25, 50, 100, 200, or 400 rad γ-WBI 1, 4, or 7 days prior to the assay date, and diluted to a final concentration of 5×10^6 cells/mL in culture medium without phenol red. Spleen-derived MNC were used as effectors rather than blood-derived MNC because the number of cells required for these assays could not be feasibly obtained from whole blood.

In a 12 x 75 mm round-bottom polystyrene test tube (Sarstedt), 50 μL of $DiOC_{18}(3)$-labelled targets were combined with 100 μL of effectors in effector to target

ratios 50:1, 25:1, 12.5:1, 6.25:1, and 3.125:1, as demonstrated in Table 2. Tubes were centrifuged at 200 x *g* for 1 minute to pellet the effectors and targets together, which were coincubated for 4 hours at 37 °C. Fifteen minutes before the end of the 4-hour incubation period, 5 μL of PI (100 μg/mL) were added to each sample, followed by flow cytometric analysis. The gating strategy for detection of lysed targets is depicted in Figure 1: total cells are electronically selected in A, and those which are DiO-positive (cursor in B) are target cells, and are analyzed for PI staining (quadrant 2 in C). The percent specific lysis at each effector: target ratio is calculated as:

$$\% \text{ specific lysis} = 100 \times \frac{\text{lysed (DiO+/PI +) targets (quadrant 2) - nonviable targets}}{\text{total (DiO+) targets (quadrant 1 + 2)}}$$

In addition to the effector/target co-cultured samples, an additional sample was analyzed for each radiation dose, at each timepoint post-exposure. SMNC stained with the NK-1.1 PE monoclonal antibody, and containing Coulter Flow Count Fluorospheres (Coulter) were analyzed to determine the number of NK cells in each sample. This method correlates well with the traditional ^{51}Cr-release method {86}.

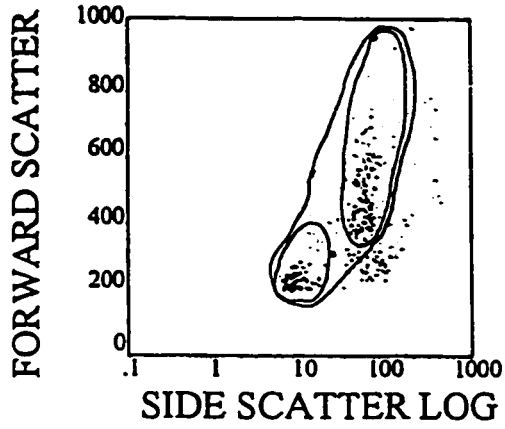
Results are reported as lytic units to reflect the number of effector cells required to lyse a specific percentage of target cells, allowing easy comparison from one experiment to another {87, 88}. Calculation of lytic units is complex, by equations described by Bryant *et al.* To assist in this task, calculation of lytic units was performed using EXPFIT software, provided by Dr. Hugh Pross.

TABLE #2: PANEL OF STAINING REAGENTS USED FOR NK CYTOTOXICITY DETERMINATION

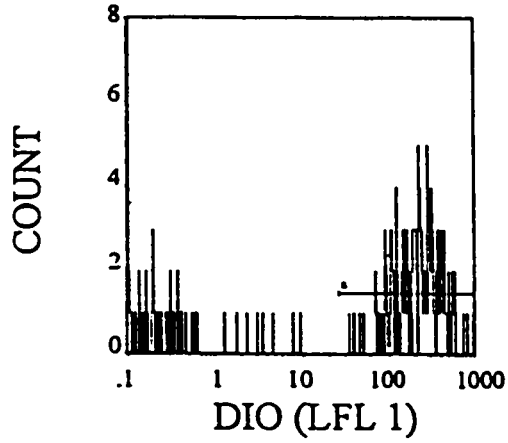
Target cells (YAC-1, P815) are stained with $DiOC_{18}(3)$ (3,3'-dioctadecyloxacarbocyanine perchlorate) prior to incubation with effector cells, and propidium iodide. (PI) staining indicates lysed or non-viable cells, as described in Methods. PI was used at a concentration of 100 μg/mL; $DiOC_{18}(3)$ was used at a concentration of 10 μg/mL target cell suspension.

Sample #	Sample Cell Content (Effector:Target Ratio)	Staining
1	Effectors	PI
2	Targets	DiO + PI
3	E:T ratio = 50:1	DiO + PI
4	E:T ratio = 25:1	DiO + PI
5	E:T ratio = 12.5:1	DiO + PI
6	E:T ratio = 6.25:1	DiO + PI
7	E:T ratio = 3.125:1	DiO + PI
8	Effectors	NK-1.1 PE mAb

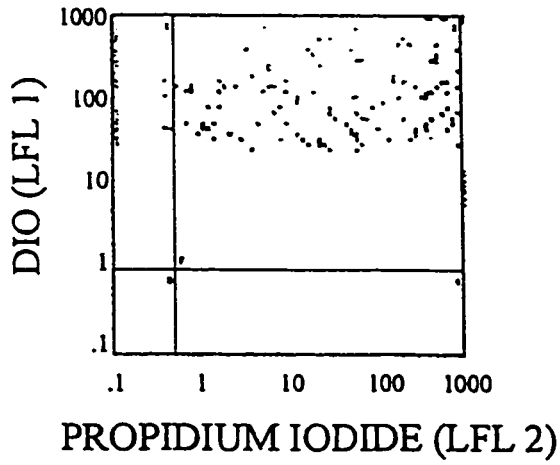
Prior to calculation of lytic units, effector to target ratios were adjusted to reflect the actual number of NK cells in each sample: # NK cells/mL (as determined by flow cytometry using Coulter Flow Count Fluorospheres) multiplied by 100 μL (volume of SMNC suspension per sample) gives the total number of NK cells added to 10^4 target cells.


FORWARD SCATTER
1000
800
600
400
200
0
.1
1
10
100
1000
SIDE SCATTER LOG

A


COUNT
8
6
4
2
0
.1
1
10
100
1000
DIO (LFL 1)

B


DIO (LFL 1)
1000
100
10
1
.1
.1
1
10
100
1000
PROPIDIUM IODIDE (LFL 2)

C

FIGURE#1: FLOW CYTOMETRIC ANALYSIS OF PERCENT SPECIFIC LYSIS OF TARGETS BY NATURAL KILLER CELLS

Target cell discrimination from total cellular events acquired (**A**) is by positive staining for $DiOC_{18}(3)$ (LFL 1), indicated in (**B**) by the linear cursor. Events under this cursor are directed for dual-parameter analysis shown in histogram (**C**).

I. STATISTICAL ANALYSIS

All data shown are given as the mean standard deviation.

The effects of WBI (0-700 rad) on the number of nucleated cells in the peripheral blood, on the relative proportions of PBMC populations in the periphery, and on dextran sulfate mobilization of MNC on days 1, 4, and 7 post, irradiation were compared by one-way ANOVA (analysis of variance). If results were significant ($p<0.05$), a Neuman-Keuls multiple comparison test was used, to demonstrate which of the means are significantly different than others at the 0.05 significance level.

The effect of WBI (0, 400 rad) on phosphatidyl serine surface expression on apoptotic cells at 0, 1, 2, 4, 8, and 24 hours post-irradiation by one-way ANOVA, and if the results were significant ($p<0.05$), a Neuman-Keuls multiple comparison analysis was performed.

Statistics were calculated using KWIKSTAT 2.00 for IBM PC compatible computers (Texasoft/Mission Technologies, Cedar Hill, TX).

VII. RESULTS

A. DECLINE IN TOTAL PBMC NUMBER POST-IRRADIATION IS DOSE-DEPENDENT

Radiation induces death in cells, especially in the exquisitely radiosensitive lymphoid cells. Reduction in the number of immune-competent cells may contribute to impaired host defence post-irradiation. An investigation of the impact of radiation on total peripheral blood mononuclear cell number was conducted, to determine the overall survival of MNC following a radiation insult. Blood samples collected from mice exposed to 0, 100, 400, or 700 rad whole-body γ-irradiation (γ-WBI) 1, 4, or 7 days post-irradiation were counted on a Coulter-ZM Cell Counter, and the total PBMC counts are shown in Figure 2. The decline in PBMC number at all times after all doses relative to the unirradiated control is significant ($p<0.05$), and PBMC number is significantly lower in mice irradiated with 400 or 700 rad than with 100 rad ($p<0.05$), although there is no significant difference between PBMC counts at any time after 400 or 700 rad.

B. ALTERATIONS IN PBMC POPULATIONS WITH RADIATION

Previous investigations into the relative proportion of different splenic MNC populations post-irradiation revealed alterations in the balance of surviving cells {1}. These alterations might contribute to an altered immune response to antigenic challenge. To determine the importance and uniformity of these alterations among lymphoid cells, the relative proportion of peripheral blood MNC populations post-irradiation was

investigated in mice exposed to ionizing radiation. Blood samples were taken at specific times post-irradiation for antibody labelling and analysis by flow cytometry. Results are presented in Figures 3A through D, and Figure 4.

Figure 3A shows the relative change in Thy-1.2+/CD4 + T cells (mainly T helper/inducer) with various doses of radiation. This figure depicts a slight but significant decrease in the proportion of these cells on day 1 after 400 or 700 rad, and a large, significant increase on day 4 after 700 rad ($p<0.05$). Figure 3B shows alterations in the relative proportions of Thy-1.2+/CD8+ cells (T cytotoxic/suppressor) with radiation. On day 1 after 100 rad, CD8+ T cells increase in their proportion of total PBMC relative to the control, and on day 4 post-irradiation, the proportion declines after 400 rad, relative to 100 rad ($p<0.05$).

Figure 3C shows a decline in the proportion of B cells relative to the PBMC population with increasing dose, and this decline was significant at all doses and on all days ($p<0.05$) compared to the control values. After 100 rad WBI, the B lymphocyte population declined to roughly half of its original proportion of the total PBMC, and after 400 and 700 rad, the proportion of B cells declined to roughly 10% of the control value by day 1, and remained as low even by day 7. In Figure 3D, natural killer (NK) cells increased markedly in proportion of the PBMC, increasing nearly 9, fold on day 4 after 700 rad. The large increase in proportion of NK cells relative to control is highly significant on day 7 after a dose of 700 rad, and on day 4 after 400 or 700 rad ($p<0.05$). 100 rad did not seem to result in any significant change from controls.

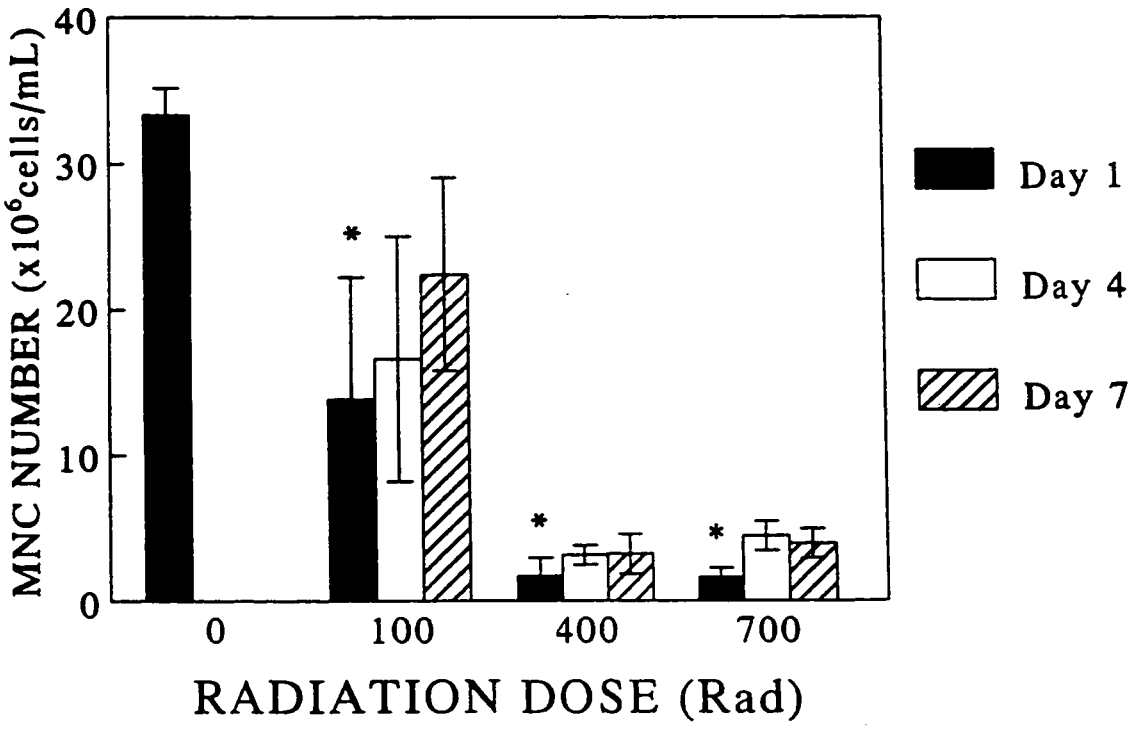
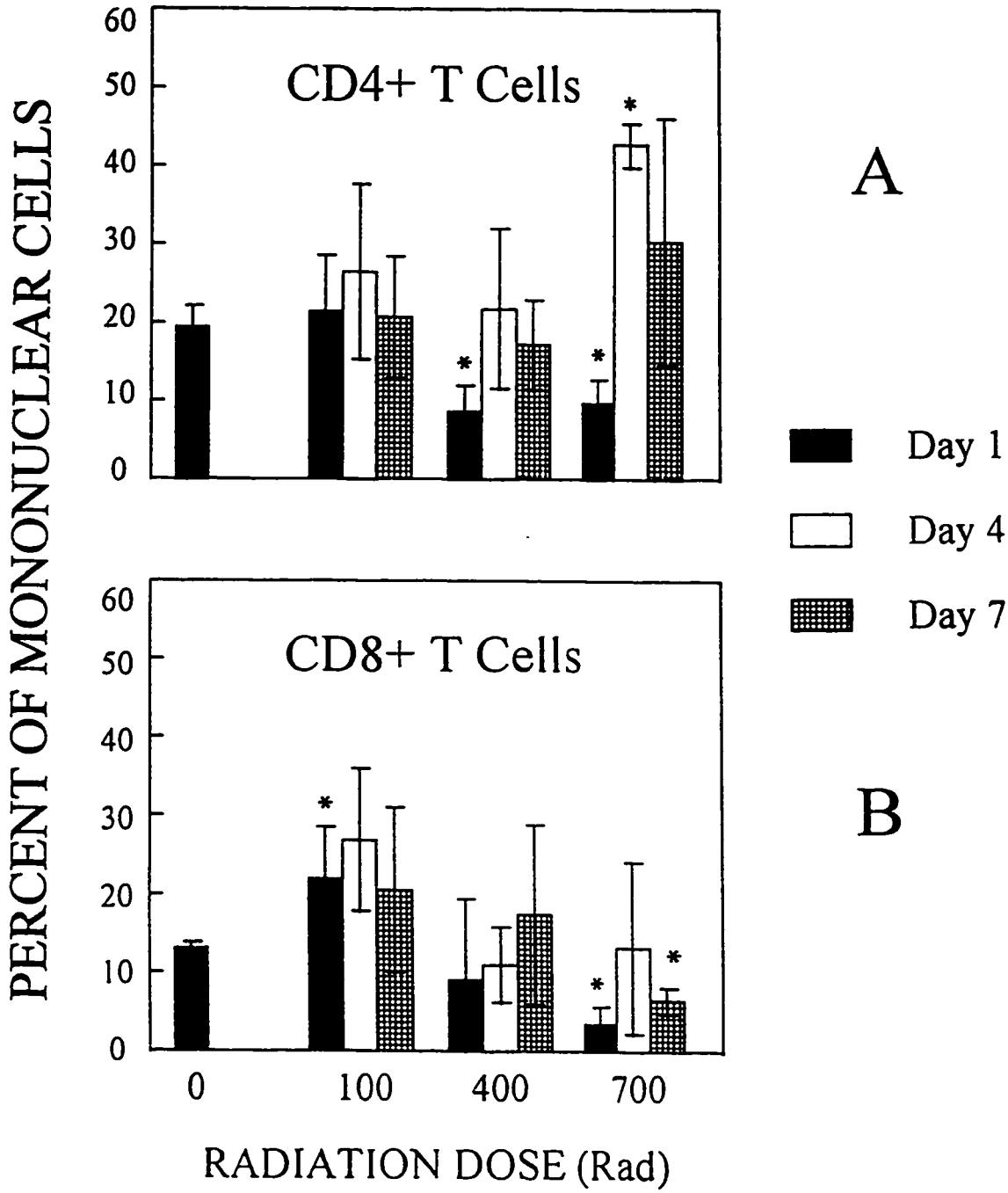

MNC NUMBER (x10^6cells/mL)
40
30
20
10
0
*
*
*
0
100
400
700
RADIATION DOSE (Rad)
Day 1
Day 4
Day 7

FIGURE #2: DECLINE IN PERIPHERAL BLOOD MONONUCLEAR CELL NUMBER AFTER WHOLE-BODY IRRADIATION

Total MNC in whole blood of C57BL/6 mice irradiated with 0-700 rad WBI counted during one week post-irradiation, as described in Methods. Mean PBMC number per mL ± SD (n=4). Bars marked with an asterisk (*) are significantly different than the unirradiated control, at the 0.05 significance level.


CD4+ T Cells
CD8+ T Cells
PERCENT OF MONONUCLEAR CELLS
RADIATION DOSE (Rad)
0
100
400
700
60
50
40
30
20
10
0
A
B
Day 1
Day 4
Day 7

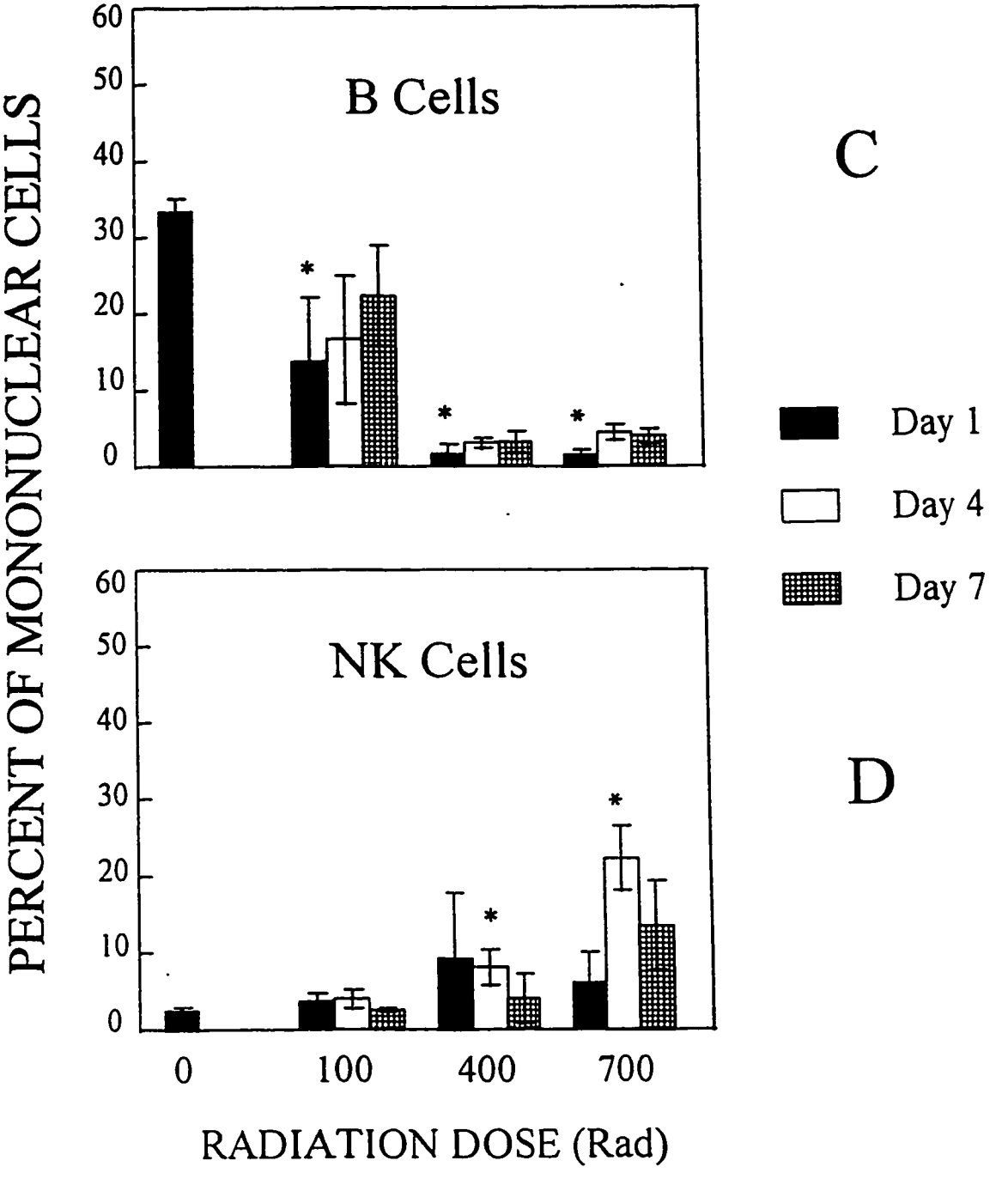

B Cells
NK Cells
C
D
Day 1
Day 4
Day 7
PERCENT OF MONONUCLEAR CELLS
RADIATION DOSE (Rad)
0
100
400
700
60
50
40
30
20
10
0
*

FIGURE #3: RELATIVE CHANGE IN BLOOD MNC POPULATIONS DURING 7 DAYS AFTER WBI

PBMC of C57BL/6 mice were analyzed by FCA and single or double antibody labelling 1, 4, and 7 days post-irradiation, as described in Methods. The relative proportions of (**A**) CD4+ T cells (Thy-1.2+/CD4+), (**B**) CD8+ T cells (Thy-1.2+/CD8+), (**C**) B lymphocytes (CD45/B220+), and (**D**) NK cells (NK-1.1+) are expressed as a percentage of total PBMC. The data represent the mean and standard deviation for 5 experiments. Bars marked with an asterisk (*) are significantly different than the unirradiated control, at the 0.05 significance level.

Results in Figures 3A to D for day 4 post-irradiation are summarized in Figure 4, which shows alterations in the different populations of PBMC with dose, relative to their values in the control mice. NK cells increased dramatically, B cells were decimated, CD4+ T lymphocytes increased approximately 2-fold, and CD8+ T lymphocytes and monocytes/macrophages remained steady.

C. VIABILITY

Following irradiation of the mice, a population of cells morphologically distinct from mononuclear cells appeared (determined by flow cytometry) as shown in Figures 5B (100 rad) and 5C (400 rad) compared to the unirradiated control (5A). This population seemed to have been the result of an increase in the granularity of blood MNC post-irradiation, as indicated by their increased side-scatter signal. The prevalence of these cells was dose-dependent and significantly declined by day 4 post-irradiation (Figure 6).

Radiation injury is associated with death of cells of the immune system, and I employed the Molecular Probes Live/Dead Viability/Cytotoxicity assay) to characterize this event. Dead PBMC will stain positively by flow cytometry for the nuclear stain ethidium homodimer, while live, viable cells will stain positively for Calcein-AM, an intracellular esterase substrate which fluoresces within LFL1 when cleaved. Experiments employing this assay were performed on cells from 10 mice one day after 0 rad or 700 rad, and the results are depicted in Table 3. Of note is the much lower overall viability in the atypical granular population of cells after 700 rad WBI compared to the peripheral

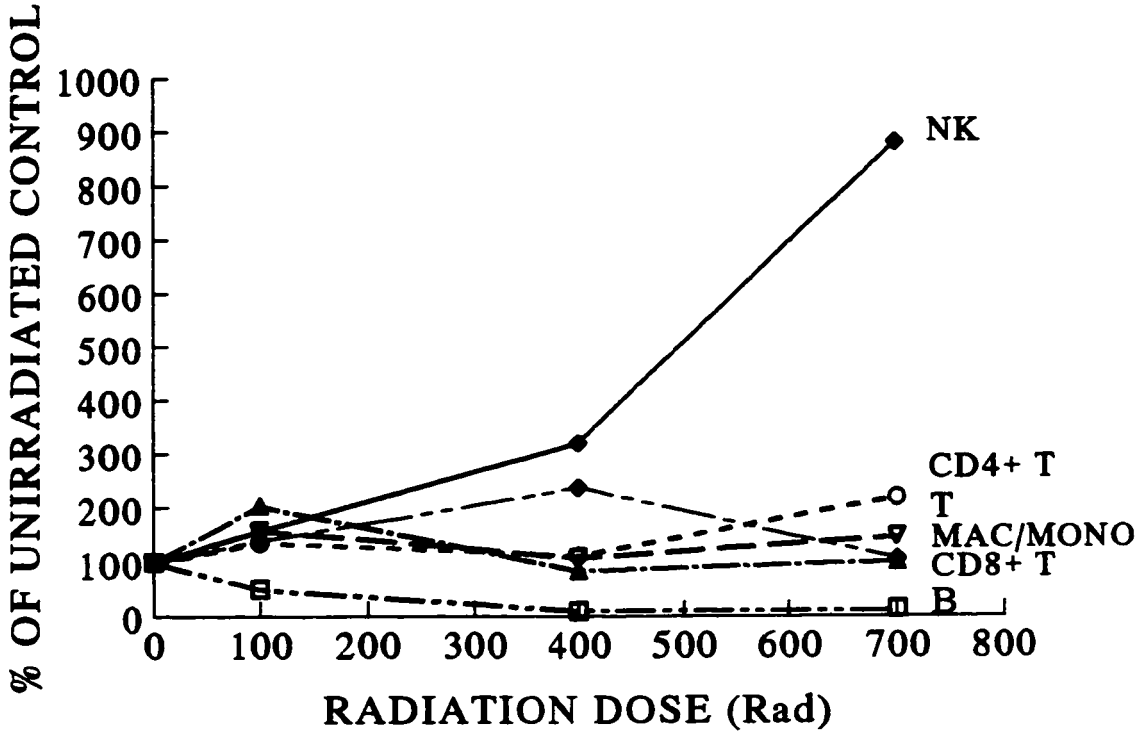

% OF UNIRRADIATED CONTROL
1000
900
800
700
600
500
400
300
200
100
0
0
100
200
300
400
500
600
700
800
RADIATION DOSE (Rad)
NK
CD4+ T
T
MAC/MONO
CD8+ T
B

FIGURE #4: SUMMARY OF ALTERATIONS IN BLOOD MNC POPULATIONS 4 DAYS POST-IRRADIATION

The effects of WBI on the balance among PBMC populations are summarized for day 4 post-irradiation. The proportion of each blood MNC population after each dose of radiation is expressed as a percentage of the unirradiated control proportions for those cell types to display the differential responses to radiation exposure.

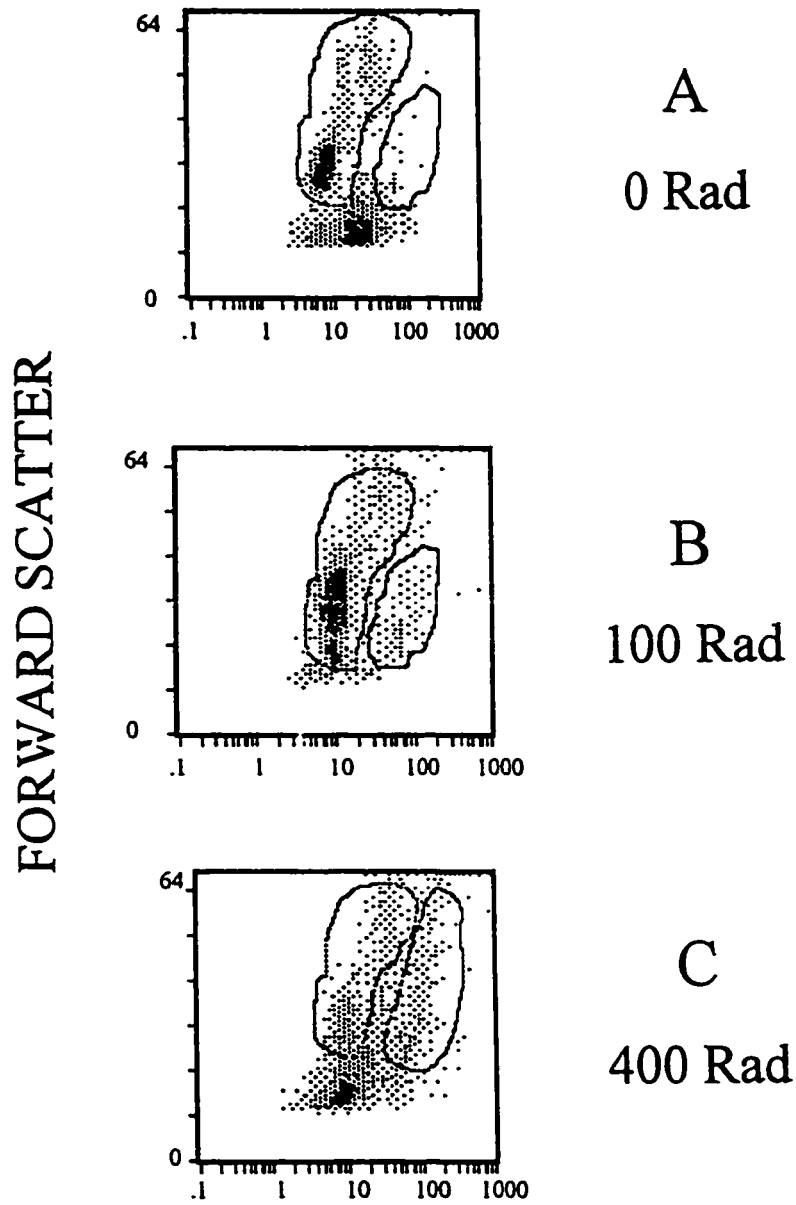

64
0
.1
1
10
100
1000
A
0 Rad
64
0
.1
1
10
100
1000
B
100 Rad
64
0
.1
1
10
100
1000
C
400 Rad
FORWARD SCATTER
SIDE SCATTER (LOG)

FIGURE #5: APPEARANCE OF AN ATYPICAL CELL POPULATION FOLLOWING IRRADIATION, AS DEMONSTRATED BY FLOW CYTOMETRY

Appearance of atypical cell population with greater granularity than blood MNC is more marked with increasing radiation dose. Forward scatter (linear) versus side scatter (logarithmic) histograms from analysis of whole blood from C57BL/6 mice after (**A**) 0 rad, (**B**) 100 rad, and (**C**) 400 rad WBI.

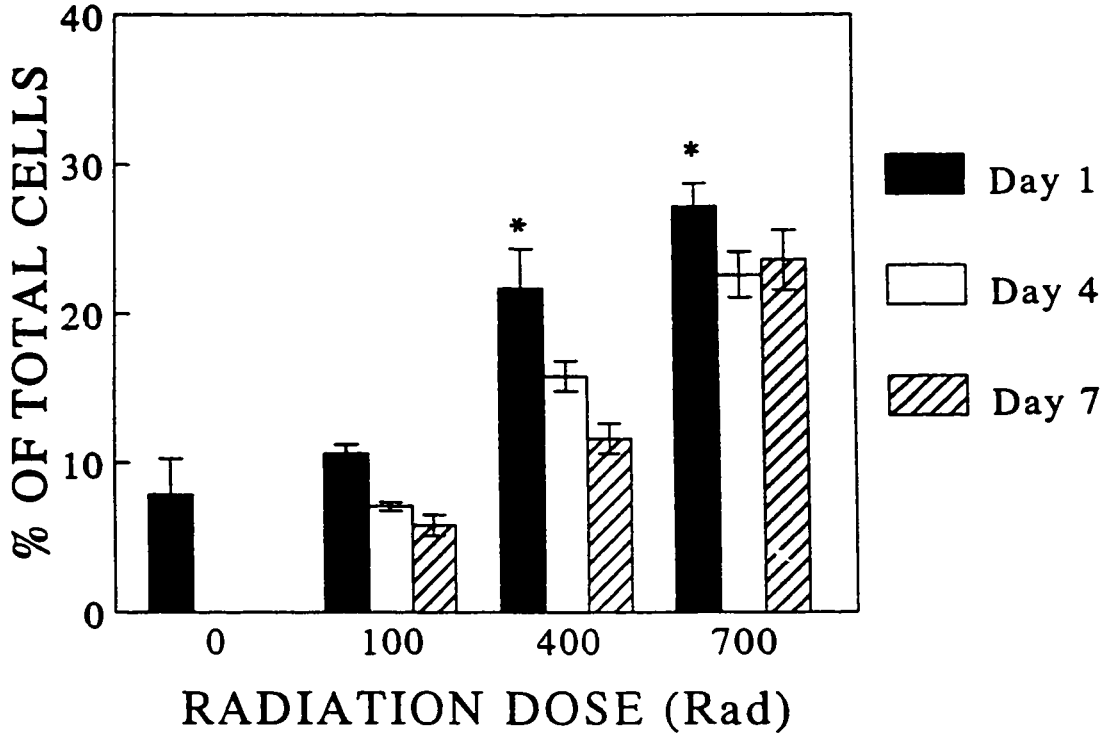

40
30
20
10
0
% OF TOTAL CELLS
*
*
0
100
400
700
RADIATION DOSE (Rad)
Day 1
Day 4
Day 7

FIGURE #6: CHANGES IN ATYPICAL POPULATION PREVALENCE WITH DOSE AND TIME

Magnitude of atypically granular blood cell population expressed as a percent of total cells surveyed. Whole blood of mice irradiated with 0-700 rad was analyzed by flow cytometry 1,4, and 7 days post-irradiation, gating on the atypical population. The data represent the mean and SD of 5 experiments. Bars marked with an asterisk (*) are significantly different than the unirradiated control, at the 0.05 significance level.

blood mononuclear cell population from the same set of animals, as demonstrated by the higher percentage of dead or dying cells and the lower percentage of viable cells in the atypical population. Damaged or dead cells do not accumulate in the PBMC population 24 hours post-exposure.

TABLE #3: VIABILITY ASSESSMENT OF ATYPICAL CELL POPULATION

Calcein-AM and ethidium homodimer staining for flow cytometric analysis of PBMC and atypically granular blood cell population after 0 and 700 rad. Viable cells are calcein-AM positive, dead cells are ethidium homodimer positive. Results are given as the mean of the percent of cells staining for each stain alone or in combination, ± (SD) for each population (n=10).

	Blood Mononuclear Cells		Atypical Cell Population	
	0 Rad	700 Rad	0 Rad	700 Rad
Calcein-AM +(live)	98.15 (0.9)	91.5 (1.5)	not observed	25.2(7.2)
Ethidium Homodimer +(dead)	0 (0)	0.1 (0.17)	not observed	1.8 (1.3)
Double + (dying)	0.95 (0.5)	1.8 (0.7)	not observed	20.5 (9.9)
Double Negative (debris)	0 (0)	6.7 (1.2)	not observed	52.5 (8.1)

D. DEXTRAN SULFATE MOBILIZATION

Dextran sulfate is a powerful lymphocyte mobilizing agent, promoting their release from lymphoid and peripheral tissues into the blood. Mobilization of MNC from reserves into the blood of mice following irradiation may reveal whether MNC loss occurs througout the body.

1. Kinetics of MNC Mobilization

Mobilization of MNC into peripheral blood by dextran sulfate is transient, with peak mobilization occurring 3 to 5 hours following drug treatment {42}. For the mobilization studies conducted in irradiated C57BL/6 mice, the time post-treatment to produce the optimal amount of mobilization was determined.

C57BL/6 mice given 50 mg/kg body weight DS via subcutaneous injection were bled by the retro-orbital sinus 0, 1, 2, 3, and 4 hours post-treatment. The total number of PBMC per mL was determined by cell counting with a Coulter-ZM Counter. Figure 7 compares PBMC number per mL at various times after DS administration with PBMC per mL in mice without DS treatment. Peak mobilization occurred 2 hours post-administration, averaging a 2.75-fold increase over the untreated control ($p<0.05$).

2. Monoclonal Antibody Labelling of PBMC in the Presence of Dextran Sulfate

Dextran sulfate may influence MNC migration by coating cells and affecting surface charge and adhesion molecules {41, 42, 44}. Given that identifying cell populations which may be mobilized by DS using flow cytometry requires surface antigen labelling with monoclonal antibodies (mAbs), the possibility of interference of

DS with labelling was explored.

SMNC harvested from one C57BL/6 mouse were incubated *in vitro* for 2 hours in various concentrations of DS (in PBS) prior to incubation with each of the monoclonal antibodies to be used in subsequent studies. Both the percent of SMNC staining for each monoclonal at each DS concentration, and the mean channel fluorescence of fluorochromes attached were measured by FCA, and are recorded in Table 4. With all three mAbs used (Thy-1.2 PE for T lymphocytes, CD45/B220-FITC for B lymphocytes, and NK-1.1 PE for NK cells) for all DS treatment groups (0, 1, and 5 mg/mL), there was no appreciable difference in mAb staining as noted by both percent positive staining and mean channel fluorescence (intensity of staining).

TABLE #4: MONOCLONAL ANTIBODY INCUBATION FOLLOWING *IN VITRO* DEXTRAN SULFATE EXPOSURE

SMNC were incubated with 0, 1, or 5 mg/mL dextran sulfate in PBS prior to monoclonal antibody (mAb) staining to determine whether the DS interferes with mAb staining. The percent of SMNC positive for each mAb, as well as fluorescence intensity (Mean Channel Fluorescence) are indicated for each dextran sulfate treatment.

Sample	Treatment mg DS/mL	% Positive Staining		Mean Channel Fluorescence	
		FITC	PE	FITC	PE
Thy-1.2 (PE)	0	64.7	26.7	84.7	314.4
CD45 (FITC)	1	65	28.8	91.9	323.3
	5	63.8	31	82.1	311.3
NK-1.1 (PE)	0	N/A	3.6	N/A	21.7
	1	N/A	3.4	N/A	26.8
	5	N/A	3.5	N/A	22.4

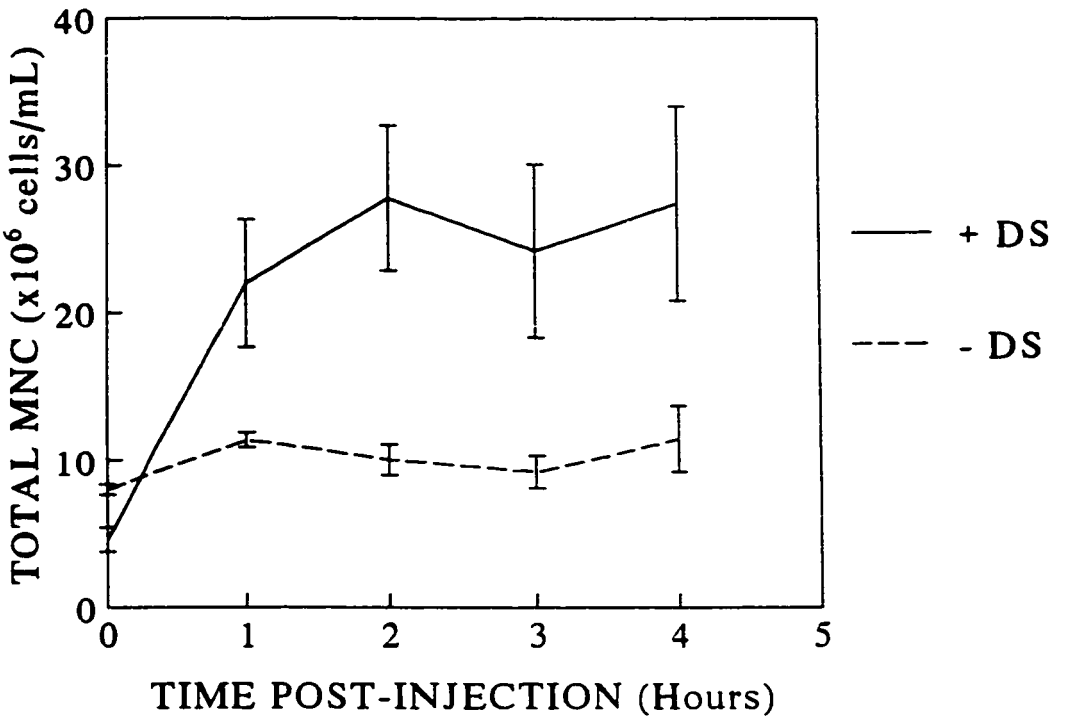

40
30
20
10
0
TOTAL MNC (x10⁶ cells/mL)
0
1
2
3
4
5
TIME POST-INJECTION (Hours)
+ DS
- DS

FIGURE #7: KINETICS OF MNC MOBILIZATION BY SUBCUTANEOUS ADMINISTRATION OF DEXTRAN SULFATE

Mice with or without dextran sulfate administration were bled, and the total number of peripheral blood mononuclear cells (PBMC) per mL was counted 0, 1, 2, 3, and 4 hours post-injection, as described in Methods. The mean PBMC number/mL at each time for each treatment condition is given, and error bars represent the SD (n=4). Points marked with an asterisk (*) are significantly different than the unirradiated control, at the 0.05 significance level.

3. Dextran Sulfate Mobilization of PBMC Subsets in Irradiated Mice

Reduction in the number of circulating lymphocytes post-irradiation is a serious consequence, contributing to immunosuppression and susceptibility of the host to opportunistic infection. Cell loss from spleen {1} and peripheral blood (Figure 2) has been demonstrated, but it is unclear whether this cytoreduction occurs in other lymphoid and peripheral tissues. Mobilization of MNC from reserves (spleen, lymph nodes, peripheral tissues) into the peripheral blood will allow observation of MNC in other tissues.

Mice were bled pre- and 2 hours post-administration of DS, and those mice which demonstrated greater than a 3-fold mobilization of MNC into the periphery were stained with monoclonals for FCA. Mobilization of B lymphocytes by dextran sulfate is shown in Figure 8A. On days 1, 4, and 7 following a dose of 100 rad WBI, B lymphocytes are mobilized by DS, showing a significant ($p<0.05$) 2 to 3 fold increase in circulating B lymphocyte number in the peripheral blood relative to DS untreated controls. At the higher dose of 400 rad, B lymphocytes are nearly eliminated in the periphery even by day 1, and DS administration on days 1, 4, or 7 post, irradiation does not significantly improve their number.

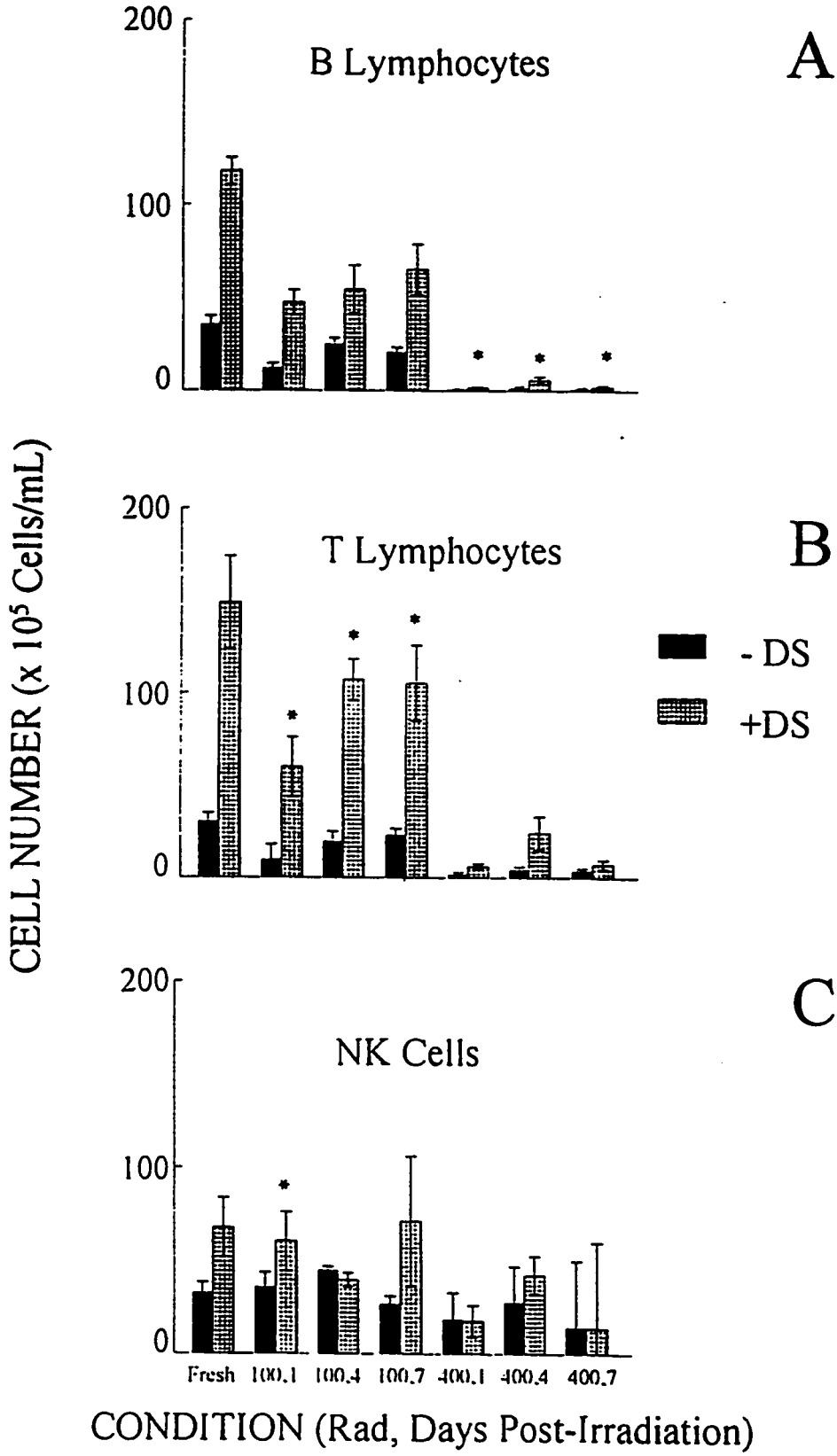

200
100
0
B Lymphocytes
A
T Lymphocytes
B
- DS
+DS
NK Cells
C
CELL NUMBER (x 10⁵ Cells/mL)
Fresh
100.1
100.4
100.7
400.1
400.4
400.7
CONDITION (Rad, Days Post-Irradiation)

FIGURE #8: MOBILIZATION OF MONONUCLEAR CELLS INTO PERIPHERAL BLOOD BY DEXTRAN SULFATE IN IRRADIATED MICE

Mice irradiated with 0, 100, or 400 rad WBI were assessed for dextran sulfate (DS) induced mobilization of B and T lymphocytes, and natural killer cells into the periphery 1, 4, and 7 days post-irradiation. Absolute numbers of B cells (**A**), T cells (**B**) and NK cells (**C**) were determined by flow cytometric analysis, using monoclonal antibodies listed in Table 1. Bars indicate mean cell number per mL of whole blood, and error bars represent the SD (n=4). Bars marked with an asterisk (*) are significantly different than the unirradiated control, at the 0.05 significance level.

The results for T lymphocyte mobilization by subcutaneous administration of DS are shown in Figure 8B. On days 1, 4, and 7 following a dose of 100 rad WBI, T lymphocytes are mobilized by DS, showing a 4 fold increase in circulating T lymphocyte number in the peripheral blood. Relative to the unirradiated control, representative of normal levels of circulating T lymphocytes in the periphery, DS administration 1, 4, or 7 days after 100 rad significantly augments T lymphocyte number above control levels ($p<0.05$). After 400 rad, DS only significantly improves T lymphocytes in the periphery on day 4, increasing T cell number to unirradiated, DS-untreated levels ($p<0.05$).

Natural killer (NK) cell mobilization by dextran sulfate administration post-irradiation was also monitored, and the results are shown in Figure 8C. There was no significant decline in NK numbers in the periphery with radiation exposure (100 or 400 rad WBI), and no significant improvement with DS treatment ($p<0.05$), except in the unirradiated control.

E. APOPTOSIS DETECTION AND QUANTIFICATION

Apoptosis has been identified as an important cell death mechanism in response to radiation injury. This investigation sought to determine the extent to which radiation plays a role in radiation injury to murine PBMC, especially in the low-dose (<100 rad) range.

1. Apoptosis Induction by TNF-α or Ionizing Radiation

The Annexin V protein used in this assay is a human recombinant protein, and

prior to its use in detection of early apoptotic events in a murine system, it was necessary to assess whether this protein would interact with murine cells induced to undergo apoptosis.

SMNC harvested from a C57BL/6 mouse were cultured for 3 hours in the presence or absence of 2 ng/mL TNF-α to induce apoptosis. Following the 3 hour incubation, cells were harvested, washed, and stained with Annexin V-FITC for flow cytometric analysis (FCA). In Figure 9, SMNC treated with TNF-α exhibit abundant Annexin V-FITC staining (9D) relative to the autofluorescence control (9C), whereas untreated SMNC show very little Annexin V staining (9B) above background autofluorescence (9A). SMNC harvested from C57BL/6 mice 4 hours after treatment with or without 400 rad WBI were stained with Annexin V-FITC and analyzed by flow cytometry. Figure 9F demonstrates that SMNC isolated from 400 rad irradiated mice do exhibit considerable Annexin V-FITC staining above the unirradiated (9B) and autofluorescence (9E) controls, indicative of early apoptosis induction by WBI.

Ultimately, this detection system was chosen for use in assessing apoptosis induction in murine PBMC. It was essential to assess the applicability of this staining system in a murine PBMC preparation protocol involving erythrocyte lysis. Four hours following treatment of mice with or without 400 rad WBI, blood was harvested, erythrocytes were lysed with ACK lysis buffer, and samples were stained with Annexin prior to FCA. PBMC from the irradiated mouse (Figure 10B) demonstrated a significant amount of positive staining with Annexin V-FITC, whereas PBMC from the unirradiated mouse (Figure 10A) show no significant Annexin V-FITC staining.

2. Confirmation of Apoptosis by DNA Laddering

Phosphatidyl serine (PS) expression on the surface of PBMC post-irradiation is detected and quantified by flow cytometry, as an indicator of early apoptotic cells. To ensure that this PS expression was coincident with apoptosis induction, and was not occurring as a result of another process or pathway independent of apoptosis, DNA laddering was assessed. SMNC were harvested from mice treated with 0, 100, or 400 rad WBI, 4 hours post-irradiation. DNA was extracted and precipitated from the SMNC, and equal amounts of DNA from each treatment condition were run on 1.5% agarose gel electrophoresis.

In Figure 11 (A), distinct DNA ladders are observed in all three conditions, with visibly more laddering in the 100 rad and 400 rad treatments (lanes 2 and 3) compared to the unirradiated control (lane 1). Flow cytometric analysis of unirradiated SMNC revealed 3.2% of cells staining positively for Annexin V-FITC, compared with 20.3% and 34.5% of cells in 100 and 400 WBI SMNC, respectively (B).

3. Detection and Quantification of Apoptotic Events in Murine PBMC

To investigate the role of apoptosis in radiation-induced immune suppression, WBI-induced apoptosis was examined in more detail, at a variety of doses and times post-irradiation.

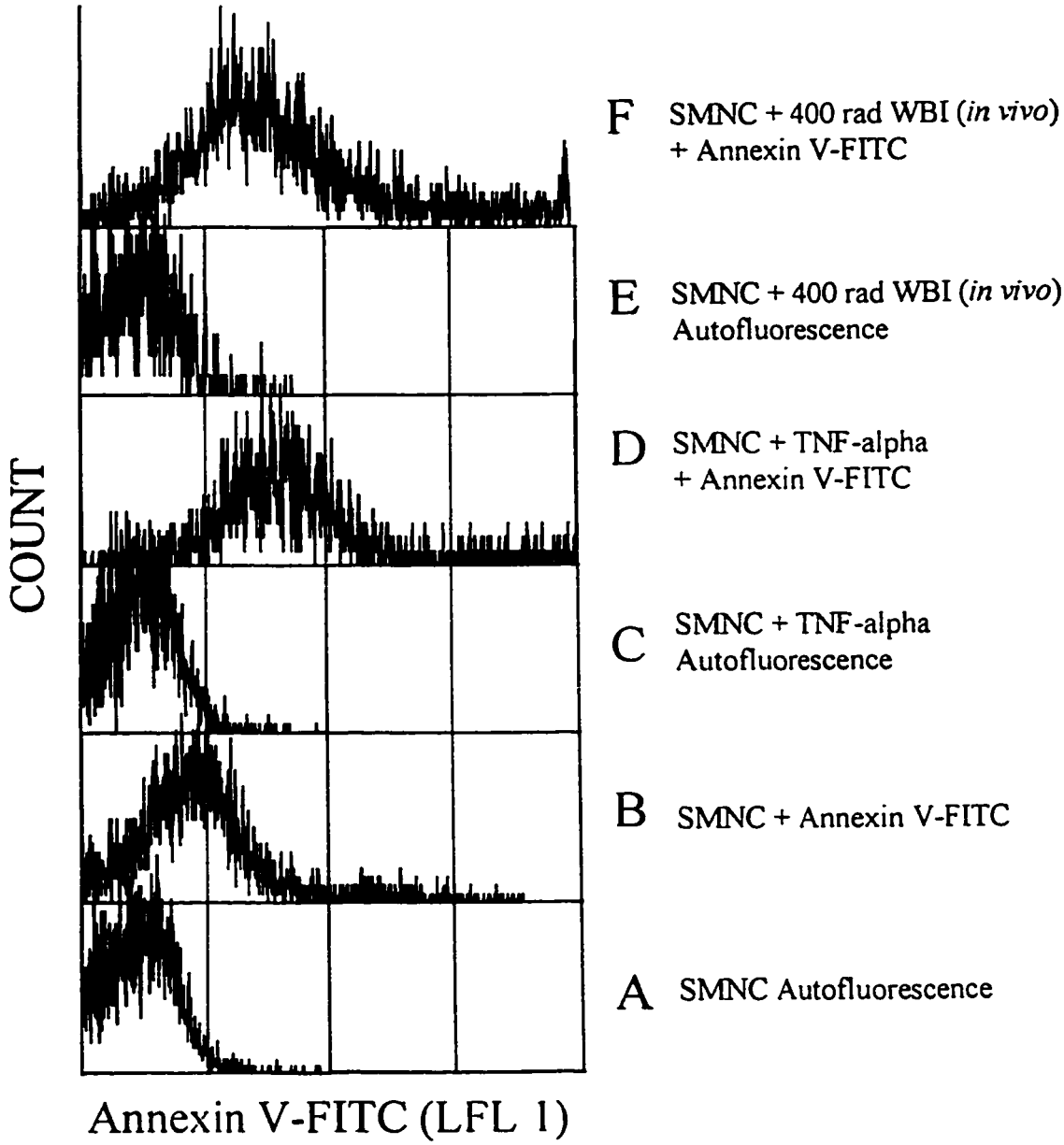

COUNT
Annexin V-FITC (LFL 1)
F SMNC + 400 rad WBI (*in vivo*) + Annexin V-FITC
E SMNC + 400 rad WBI (*in vivo*) Autofluorescence
D SMNC + TNF-alpha + Annexin V-FITC
C SMNC + TNF-alpha Autofluorescence
B SMNC + Annexin V-FITC
A SMNC Autofluorescence

FIGURE #9: ANNEXIN V STAINING OF SMNC TREATED WITH TNF-α OR WBI

SMNC harvested from an unirradiated mouse were cultured in the presence or absence of 2 ng/mL TNF-α for 3 hours prior to staining with Annexin V-FITC and/or PI, as described in Methods. (**A**) Autofluorescence control for untreated SMNC; (**B**) Annexin V-FITC staining of untreated SMNC; (**C**) Autofluorescence control for TNF-α-treated SMNC; (**D**) Annexin V-FITC staining of TNF-α-treated SMNC. Additionally, SMNC were harvested from a mouse 4 hours after *in vivo* γ-irradiation (400 rad), and analyzed by flow cytometry following staining with Annexin V-FITC£ (**F**). Autofluorescence control for SMNC from irradiated mouse (**E**).

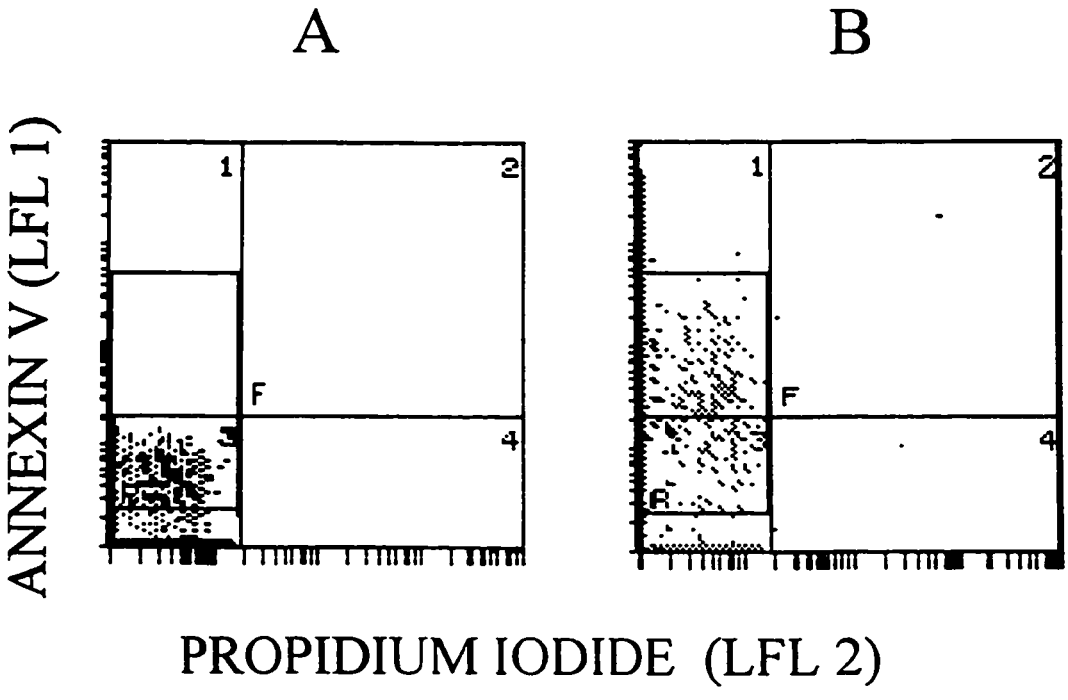

A
B
ANNEXIN V (LFL 1)
1
2
F
4
1
2
F
4
PROPIDIUM IODIDE (LFL 2)

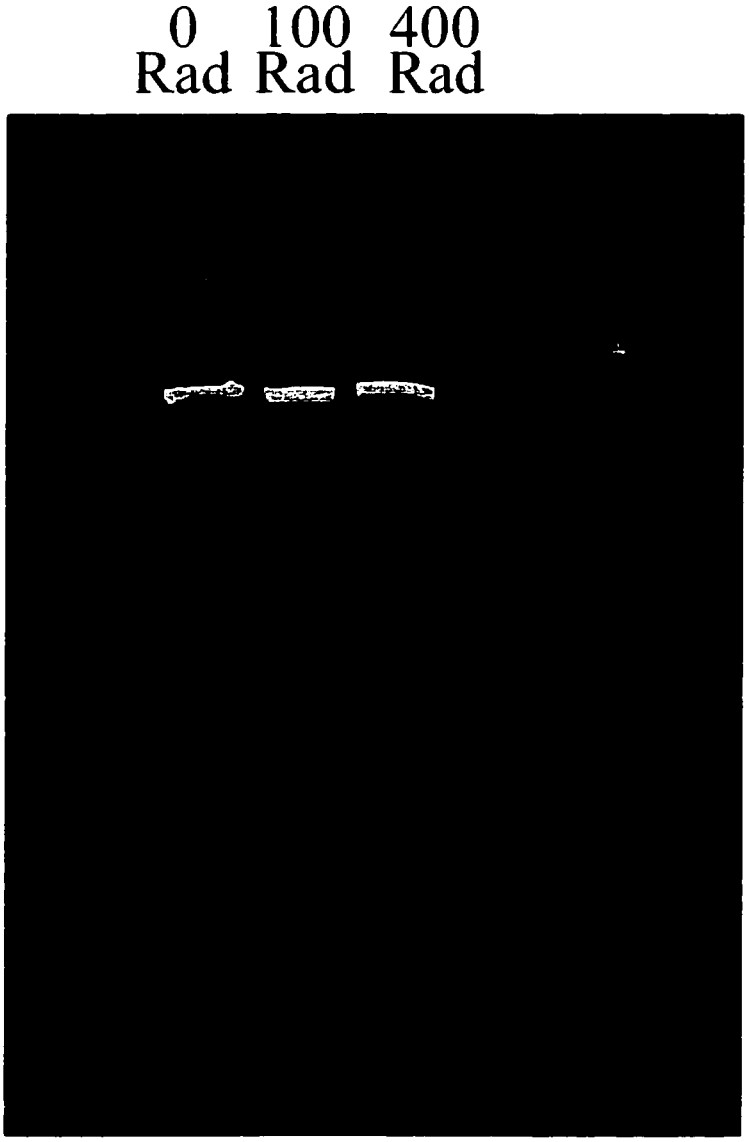
FIGURE #10: ANNEXIN V STAINING OF PERIPHERAL BLOOD MONONUCLEAR CELLS OF MICE IRRADIATED WITH 400 RAD

Single parameter flow cytometric analysis of PBMC stained with 0.5 μg Annexin V-FITC. (**A**) PBMC derived from unirradiated mice; (**B**) PBMC from 400 rad γ-WBI mice, 4 hours post-irradiation.

C57BL/6 mice were treated with 0, 25, 50, 75, 100, 200, or 400 rad WBI, and blood samples were collected 0, 1, 2, 4, 8, and 24 hours post, irradiation. Figure 12 demonstrates a general trend of increased Annexin V staining of murine PBMC with increasing dose, as depicted by a higher peak staining with higher doses. At 2 hours post-irradiation, there is no significant difference in the percent of PBMC staining positively for Annexin V within 25 to 75 rad or 100 to 400 rad exposure ranges. However, all doses produce significantly greater staining than the unirradiated control, and high dose exposures (100, 400 rad) induce apoptosis in more PBMC than lower doses (25-75 rad) ($p<0.05$). Of note is the significant increase in early apoptosis detection in murine PBMC at a dose as low as 25 rad (approximately 25% of PBMC are Annexin V-FITC positive 1 hour post-irradiation).

The same data are presented in Figure 13 to show progression with time for any given dose. Here, it is clear that apoptosis in PBMC of irradiated mice can be detected very rapidly (2 to 4 hours post-irradiation at any of the doses tested). As dose increases, peak Annexin V-FITC staining increases and/or occurs sooner post-irradiation. For example, 4 hours after 75 rad, roughly 30% of PBMC are stained with Annexin V-FITC, whereas 2 hours after 400 rad WBI, a peak of approximately 48% Annexin V positive PBMC is reached. A rapid decline in detection of apoptotic events in murine PBMC is revealed after 8 hours post-irradiation, regardless of dose, ultimately reaching levels near background (0 rad) after 24 hours. This is accompanied by an increase in the appearance of the atypical cells of reduced viability and increased granularity, as detected in the early

A



B

	0 Rad	100 Rad	400 Rad
% Annexin + Cells	3.2	20.3	34.5

FIGURE #11: DNA LADDERING IN MURINE SMNC IS INDICATIVE OF APOPTOSIS

SMNC were harvested from mice 4 hours after WBI of 0, 100, or 400 rad, and fragmented DNA was extracted and precipitated. (**A**) 3 μg of DNA from each SMNC sample were separated electrophoretically on 1.5% agarose gel: (lane 1) unirradiated; (lane 2) 100 rad; and (lane 3) 400 rad. SMNC from 0, 100, or 400 rad WBI mice were stained with Annexin V, FITC and analyzed by flow cytometry, prior to DNA extraction. (**B**) Percent of total SMNC staining positively for Annexin V in each radiation treatment are reported.

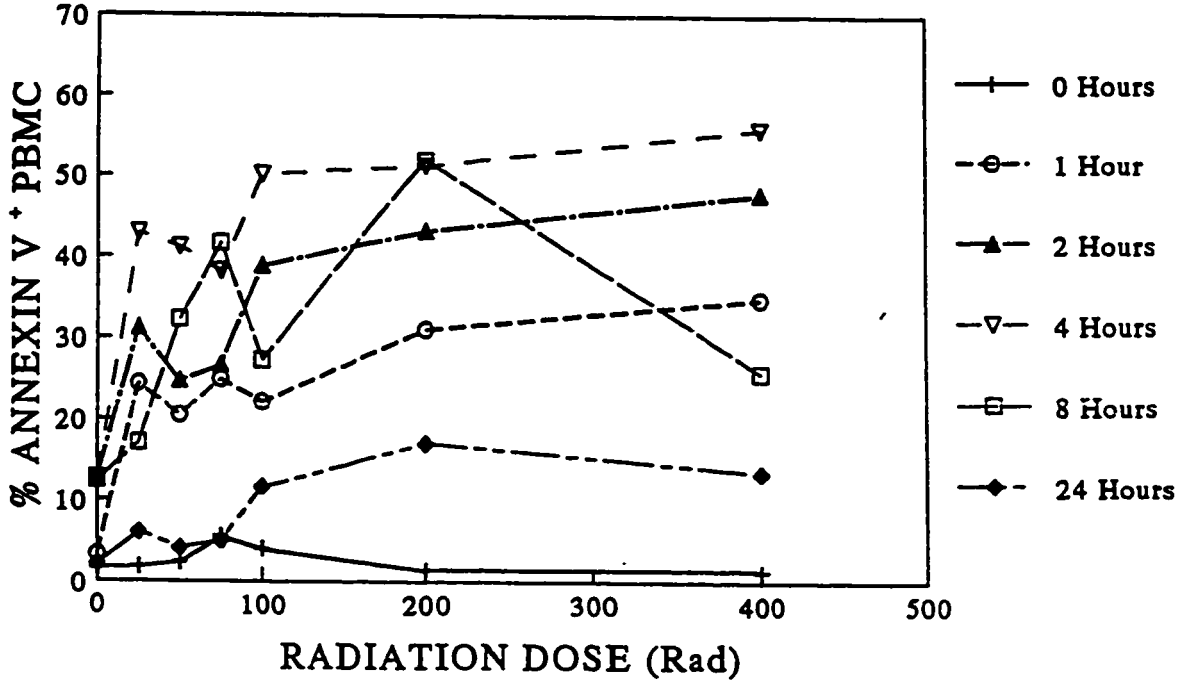

70
60
50
40
30
20
10
0
% ANNEXIN V + PBMC
0
100
200
300
400
500
RADIATION DOSE (Rad)
0 Hours
1 Hour
2 Hours
4 Hours
8 Hours
24 Hours

FIGURE #12: APOPTOSIS DETECTION BY ANNEXIN V IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF IRRADIATED MICE

PBMC were harvested 1, 2, 4, 8, or 24 hours following 0, 25, 50, 75, 100, 200, or 400 rad WBI. Samples were stained with Annexin V-FITC and propidium iodide, and the percent of PBMC staining positively for Annexin V alone was determined by flow cytometry. Each point represents the mean percent Annexin V-positive PBMC (n=5). Error bars were eliminated to simplify presentation of the data.

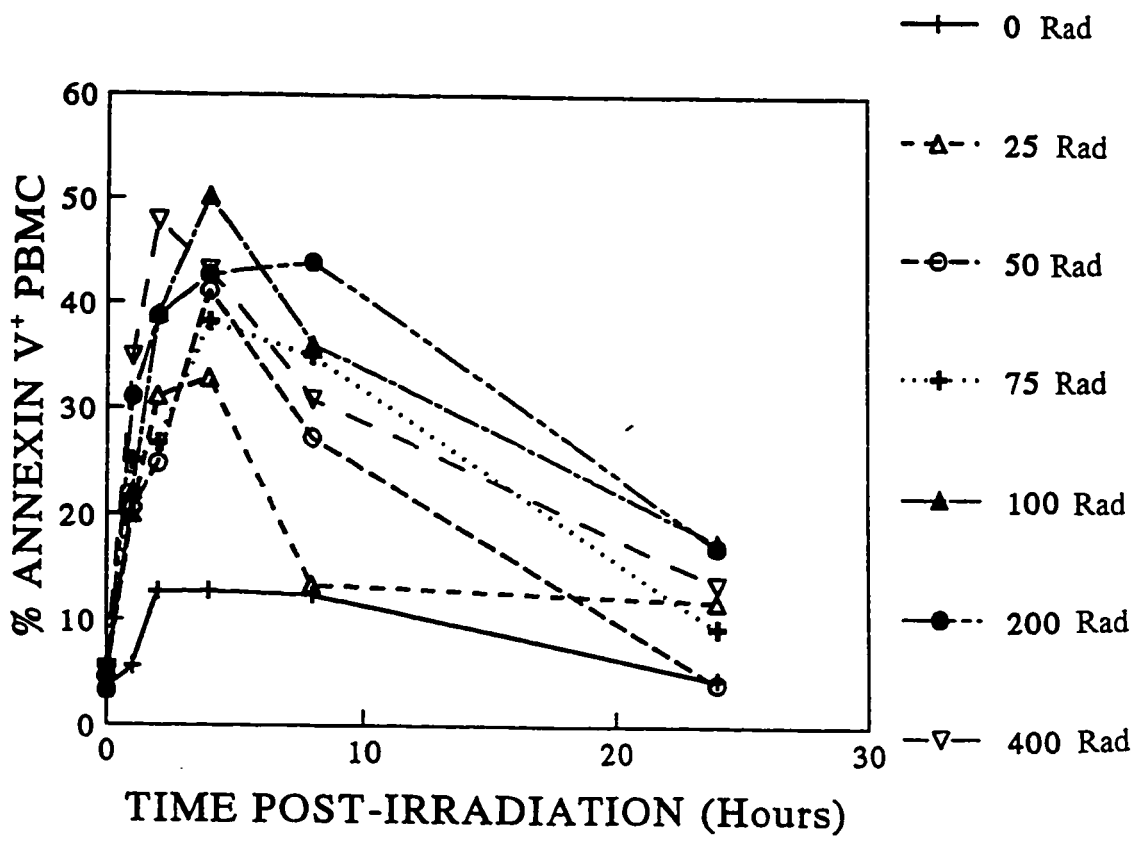

0 Rad
25 Rad
50 Rad
75 Rad
100 Rad
200 Rad
400 Rad
% ANNEXIN V+ PBMC
60
50
40
30
20
10
0
0
10
20
30
TIME POST-IRRADIATION (Hours)

FIGURE #13: KINETICS OF APOPTOSIS INDUCTION BY γ-WBI IN MURINE PERIPHRAL BLOOD MONONUCLEAR CELLS

PBMC were harvested from mice treated as in Figure 12 and Methods. Data reflect changes in the proportion of PBMC staining positively for Annexin V over 24 hours following γ-WBI. Each point represents the mean percent Annexin V positive PBMC, n=5. Error bars were eliminated to simplify presentation of the data.

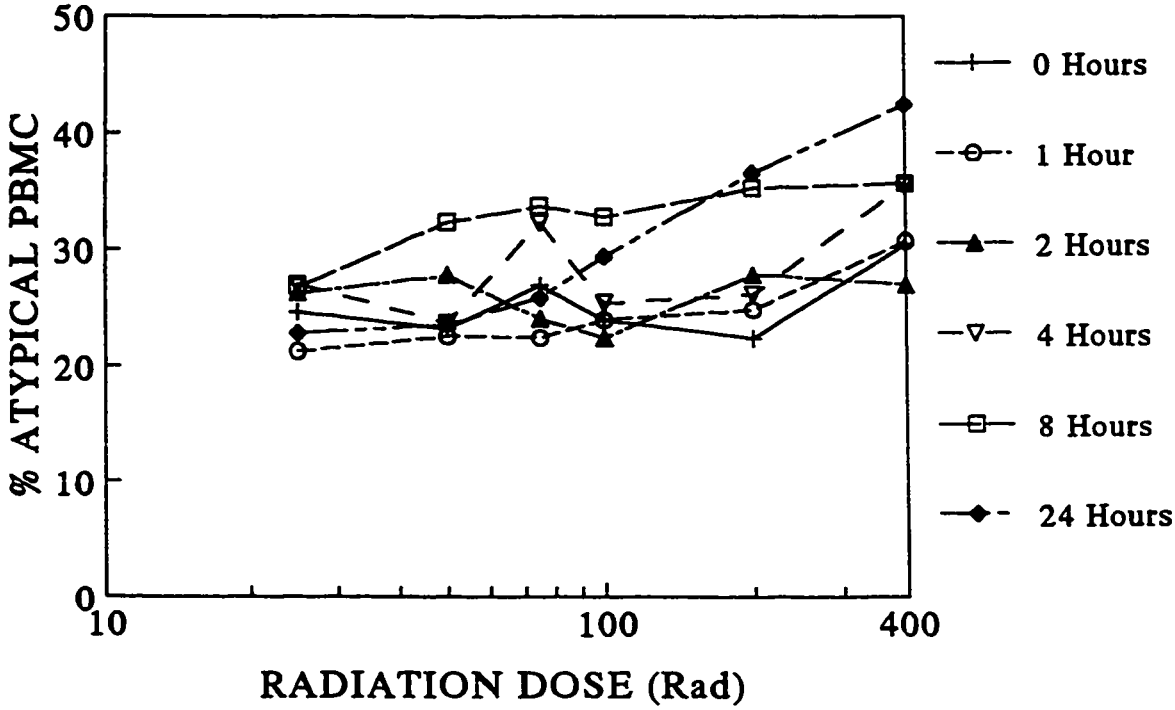

50
40
30
20
10
0
% ATYPICAL PBMC
10
100
400
RADIATION DOSE (Rad)
0 Hours
1 Hour
2 Hours
4 Hours
8 Hours
24 Hours

FIGURE #14: PERIPHERAL BLOOD MONONUCLEAR CELLS WITH ATYPICAL MORPHOLOGY FOLLOWING WBI

PBMC were harvested from mice treated as in Figures 12 and 13, and as described in Methods. Atypically granular blood cells were quantified from forward scatter versus side scatter representations of this population, by flow cytometry. Each point represents the percent of cells in each sample which have increased granularity and reduced viability as previously described in Figure 5 and Table 3. Error bars were eliminated to simplify presentation of the data.

investigations into altered proportions of PBMC populations post-irradiation. In Figure 14, a significant increase ($p<0.05$) in the prevalence of this population can only be observed 24 hours after radiation exposures of 100, 200, or 400 rad WBI.

F. NATURAL KILLER CELL CYTOTOXICITY

The profound augmentation in the relative proportion of NK cells occurring in the PBMC (and in SMNC, in previous studies by Harrington) prompted an investigation into the cytolytic potential of these apparently radioresistant cells {1, 20, 95}. Lysis of YAC-1 sensitive NK targets by murine NK cells in these following experiments was specific, since lysis of P815 NK, resistant targets was approximately 50 % less than that of YAC-1 NK-sensitive cells (Figure 15), as predicted in the literature {75}.

Splenic mononuclear cells from C57BL/6 mice irradiated with a single exposure of 0, 25, 50, 100, 200, or 400 rad WBI were harvested 1, 4, or 7 days post-irradiation. SMNC were serially diluted and added to YAC-1 murine lymphoma cells (NK-susceptible targets) pre-labelled with $DiOC_{18}(3)$ at effector to target ratios of 50:1, 25:1, 12.5:1, 6.25:1, and 3.125:1. Following a 4 hour incubation at 37 °C, PI was added and samples were analyzed by flow cytometry. A sample of effectors (SMNC) at each dose during each experiment was stained with NK-1.1 PE monoclonal antibody and combined with Coulter Flow Count Fluorospheres to determine the concentration of NK cells within the effector population for each condition. The cytolytic potential of NK cells at each time after each dose of radiation is indicated in lytic units: cytolytic potential is directly proportional to lytic units. In the results shown in Figures 16A to C, changes in

the number of lytic units for each dose are expressed as a percentage of the unirradiated control. Lytic units were calculated after correction of the effector to target ratios used for the actual number of NK cells in each sample (since the proportion of NK cells within SMNC changes post-irradiation).

24 hours post-irradiation (Figure 16A), 25 rad WBI resulted in depressed cytolytic capacity of NK cells in the spleen in all four experiments depicted, to levels near or below 50% of the potency of the unirradiated controls. Doses of 100 rad or more resulted in depression of cytolytic activity in 2 experiments (3 and 4) and little change in cytotoxicity in experiments 1 and 2.

Figure 16B summarizes the results of cytotoxicity experiments conducted 4 days after radiation exposure. Doses below 100 rad enhanced cytolytic activity of NK cells in the spleen against YAC-1 targets in three of four experiments (#1, 2, and 4). In one experiment (#3), a 200 rad exposure enhanced cytolytic activity relative to the unirradiated control, but otherwise, doses $\geq$ 100 rad suppressed cytotoxicity in the remaining three experiments. Seven days post-irradiation (Figure 16C), the cytolytic potential of spleen-derived NK cells against YAC-1 was augmented by a dose of 400 rad. Doses of 50 to 100 rad resulted in no appreciable difference in the cytotoxic activity of NK cells, while a 200 rad exposure depressed NK function.

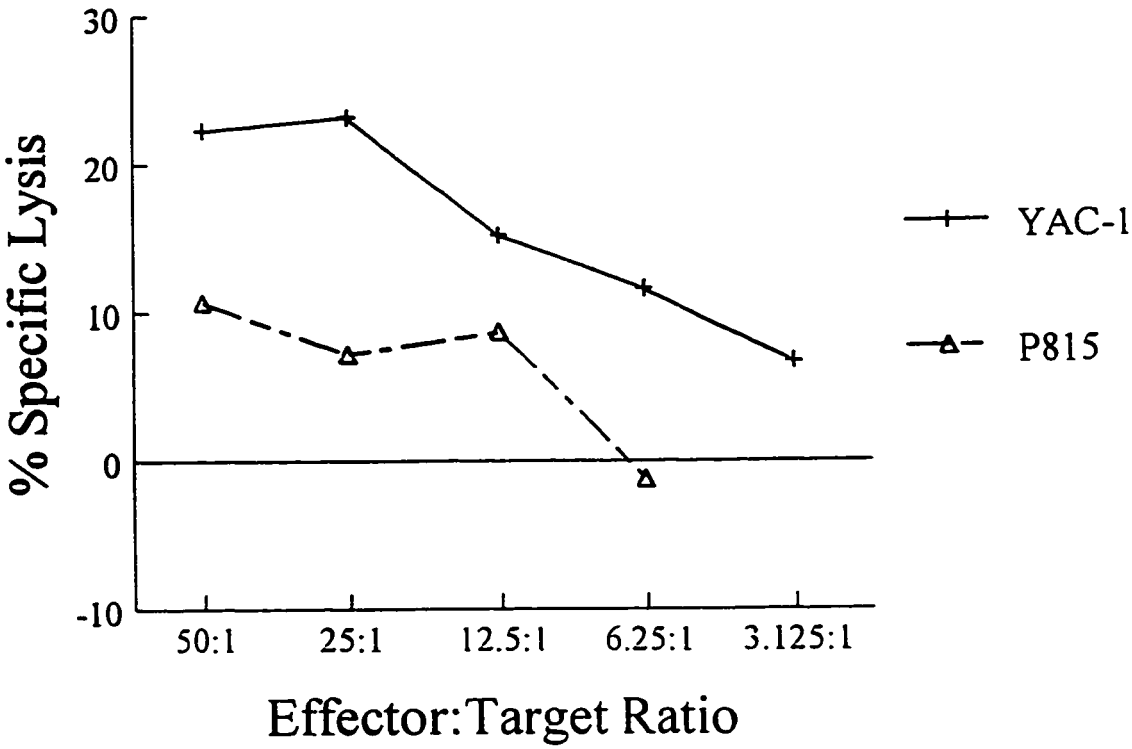
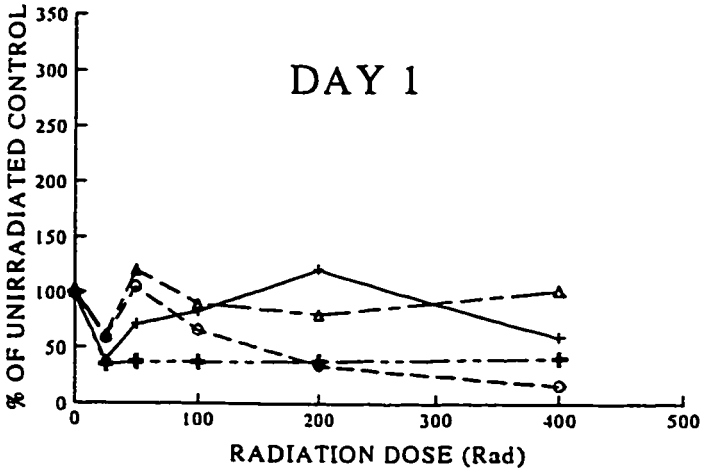

30
20
10
0
-10
% Specific Lysis
50:1
25:1
12.5:1
6.25:1
3.125:1
Effector:Target Ratio
YAC-1
P815

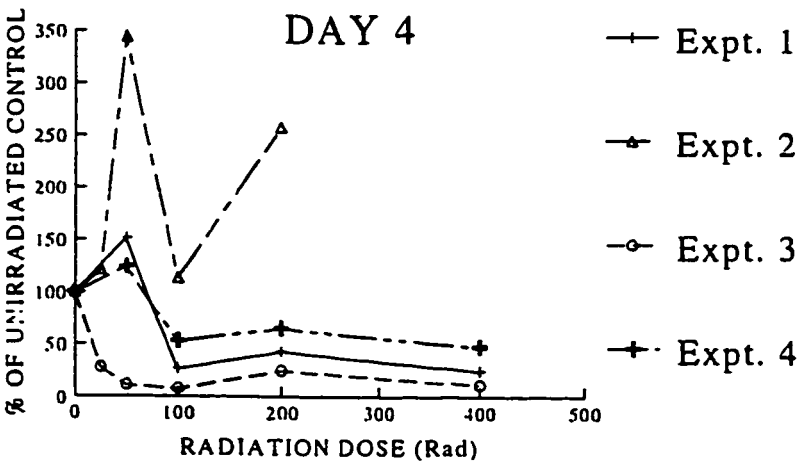
FIGURE #15: SPECIFICITY OF MURINE NK CELLS FOR YAC-1 TARGETS

Comparison of NK-mediated cytotoxicity against YAC-1 and P815 targets. One C57BL/6 mouse was sacrificed, and SMNC were co-cultured with $DiOC_{18}(3)$-labelled NK-sensitive YAC-1 target cells or P815 NK-resistant cells for 4 hours at effector to target ratios of 50:1, 25:1, 12.5:1, 6.25:1, and 3.125:1. Percent specific lysis of each target population at each E:T ratio was determined by flow cytometric analysis. At the effector:target ratio 6.25:1, lysis of P815 cells by NK cells was actually lower than the spontaneous lysis control.

A


DAY 1
% OF UNIRRADIATED CONTROL
0
50
100
150
200
250
300
350
0
100
200
300
400
500
RADIATION DOSE (Rad)

B


DAY 4
% OF UNIRRADIATED CONTROL
0
50
100
150
200
250
300
350
0
100
200
300
400
500
RADIATION DOSE (Rad)
Expt. 1
Expt. 2
Expt. 3
Expt. 4

C

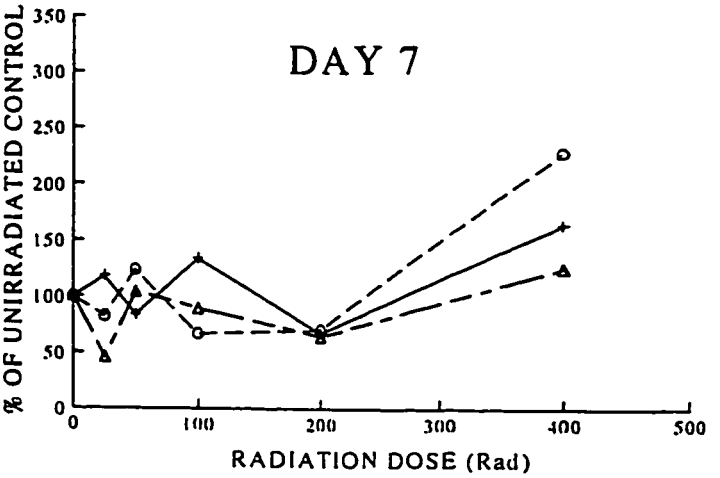

DAY 7
% OF UNIRRADIATED CONTROL
0
50
100
150
200
250
300
350
0
100
200
300
400
500
RADIATION DOSE (Rad)

FIGURE #16: CYTOLYTIC POTENTIAL OF NATURAL KILLER CELLS FOLLOWING WBI

C57BL/6 mice irradiated with 0, 25, 50, 100, 200, or 400 rad WBI were sacrificed, and SMNC were co-cultured with $DiOC_{18}(3)$, labelled YAC-1 target cells at effector to target ratios of 50:1, 25:1, 12.5:1, 6.25:1, and 3.125:1. Percent specific lysis was determined at each ratio by flow cytometric analysis, and converted to lytic units, corrected for the number of NK cells in the effector population. The results of 4 individual experiments are shown for days 1 and 4, and 3 experiments for day 7. Cytotoxicity of murine NK cells against YAC-1 targets: (A) 1 day post-irradiation; (B) 4 days post-irradiation; and (C) 7 days post-irradiation.

VIII. DISCUSSION

Exposure to ionizing radiation, whether accidental or for therapeutic purposes, can lead to severe immune suppression and resultant opportunistic infections and disease. Much of the suppression can be attributed to cell damage or death caused directly by the irradiation, by fragmentation of polypeptide chains, destruction of amino acids, and especially by damaging nucleic acids {3, 5}. Nucleic acids are exquisitely sensitive to radiation damage including single or double strand breaks, or formation of purine dimers, and this sensitivity results in inhibition of DNA synthesis and cell replication. In - addition, even relatively low doses of radiation can cause mitotic or reproductive cell death by inhibiting the ability to undergo rapid and unlimited cellular proliferation, while at high doses, radiation causes the cessation of cellular metabolism and the disintegration of the cell {3, 5}. In addition to leukocytes, the young, rapidly proliferating and undifferentiated stem cells of the bone marrow are highly susceptible to radiation injury, thereby hindering the regeneration of circulating leukocytes and repopulation of the immune system to counteract the cytoreduction caused by the initial exposure.

Aside from the contribution of cell death to reduction in the numbers of immune-competent cells available to meet antigenic challenge, radiation-induced impairment of leukocyte migration and homing may play a role in immune incompetency post-irradiation {36}. Whether by inflicting damage or loss of critical membrane-bound adhesion molecules, or damage to endothelia with which lymphocytes must interact in order to migrate from one compartment to another, disruption in lymphocyte migration may lead to inadequate distribution of immune-competent cells.

In addition to an overall reduction in leukocytes, the immune system is further down-regulated such that surviving leukocytes are less responsive to antigenic challenge. Therefore, the immunosuppression observed following radiation exposure cannot be attributed solely to cell loss or inappropriate distribution {20, 45}. Several mechanisms, albeit poorly understood, may act alone or in combination to produce this suppressed state following radiation exposure. Effective immune function is largely dependent on interactions among the different cells of the immune system. Imbalance in the proportions of interacting populations or loss of effector function by a critical cell may down-regulate these functions. Radiation exposure may alter the cytokine profile of the microenvironment indirectly by affecting the numbers of cytokine-producing cells, or directly by inducing changes at the molecular level which in turn also affect cytokine production {50}. Immune dysregulation, generation of an immunosuppressive cytokine milieu, or the generation of antigen-specific suppressor cells further downregulate the function of lymphoid cells which survived the initial radiation insult {20, 64, 65}. Whether one or all of these mechanisms are involved in radiation-induced immune suppression, the net result is at least debilitating, and possibly lethal. The major concern following radiation injury is opportunistic infection by pathogens such as *Escherichia coli* or *Staphylococcus aureus*{6}, unless the dose and damage sustained are so severe that other systems (ie. gastrointestinal) are irreparably injured, leading to earlier potentially lethal developments.

Considering the severe, detrimental impact of radiation exposure on host defence, elucidation of mechanisms underlying immunosuppression would be of tremendous benefit in developing strategies to counteract that suppression and improve host survival

against the threat of opportunistic infections. The purpose of this current investigation has been to contribute to the understanding of some of the proposed mechanisms of radiation, induced immune deficiency. This study was limited to a murine model to allow control of the magnitude and rate of radiation exposure, and to reduce certain psychological, emotional, or academic stresses which may alter results of immunological investigation in a human trial. Evidence of imbalance among key mononuclear cell populations in the blood or spleen in various animal models {1, 20, 21} and human models {88-92} has been reported, and therefore, post-irradiation immune dysregulation on the basis of alterations among interacting subpopulations is a likely mechanism. The cause of these alterations is unknown, although two alternatives may be proposed. Mononuclear cells of the immune system may differ in their susceptibility to radiation induced elimination, or certain cell types may be mobilized from lymphoid compartments to the periphery to a greater extent than others, leading to the observation of altered proportions among cells when one compartment is sampled. This current work attempted to quantify alterations in peripheral blood mononuclear cells, and to correlate those changes with previously observed alterations among splenic mononuclear cells {1}, in an effort to determine the cause, relevance, and consistency of the observed alterations.

In addition to investigating the root of alterations among cell populations which may in turn promote dysregulation of the immune system, I also investigated the role of apoptosis in radiation-induced cell loss. Cells of the immune system are extremely sensitive to radiation injury and death. Susceptibility to radiation damage has been well characterized in intermediate to high-dose radiation exposures (>100 rad), although the extent of injury imparted to lymphoid cells by low-level irradiation is under much

investigation currently. Low-dose studies are particularly relevant in assessing risks of low level, chronic radiation exposure which may be encountered in a occupational radiation environment, or where there has been accidental dispersal of radioactive material. Measurement of apoptosis induction in blood MNC, using flow cytometry and Annexin V-FITC labelling of cells undergoing early stages of apoptosis, may be a sensitive assay for detecting early radiation injury which may be more subtle at low dose levels than cell loss and altered populations occurring at intermediate to high doses.

Finally, the effect of radiation exposure on the cytotoxic activity of natural killer (NK) cells was investigated, to further explore the impact of radiation on functional immune competence. Given the reported resistance of NK cells to radiation-induced death {1, 20}, and the role of NK cells in anti-tumor and anti-viral immunity{69, 78}, this particular immune function was of great interest. As a result of this work, future studies will likely expand the scope of investigation into other specific immune functions affected by radiation.

A. ALTERATIONS IN MURINE PERIPHERAL BLOOD MONONUCLEAR CELL POPULATIONS AFTER IRRADIATION

Previous investigations had revealed differential radiosensitivity of various splenic mononuclear cell (SMNC) populations in mice; specifically, there was a 4-fold increase in NK cells after 400 rad γ-whole body irradiation, a dramatic decline in B cells, and a two-fold increase in CD4+ T lymphocytes in the SMNC population {1}. These altered proportions occurred in the face of a decline in the absolute number of all SMNC in a dose-dependent manner {1}.

The results show that although all PBMC declined in absolute number after WBI (Figure 2), an effect previously described in the literature {20, 93}, some cell types decreased faster than others, leading to marked alterations in the proportions of various blood MNC populations relative to their original proportions in the unirradiated control. NK cells increased markedly (3-fold on day 1 after 400 rad, and as high as 9-fold on day 4 after 700 rad), as shown in Figures 3D and 4. B cells declined dramatically (Figures 3C and 4) to as low as roughly 10% of their proportion in the unirradiated control, while CD4+ T cells showed a significant rise in their proportion, approximately 2-fold on day 4 after 700 rad (Figures 3A and 4). CD8+ T cells failed to show any such notable alterations post, irradiation (Figures 3B and 4).

Comparing the results obtained in this study of alterations in PBMC populations induced by radiation with Harrington's results in the SMNC study, it is clear that the same cell types in the different compartments (blood and spleen) follow similar trends, either increasing or decreasing in proportion relative to the other MNC in that compartment. If the proportion of B cells were declining dramatically in the spleen after irradiation, but were increasing in the blood, then net trafficking of B cells out of the spleen could explain the change in proportions relative to the unirradiated control. Since the cells seem to follow the same trends, whether in the spleen or in the blood, it can be concluded that directional trafficking does not apparently play a major role in the cellular imbalances observed by Harrington in his study of the spleen. Therefore, alterations in cellular balance among lymphoid cells occurring post-irradiation can be attributed to differential radiosensitivity among the various cell types.

B. DIFFERENTIAL RADIOSENSITIVITY OF MNC POPULATIONS

Several published reports {20, 94, 95} support the conclusion that cellular imbalances in the PBMC are due to differential sensitivity of the various cell types to ionizing radiation. A study of the radiation sensitivity of human NK cell activity revealed that low-dose irradiation (200-500 rad) *in vitro* resulted in an enhancement of activity {20, 95}, indicating that NK cells are relatively radioresistant, which could explain the relative increase in NK cell prevalence after irradiation. NK resistance to radiation, induced death is also supported in studies of NK activity in PBMC from human patients treated with total-lymphoid irradiation for Hodgkin's disease, which demonstrated an increase in both proportion and cytotoxicity of NK cells {59}. B cells have been shown to be extremely radiosensitive; they are very susceptible to interphase death (apoptosis) at low doses, with reduction of primary migration to lymphoid tissues and abolishment of secondary migration after only 100 rad {94}. T lymphocytes are also susceptible to interphase death, but death is much more protracted than in B cells, and activated T lymphocytes are more resistant to radiation damage {20}. Within the T lymphocytes, CD4+ T cells are more radioresistant than CD8+ T cells {20}. Overall, those cells that are more radioresistant will be expected to occupy a greater proportion of the total MNC population after irradiation than those that are radiosensitive (as observed), possibly influencing the immune response by altering the contributions of the various cell types to critical functional elements of the immune response.

C. DEXTRAN SULFATE MOBILIZATION

Dextran sulfate is a non-toxic, heparinoid polyanion capable of mobilizing stores of MNC, particularly lymphocytes, from reserves in the spleen and lymph nodes into the circulation via the thoracic duct. This compound has been employed in past studies to indirectly assess immune competence in mice post-irradiation, as indicated by the number of MNC available to be mobilized into the periphery. In this study, dextran sulfate was employed to assist in monitoring immune competence by quantifying the extent of mobilization of particular MNC types into the blood from other lymphoid and peripheral tissues. The presumption was that if there are more cells available for mobilization into the blood, that is directly related to the capacity of the system to mount an immune response.

Mice treated with γ-whole body irradiation (WBI) were surveyed for mobilization of MNC into the peripheral blood by subcutaneous administration of dextran sulfate (DS). Following 100 rad WBI exposure, DS increased the number of circulating B and T lymphocytes on days 1, 4, and 7 post-irradiation, to levels significantly greater than in unirradiated controls without dextran sulfate administration ($p<0.05$). However, treatment with DS did not increase peripheral B or T cell number at any time after 400 rad WBI, except for a significant improvement in peripheral T cell number 4 days post-irradiation, up to a control level, not significantly different from unirradiated, DS-untreated mice ($p<0.05$). This supports the uniformity of radiation-induced depletion of particular cell types throughout lymphoid tissues, concluded earlier because of the similarities in alterations of PBMC and SMNC. That is, radiation does not just deplete

cells from the blood and spleen, but also from other lymphoid and peripheral tissues, since significant mobilization of cells from these sources is not observed following 400 rad. The cells mobilized presumably represent the cells available for release into the circulation, a definite portion of the total cellularity in all lymphoid tissues. If the total population is depleted, proportionally fewer cells will be available.

Dextran sulfate demonstrated a greater capacity to mobilize T lymphocytes (~ 5-fold after 100 rad) over B lymphocytes (~ 2-3 fold after 100 rad), not accounted for by interference of dextran sulfate with monoclonal antibody detection (Table 4). This may reflect the differential radiosensitivity of these cells as described earlier in this report, and by many other authors {20, 21, 92}. Since T cells are more resistant to radiation injury than B cells, it is logical that more T lymphocytes will survive and be available for mobilization into the peripheral blood by dextran sulfate administration. T and B lymphocytes may not only differ in their susceptibility to radiation-induced death, but also to radiation-induced membrane damage, loss of surface glycoproteins, or other alterations in surface structures necessary for migration or adherence {21}. If the mobilizing function of dextran sulfate is dependent on cell surface markers, it is also possible that DS acts preferentially on T cells over B cells, if they differ in the level of expression of key markers involved. In any case, the observation of greater improvement in T cell numbers in the periphery over B cells post-irradiation is supported in the literature. Following 800 rad WBI, a greater number of T lymphocytes than B lymphocytes were mobilizable via thoracic duct cannulation {20}.

Arising from investigations into the effect of ionizing radiation on PBMC populations in mice, was the observation of a population of cells morphologically distinct from mononuclear cells (determined by flow cytometry) shown in Figures 5B (100 rad) and 5C (400 rad) compared to the unirradiated control (5A). This population seemed to have been the result of an increase in the granularity of blood MNC post-irradiation. The viability of these atypical cells was assessed by the Molecular Probes Live/Dead Cell Viability/Cytotoxicity assay. Viability in the atypical population was reduced post-irradiation relative to the blood MNC after the same exposure, and a very small difference in viability within the PBMC with or without radiation exposure (Table 3). This may indicate that radiation damage to cells produces increased granularity in those cells prior to death, and that those cells accumulate in the so, called atypical region of the forward scatter versus side scatter histogram, and disappear soon thereafter. This is consistent with apoptosis induction in some cells by ionizing radiation, as the subsequent increase in granularity among PBMC prior to death and engulfment by neighbouring phagocytes may be indicative of DNA fragmentation, characteristic of apoptotic cells. Moreover, the observation of cells staining positively for both Calcein-AM and ethidium homodimer, indicative of cells at some stage of the death process, is consistent with the notion of the atypical cell population being composed of some cells undergoing late stages of apoptosis. Other reports describe cells with this atypical morphology (increased granularity or side scatter flow cytometry profile) occurring post, irradiation, the entire population continuing on to death as determined by ethidium bromide uptake {96}. These observations, although originally unanticipated, highlight the importance of investigating the role of apoptosis in radiation injury to the immune system.

D. APOPTOSIS AND RADIATION INJURY

Apoptosis (programmed cell death) is an important process in the development and proper functioning of the immune system, among others. This process has numerous triggers, including membrane signals, physiological stimuli, physical injury, or chemical insults. Apoptosis comprises a series of specific, rapid, and distinct biochemical events culminating in engulfment of the cell by neighboring phagocytes and eventual death.

A new flow cytometry based method for detection of early events of apoptosis induction, specifically the loss of plasma membrane phospholipid asymmetry, has allowed rapid and convenient investigation of radiation-induced apoptosis in peripheral blood mononuclear cells of irradiated mice. Normally, phosphatidyl serine (PS) is localized to the cytoplasmic face of the plasma membrane. In cells undergoing apoptosis, PS is exposed to the outer surface in an active process involving an aminophospholipid translocase. In these investigations, apoptotic murine PBMC were identified following radiation exposure by detection of surface expression of PS using Annexin V-FITC.

1. Apoptosis Detection and Quantification in PBMC from Irradiated Mice

A low dose (25 rad) of ionizing radiation has a significant impact on the murine immune system at the level of induction of DNA damage. Although Figure 12 shows a significant increase in apoptotic events in PBMC following 25 rad relative to the unirradiated control, I was unable to discern any significant difference in apoptosis induction in the periphery for doses from 25 to 75 rad, or in the 100 to 400 rad range of exposures, at any timepoint. In part, this may be explained by the large amount of

variability among the results at each data-point, where the standard deviation ranges from 2.33 to 17.83 at the 2-hour time point, and from 4.92 to 12.54 at 4 hours post-irradiation. This wide range in standard deviations occurring in the data, where n=5, highlights the fact that individuals vary widely in their susceptibility to radiation injury {97, 98}. Although there is no statistically significant difference between the percent of PBMC which are Annexin V positive in the 25 to 75 rad dose range or in the 100 to 400 rad range, there is a significantly higher percentage of apoptotic PBMC in the high-dose range compared to the low-dose range ($p<0.05$). This general dose response coupled with the significant occurrence in apoptotic events above background in the peripheral blood following a very low dose of 25 rad, clearly demonstrates a role for apoptosis in radiation-induced injury to the immune system at low to intermediate doses.

2. Kinetics of Apoptosis Induction

For all doses tested, peak apoptosis detection occurs 2 to 4 hours post-irradiation, and this peak is significantly higher than at all other sample times, for all doses tested, except for the unirradiated control ($p<0.05$). Clearly, apoptosis induction in murine PBMC is a very rapid manifestation of radiation injury. Eight hours after single WBI exposures (25, 50, and 100 rad), apoptosis detection in PBMC declines significantly, and 24 hours after all doses, apoptosis detection is not significantly different from background levels ($p<0.05$). This is not a surprising observation, since phosphatidyl serine exposure on the surface of apoptotic cells is an early event, followed by engulfment of apoptotic cells by phagocytes and subsequent cell death {27}. This is further substantiated by the appearance of cells with atypical morphology in the

peripheral blood post-irradiation, as defined earlier. Again, by their increased granularity and reduced viability, they may represent cells at some later stage of the apoptotic process. It was only 24 hours after radiation exposures of 100, 200, or 400 rad WBI that a significant increase in the prevalence of this population could be observed ($p<0.05$). It was surprising that increases in the occurrence of this population after lower doses was not significant, although there are many factors at play which may explain this. Blood samples were harvested and monitored for apoptotic PBMC at times 0, 1, 2, 4, 8, and 24 hours post-irradiation. Considering that cells in later stages of apoptosis are sequestered by phagocytes, the 16-hour lapse between the 8 and 24-hour sample times may be too large, during which a significant rise in this atypical population may occur at other radiation exposures.

Aside from gaps in sampling, we must also consider the role of repair. As in many biological systems, a certain level of change for a given stimulus is often tolerated, but beyond this threshold, requisite mechanisms are called into action {99}. DNA damage resulting from radiation exposure elicits a cascade of events which may initiate apoptosis, or may activate DNA repair mechanisms {100}. Recent speculation regarding the activity of p53 suggests that this tumor suppressor acts to regulate the cell cycle, inducing the G1 checkpoint following radiation exposure to stabilize DNA damage and prevent progression through the cell cycle until the DNA repair machinery can act {100, 101}. Detection of DNA damage may involve a second protein, ATM (ataxia-telangiectasia mutated). which may be required for induction of p53 accumulation {100}. The decision whether to induce repair or initiate apoptosis may be related to the rate or magnitude of nuclear accumulation of p53 {100, 101}. It is possible that in our

investigations, low doses of radiation (25 to 75 rad) are enough to elicit DNA repair mechanisms, having induced DNA damage not in excess of the DNA repair capabilities of the cells. Radiation exposures above 100 rad may overwhelm DNA repair machinery trying to salvage the cells, resulting in rapid accumulation of nuclear p53 protein and the initiation of apoptosis {100}. This would explain the insignificant alterations in the appearance of the atypical PBMC population over 24 hours at doses below 100 rad. We must not assume that just because early signs of apoptosis (eg. loss of PS asymmetry) are evident following low-dose irradiation, that all these cells will necessarily progress to cell death. In fact, it is quite possible that a single, low dose (<100 rad) of ionizing radiation produces no significant acute, short-term health risks at all, lending support for further investigation into health risks of chronic low-dose exposures in both the short and long-terms following irradiation.

Many recent investigations have revealed that apoptosis can be induced by multiple pathways. Godar *et al* have recently described two pathways for apoptosis induction, triggered by ultraviolet radiation of different qualities (wavelengths), and exhibiting different kinetics for apoptosis induction {102, 103}. Immediate apoptosis was initiated by ultraviolet A radiation (320, 400 nm) and took place within four hours of exposure, while delayed apoptosis was initiated by ultraviolet B (290, 320 nm) and C (200, 290 nm) insults, initiating apoptosis more than 20 hours post-irradiation {102, 103}. In light of these observations, it is possible that in our scenario, low doses of γ-whole-body irradiation induce a delayed apoptosis mechanism, the completion of which might occur outside of the 24-hour sampling window, accounting for the lack of any significant increases in the incidence of atypically granular PBMC in the low-dose range.

E. NATURAL KILLER CELL CYTOTOXICITY

Due to the selective enrichment in NK cells in the peripheral blood of mice post-irradiation observed in earlier studies, and the importance of NK cells in anti-viral and anti-tumor defence, investigation of the cytotoxic capacity of these NK cells was indicated. Much of the literature in this area is conflicting; the number of exposures, dose of radiation, and type of radiation absorbed all play a role in determining alterations in cytotoxicity of NK cells. Literature regarding studies of the consequences of γ-whole-body irradiation on NK cell cytotoxicity function is lacking.

Results of this investigation into the impact of radiation exposure on NK cell function are depicted in Figures 16 A to 16 C. Overall, the impact of ionizing radiation on NK cytolytic potential is heterogeneous: on day 1, responses are depressed by radiation doses less than 50 rad, while by day 7, NK potency is diminished or unaffected by doses of 200 rad or lower, but augmented by 400 rad. Alterations in NK cytolytic capacity are not due to alterations in the proportion of NK cells in SMNC surviving radiation since calculation of lytic units had been corrected for the size of the surviving NK cell population.

Several literature reports investigating the impact of various dose ranges and qualities of radiation exposure on NK activity have reported heterogeneous responses by the NK population. Rana *et al* {78} reported a heterogeneous response to radiation injury by human natural killer cells elicited by *in vitro* γ-irradiation, attributed to an impact of the radiation exposure on lytic machinery. Mean radiation exposures of 918 +/ 149 rad in 80 to 100 rad fractions, given to allograft recipients conditioned with total lymphoid

irradiation (TLI), resulted in enhanced NK cytolytic activity {72}. This report was supported by the prolonged augmentation of NK activity by TLI observed by Weiss *et al* {79}, and the augmented toxicity by *in vitro* irradiation of PBMC in the dose range 100 to 1600 rad {104}. Alternatively, *in vitro* ultraviolet B irradiation suppressed NK cytotoxicity against susceptible targets {80-82}.

Aside from the differential impact of different types of radiation (ie. gamma versus ultraviolet, *in vivo* versus *in vitro*), investigators have demonstrated heterogeneity between the effects of low and high exposures of the same type of irradiation {70, 78}. A potential mode of radiation-induced enhancement of cytolytic function of NK cells may involve the selective enrichment of a radioresistant subpopulation of NK cells with increased cytotoxicity by increased binding and synthesis of cytotoxic granules. This was demonstrated in hepatic NK cells 16 hours after *in vitro* irradiation with 200 to 1200 rad {70}. Perhaps in our experiments, the overall depression in cytolytic capacity of NK cells from irradiated mice 24 hours after 25 to 400 rad WBI occurs because 24 hours is not enough time for the effects of radiation to be manifest as a significant increase in cytotoxicity by an increase in synthesis of cytotoxic granules. The augmented cytotoxicity 7 days after 400 rad WBI may occur because there has been a sufficient amount of time to produce a significant increase in cytolytic granule production.

The apparent resistance of NK cells to radiation-induced death does not necessarily translate into resistance to radiation injury altogether. Perhaps the moderate increase in cytotoxicity by NK cells after low doses of radiation in 3 of 4 experiments (by day 4) may reflect a transient activation of cytotoxic granule synthesis, while NK cells from mice given higher doses of radiation may exhibit depressed cytotoxicity on day 4

because cells are still contending with radiation-induced damage to the cell. At 7 days post-exposure, the consistent pattern of depressed cytotoxicity at low doses (200 rad or less) and enhanced cytotoxicity at a higher dose (400 rad) clearly reflects the heterogeneity of radiation-induced impact on NK function. High doses augment cytotoxicity, possibly involving increased synthesis of cytotoxic granules by a mechanism as yet not understood, while low doses seem inadequate in enhancing cytotoxicity, and actually depress cytotoxic function of NK cells.

An additional proposed contributor to alterations in NK cytotoxic potential by radiation is cytokine dysregulation. Murine hepatic NK cells irradiated with 200 to 1200 rad *in vitro* produce IL-1, IL-2, IL-4, IL-5 and TNF-α, which may serve as a potent autocrine system to increase cytolytic activity post-irradiation {70}. IFN-γ is another potent inducer of NK activity {69}. The differential effects of low and high dose irradiation observed herein may reflect differential effects of low and high dose irradiation on cytokine secretion by NK or other cells surviving irradiation. Future investigation about potential cytokine alterations after low and intermediate doses of radiation may shed light on the depression of NK cytotoxicity by low dose irradiation, and enhancement by high dose exposure.

IX. SUMMARY AND CONCLUSIONS

Following exposure to whole-body ionizing radiation, the host immune system may be severely impaired (depending on dose), rendering the individual susceptible to opportunistic infection and disease. Many mechanisms have been proposed, acting alone or in concert, to produce the profound immune suppression following radiation exposure. Understanding the underlying mechanisms may reveal avenues for intervention, to minimize the initial damage, to enhance repair, or to improve host immune function following a radiation insult. The purpose of this work has been to contribute to the understanding of some aspects of immune suppression induced by radiation. The findings of this work are summarized in Figure 17.

The results of these investigations have demonstrated that alterations in the relative proportions of mononuclear cell populations previously observed in the spleens of mice are due to the differential radiosensitivity of the different cell types. Observation of similar patterns of fluctuation in mononuclear cells of the peripheral blood at the same times following the same doses of radiation eliminated a potential role of differential trafficking of MNC types between the spleen and blood, or other lymphoid compartments. The observed alterations among MNC populations support a role for radiation-induced immune dysregulation, resulting from altered cell-cell interactions, or alterations in the cytokine profile of the internal environment. Whether the altered proportions of MNC observed have an impact on immune regulation remains to be seen.

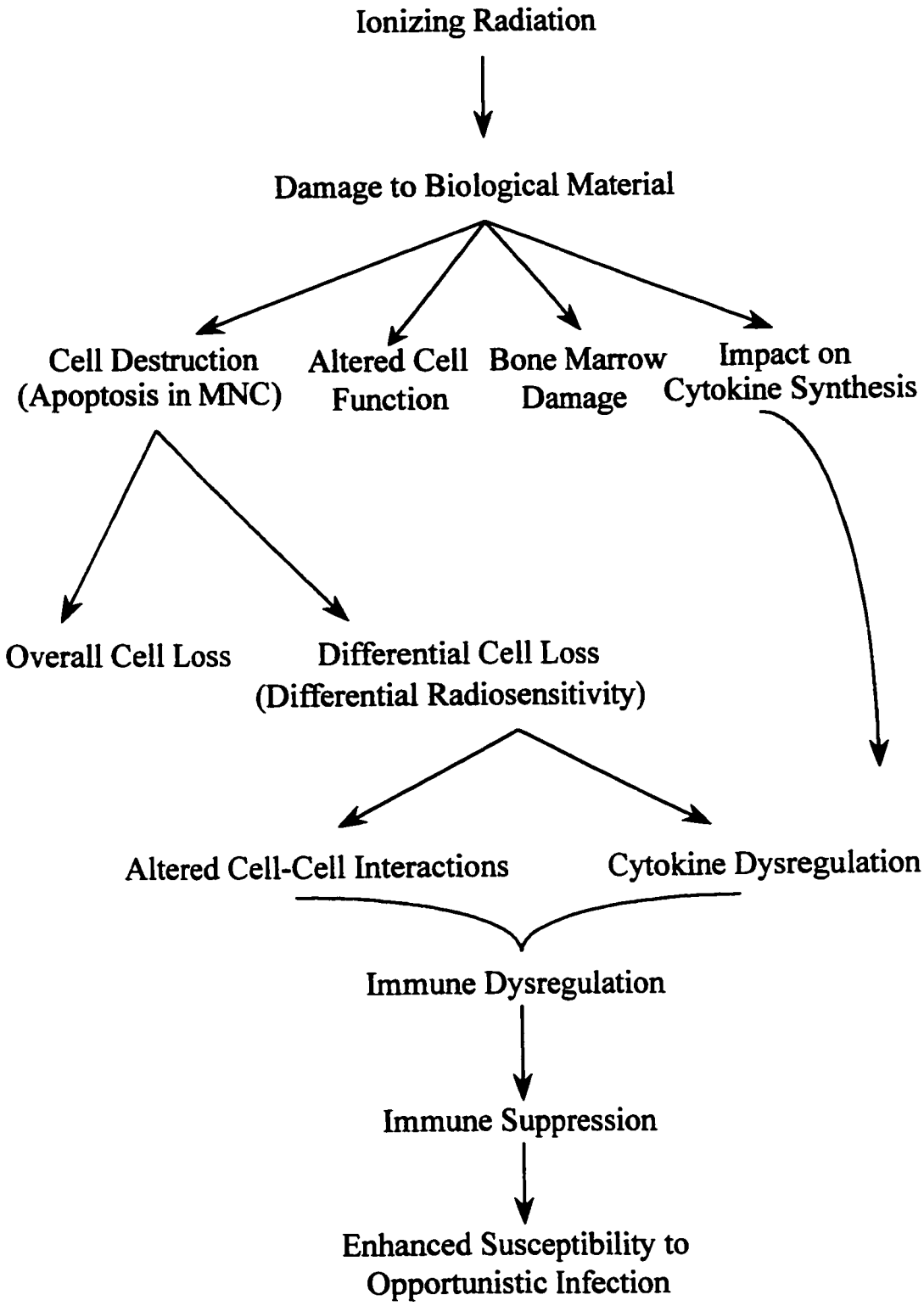

Ionizing Radiation
Damage to Biological Material
Cell Destruction (Apoptosis in MNC)
Altered Cell Function
Bone Marrow Damage
Impact on Cytokine Synthesis
Overall Cell Loss
Differential Cell Loss (Differential Radiosensitivity)
Altered Cell-Cell Interactions
Cytokine Dysregulation
Immune Dysregulation
Immune Suppression
Enhanced Susceptibility to Opportunistic Infection

FIGURE #17: CONTRIBUTION OF THIS WORK TO A MODEL OF RADIATION-INDUCED IMMUNOSUPPRESSION

Ionizing radiation may elicit a variety of effects on the immune system, which may in turn contribute to depressed immune responses to antigenic challenge observed post-irradiation. Radiation damages biological material, including proteins and nucleic acids, and influences their synthesis and regulation. Much of the suppression can be attributed to cell destruction. Peripheral blood mononuclear cells (PBMC) exhibited a dose-dependent decline in absolute number. Dextran sulfate mobilization of MNC into the peripheral blood from lymphoid tissues revealed that cytoreduction occurs in a dose-responsive manner throughout the lymphoid tissues. This overall cytoreduction of the immune system limits the availability of immune-competent cells for responding to antigenic challenge. Apoptosis plays a key role in imparting radiation injury to the immune system, even at doses as low as 25 rad γ-WBI. Due to differential radiosensitivity of different cell types, alterations in the cellular balance among MNC populations were observed. These alterations (↑↑ NK, ↑ CD4+ T lymphocytes, ↓↓ B lymphocytes) reflected similar changes observed in splenic MNC populations {1}. Cellular imbalances may affect cell-cell interactions, or alter synthesis and release of cytokines. Additionally, radiation has an impact on cytokine synthesis directly at the molecular level, or may alter effector function of particular cells. Altered cell-cell interactions and/or altered cytokine patterns may result in dysregulated immune responses, influencing the context in which an immune response may occur, thereby altering the outcome. In this manner, radiation may potentially impact hematopoiesis, NK cytotoxicity and other immune cell functions, and may promote generation of suppressor cells and/or an environment favorable to immunological tolerance to antigens. Ultimately, immune responses are hindered, and susceptibility of the host to opportunistic infection and disease is enhanced.

This investigation has also confirmed a role for apoptosis as a mechanism responsible, in part, for the cytoreduction of the immune system observed post-irradiation. Even at doses as low as 25 rad, apoptosis was detected within 2 hours following radiation exposure, being significantly greater than in the unirradiated controls. Not all of the cells exhibiting the early signs of apoptosis were necessarily eliminated. DNA repair may have occurred in some MNC following low doses of radiation early enough to prevent death of some cells. Regardless, detection of early events of apoptosis (loss of plasma membrane phosphatidyl serine asymmetry) is indicative of the occurrence of significant DNA damage even after low doses. This may indirectly support cytokine dysregulation following radiation exposure, since DNA damage has been implicated as a mechanism for triggering production of cytokines, such as IL-10, which are immunosuppressive.

The impact of ionizing radiation on functional immunity was studied by monitoring cytotoxicity by NK cells. Of note was a general depression in the cytolytic potential of NK cells against YAC-1 murine NK-susceptible targets by WBI 24 hours post-irradiation, and an enhancement in cytotoxicity by day 7 after 400 rad, while cytotoxicity was depressed by lower doses. The dichotomy observed between low and high dose effects on NK cytotoxicity may be secondary to differential effects on cytokine production by NK or other mononuclear cells after low and high dose exposures, which may, in turn, differentially influence NK activity. Alternatively, low doses may not be sufficient to activate mechanisms for increasing production of cytotoxic granules (which may occur at higher doses), or other steps in the cytolytic process may be adversely affected, such as binding, recognition, and NK activation.

In conclusion, this study has succeeded in furthering our understanding of mechanisms which may play important roles in depression of immune responses post-irradiation. Specifically:

1. Alterations among interacting mononuclear cells occur in the blood and spleen of mice post-irradiation, and may promote immune dysregulation via altered cell-cell interactions or altered cytokine levels in the microenvironment.

2. Cell loss occurs in all lymphoid compartments, not just from the spleen and blood that have already been measured.

3. Apoptosis plays a role in radiation injury to the immune system, even at low doses, and can be detected by measuring phosphatidyl serine exposure to the external surface of apoptotic cells, using a new flow cytometry-based technique.

4. Natural killer cell cytolytic activity is impaired within 1 day following low-dose radiation exposure (25 rad), and enhanced by an intermediate dose (400 rad) one week after a single acute exposure.

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XI. APPENDIX

A. APPLICATIONS

1. Biological Indicators of Radiation Injury to the Immune System

Due to the health risks associated with radiation exposure, the differential radiosensitivity of individuals, and the combined injuries or stresses that often occur in conjunction with radiation exposure, a method for the early detection of radiation injury on an individual basis would be desirable {97, 98}. Monitoring peripheral blood mononuclear cells, both for changes in the proportions of MNC subpopulations or in apoptosis induction, may be very useful in assessing radiation injury to the immune system. Methods employed for investigating PBMC are easy, rapid, and can be conducted in a practical and relatively non-invasive manner, using a small blood sample. Radiation induces injuries which are manifest rapidly in PBMC; specifically, a rapid, dramatic decline in the B lymphocyte population, a marked rise in the NK population, and a significant increase in early apoptotic events in PBMC even with low doses of radiation. The changes in the relative proportions of B lymphocytes and NK cells in the surviving PBMC fraction were dose-responsive, and to some extent, so was the induction of apoptosis between the low dose range (25-75 rad) and the intermediate dose range (100-400 rad).

The substantial degree of variability in the investigation into apoptosis detection and quantification in murine PBMC post-irradiation further supports the requirement for biological dosimetry in assessing radiation injury. Clearly, individuals are differentially

susceptible to injury. Although the observed variability limits the use of blood apopotosis detection in assessing the dose an individual has received, the values measured may be a true reflection of damage to the individual and therefore, very useful for triage and prognosis, rather than having to rely on group responses to absorbed doses. Even though a cell receiving 25 rad may exhibit early signs of apoptosis, it may not proceed to death, yet the percentage of cells exhibiting these signs is still indicative of damage.

The experiments reported herein have focused on the impact of single, acute whole-body radiation exposures on the immune system. Future experiments should focus on the effect of various dose rates of exposure, and the effect of receiving a cumulative dose in small fractions. Much evidence suggests that low dose rates are less lethal to cells than high dose rates delivering the same overall exposure {104}. The lower rate of exposure likely induces DNA damage at a slower rate, and may allow affected cells greater opportunity to initiate repair mechanisms before the system is overwhelmed. Fractionated whole-body irradiation results in less extensive of radiation-induced toxicities and immunosuppressive effects compared to single-dose WBI {105}. If the fractions are very small, initial exposures may actually be adaptive in protecting a cell against DNA damage inflicted by subsequent higher exposures {105, 106}.

Investigation of low-level chronic radiation exposure would be highly applicable to real scenarios encountered in a radiation workplace or at the site of a radiation accident, and are of valid concern. For example, extensive studies into the effects of spaceflight on astronauts have revealed a general blunting of cell-mediated immune responses during missions {88}. Effects of the stresses imparted on the immune systems

of astronauts during spaceflight, including low-level ionizing radiation, encompass post-flight depression of blast-cell transformation in response to PHA, changes in cytokine production, alterations in numbers and proportions of immune-competent cells, altered NK cytotoxicity {88}, decreased mitogen-induced IL-2 production, and a reduction in T lymphocyte proliferation in graft-versus-host reactions {91}. Personnel in any type of radiation environment risk chronic low-level radiation exposure, and subsequent potential effects on health.

2. In Vitro Applications

Monitoring differential cell loss and apoptosis induction *in vitro* by the methods outlined may be of tremendous benefit in advancing other avenues of interest. Evidence in the literature supports a synergistic role for ionizing radiation in enhancing immune suppression in conjunction with antigenic challenge by particular infectious agents, although the mechanisms are unclear for the most part. Monitoring alterations in the proportions of immune-competent cells or apoptosis induction in those cells in the presence of suppressive antigen concomitant with radiation exposure *in vitro*, may aid in investigating underlying mechanisms of suppression. Of further interest is assessing the efficacy of radioprotective compounds. *In vitro* monitoring of apoptosis induction in immune-competent cells, or *in vivo* monitoring of PBMC in animals treated with radioprotectors prior to radiation exposure, may be a very useful tool in these investigations.

B. FUTURE AVENUES OF INVESTIGATION

Although this investigation has answered a few questions, many more have arisen, providing intriguing avenues for future investigation into mechanisms of radiation depression of host immunity.

1. Apoptosis in Specific Mononuclear Cell Types

By investigating early stages of apoptosis induction in PBMC, specifically the loss of plasma membrane phosphatidyl serine asymmetry, we have identified a role for apoptosis in radiation-induced immune suppression, even at low doses. However, the possibility exists that not all cells showing early manifestations of apoptosis necessarily progress to cell death; some cells may have been able to repair damage to DNA and thus avoid progressing to later stages of apoptosis, particularly those cells exposed to low doses of radiation. However, DNA damage itself has been implicated as a trigger for cytokine production {50, 51, 52, 107}. Investigating early events of apoptosis in particular mononuclear cell types in the peripheral blood (by Annexin V and flow cytometry) may implicate particular cells as contributing to an alteration of the cytokine makeup of the internal environment.

2. Lymphocyte Mobilization by Dextran Sulfate Post-Irradiation

Radiation injury to the immune system has debilitating and possibly lethal consequences. There are many proposed explanations for the immune suppression observed post-exposure including altered cytokine levels in the internal

microenvironment, altered cell-cell interactions due to differential radiosensitivity of various immune competent cells, and a lack of circulating lymphocytes.

Reduction in the number of circulating lymphocytes post-irradiation may result from overall cytoreduction of the immune system (damaged cells, expended cells), changes in expression of adhesion molecules necessary for migration {39}, or damage to the cell membrane, which may explain previous reports of decreased lymphocyte homing ability post-irradiation {36}. Whether one or several of these mechanisms are at play, the reduction in circulating lymphocytes is extremely detrimental. Any treatment regimen which may increase the number of lymphocytes available and circulating in the peripheral blood and lymphoid tissues following radiation exposure may be beneficial in warding off infection.

As demonstrated in this report, subcutaneous administration of dextran sulfate into mice exposed to a single radiation exposure of 100 rad (but not 400 rad) induced significant mobilization of T and B lymphocytes into the peripheral blood. Whether or not inducing recirculation of lymphocytes actually improves host survival remains to be seen. A next step in this avenue may be to monitor overall survival, and various aspects of functional immunity in irradiated mice treated with dextran sulfate, or other mobilizing agents for that matter, to assess whether any of these facets are improved. If so, then perhaps development of a treatment regimen involving intermittent dextran sulfate (or another mobilizing agent) administration to radiation patients may be indicated.

3. Natural Killer Cell Cytotoxicity

This investigation into the effect of *in vivo* exposure of mice to ionizing radiation

in the range 25 to 400 rad whole-body irradiation revealed a depression in NK activity by low doses and augmentation of NK activity by high doses one week after exposure. Considering the correlation of radiation exposure with tumor development in vivo {4} and the role of NK cells in antitumor defence, long-term studies into the impact of radiation on NK function (in a murine model or in human patients) are in order. Patients treated with total lymphoid irradiation (TLI) as a conditioning procedure for allograft transplantation, demonstrated increased NK cytotoxicity to supranormal levels 6 to 12 months after irradiation {72}. TLI treatment for patients with Hodgkin's disease resulted in long-term enhancement of cytotoxicity by NK cells {59}. In investigating human NK cytolytic potential, confounding effects of disease, such as malignancy and autoimmune diseases, must be taken into consideration. Studies of low-dose, chronic exposure to radiation and its impact on NK activity, such as may occur in a potentially hazardous radiation workplace, may reveal the risks associated with the relevant levels and conditions of exposure. Low-dose ultraviolet exposure has been demonstrated to suppress immunity to skin tumor development in an antigen-specific manner {107}, but a role for NK cells in this scenario has not been examined.

Investigation of the effect of radiation on NK surface glycoproteins essential for target binding and recognition, conjugate formation, and lytic granule synthesis and release, may further our understanding of radiation-induced alterations in NK function. *In vitro* irradiation of human NK cells mostly affected lytic function (granule production and release) in influencing NK cytotoxic potential {78}. Ultraviolet irradiation of human PBMC *in vitro* resulted in reduced lysis of bound targets and recycling of active NK cells, but binding of NK cells to targets was unaffected {81}. Following recognition and

binding of target cells, NK cells are activated to release cytotoxic granules {69}. Given that macromolecules may be adversely affected by ionizing radiation, this NK activation step may be affected by radiation, thereby influencing cytotoxicity.

Aside from investigating the cytolytic potential of NK cells from mice or humans exposed to ionizing radiation, investigating the cytokine profile secreted by them is also of interest. Considering the cytokines secreted by NK cells, such as IL-1, IL-2, IL-4, IL-5, TNF-α, and IFN-γ, the influence of these mediators on immune function, and the augmented presence of NK among surviving mononuclear cells post-irradiation, investigation of cytokine production and secretion may lead to the identification of key soluble mediators in immune suppression following irradiation.

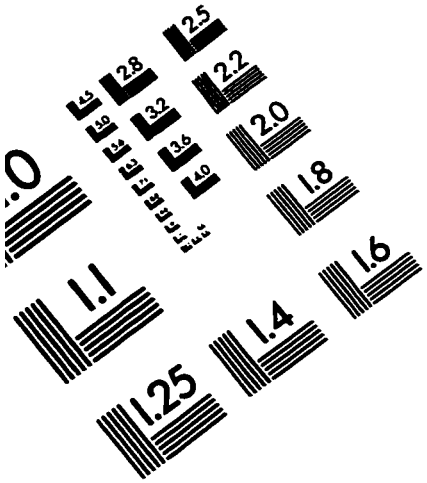
4. Other Prospects for Functional Immunity Assessment Post-Irradiation

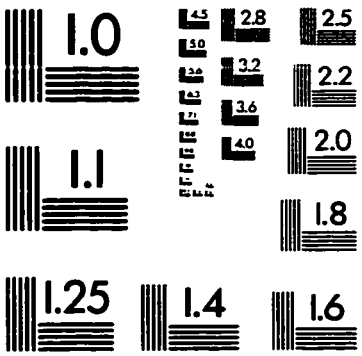
Many mechanisms may be at play in radiation induction of immune suppression. One contributor may be deficient antigen-presention function by antigen-presenting cells (APC). Many studies have investigated the effects of ultraviolet radiation on APC, and have demonstrated reduction in HLA-DR expression {108}, alteration in the ability to stimulate Th1 or Th2 responses {109}, altered cytokine profile in the microenvironment including upregulation of IL-10 {110}, or deficiency in providing adequate costimulation for T lymphocytes (B7.1 and B7.2) {111}. Any of these influences reduced the ability of APC to adequately stimulate T cell responses following ultraviolet radiation. Investigation into the effects of ionizing radiation exposure on APC function is lacking.

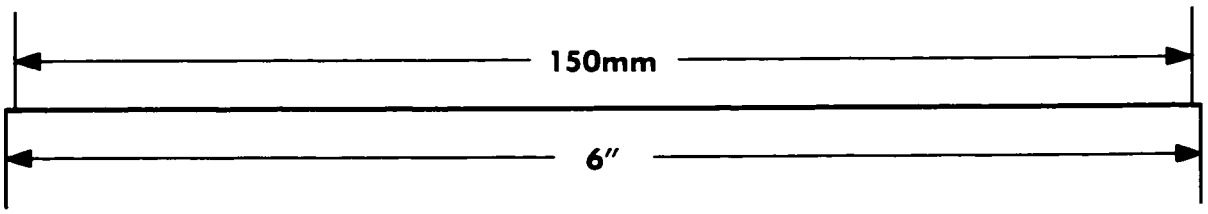
In the interest of furthering the characterization of various aspects of immune function, study of radiation effects on APC function, whether after a single acute

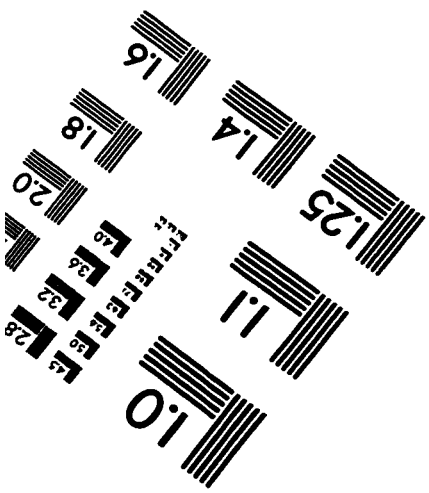
exposure or low-level chronic exposure, should be pursued. In addition to assessing proliferation of T lymphocytes to recall-antigen presented by APC (such as B lymphocytes), flow cytometric analysis of the APC could be conducted to determine if any alteration in APC function is due to changes in class II MHC expression, or expression of costimulatory molecules such as B7.1 and B7.2. The role of soluble mediators in radiation-induced alteration in APC function could easily be demonstrated by supernatant transfer from one culture system to a fresh culture system, in parallel with ELISA testing of supernatants for key cytokines such as IL-10, IL-12, IL-2, and IFN-γ.

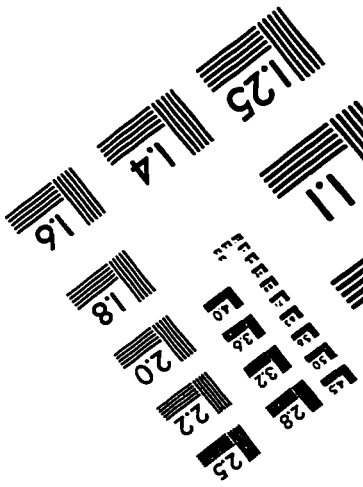
IMAGE EVALUATION
TEST TARGET (QA-3)











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