THE EFFECTS OF NITROSOUREAS ON THYMOCYTE DIFFERENTIATION AND T CELL ACTIVATION.

by

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(ABSTRACT)

Earlier studies have demonstrated that nitrosoureas such as 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) and chlorozotocin (CLZ) can cure almost 100% of C57BL/6 mice bearing syngeneic LSA tumor. In contrast, similar or higher doses of streptozotocin (STZ) completely failed to cure LSA-bearing mice. Further studies revealed that the efficacy of nitrosoureas may depend on their immunomodulating properties. In the current study, therefore, attempts were made to investigate the effects of these nitrosoureas on the immune system of normal and LSA tumor-bearing mice. Treatment of normal C57BL/6 mice with 5 intraperitoneal injections of 20 mg/kg body weight of BCNU or CLZ caused an increase in the percentage of CD4^- CD8^- T cells and a decrease in the percentage of CD4^+ CD8^+ T cells in the thymus. In addition, such treatment also caused an increase in the percentage of CD4^+ T cells without significantly affecting the CD8^+ T cells in the thymus. However, when total cellularity of the thymus was studied, BCNU and CLZ were found to decrease the total number of CD4^+ CD8^+ T cells without significantly affecting the other subsets. In contrast, similar or higher (100mg/kg body weight) doses of STZ had no significant effect on the total number and percentages of T cell subsets in the thymus. Also, BCNU and CLZ but not STZ-treatment caused a 50% decrease in the total number of CD4^+ and CD8^+ T cells in the spleen. Interestingly in tumor-bearing mice, BCNU treatment was followed by a ten-fold increase in the percentage of CD4^+ T cells found in the peritoneal cavity. The percentages of CD8^+ cells increased also, but to a lesser degree. These changes were limited to the peritoneal cavity which is the site of tumor growth. When T cells in the spleens of nitrosourea-treated normal mice were functionally analyzed, it was observed that BCNU and CLZ caused a dramatic de-
crease in the T cell responsiveness to Con A, anti-CD3, and PMA + calcium ionophore stimulation. In contrast, STZ treatment failed to significantly inhibit the T cell responsiveness to these activation signals. Using the accessory cell dependent and independent assays, BCNU and CLZ were found to suppress the functions of both T cells and macrophages in normal mice. BCNU and CLZ also suppressed the B cell responsiveness to lipopolysaccharide (LPS). Also, addition of growth factors such as IL-1, IL-2, IL-4 and IL-6 failed to reconstitute the defective responsiveness of BCNU and CLZ-treated T cells and macrophages. Together these data suggest that nitrosoureas have varying immunomodulating properties and this may in turn determine their efficacy in the treatment of cancer.
I first wish to acknowledge and dedicate this work to the memory of the man who inspired in me the joy of learning and a fascination for science; my father, D. Bain Reed. I also wish to thank my mother and my sister for many years of support and advice, and Sgt. A.B.S. Clary for his gentle persuasion and the generous giving of his time and financial resources to support my endeavors.

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1.0 GENERAL INTRODUCTION AND REVIEW OF LITERATURE

1.1 Immune System

The concept of immunity was described as early as 430 B.C. by Thucydides in his description of a plague which ravaged the city of Athens during which he noticed that "for the same man was never attacked twice" (Paul, 1989). A thousand years later this phenomenon came to be known as immununitas which describes an individual exempt from duty to the state (Golub, 1981).

Knowledge of the body's ability to protect itself under various circumstances began to develop after 1798 when Edward Jenner injected the fluid from a cowpox pustule into the arm of a healthy boy. Jenner then later challenged the boy with the fluid from a smallpox victim and
no disease developed due to the protection provided by the first inoculation. Further insight into the basis of the immune response was provided after the acceptance of the germ theory of disease demonstrated by Louis Pasteur and Robert Koch in the late 1880’s.

It was Pasteur who firmly and publicly established the efficacy of altered cultures of a pathogenic organism in the protection against dreaded diseases such as anthrax and rabies. Pasteur assumed that the introduction of an attenuated organism had induced immunity which protected the host from further exposure to the pathogen.

Today the basis of the immune response is known to be a highly complex and dynamic set of interactions. The immune response is defined as “altered reactivity to a specific molecular configuration that develops following contact with it” and immunology as a “science of self-nonself discrimination.” A functional definition of immunity would be characterized as the sum total of mechanisms within a host which aid in the defense against bacteria, viruses, parasites and transformed cells.

A primitive system of defense found in all animals consists of natural barriers against pathogenic invasion such as skin, mucosal surfaces, and circulating protective factors such as lysozyme and lactic acid. Another relatively primitive defense system is provided by phagocytic cells which are able to non-specifically engulf and destroy foreign particles.

Vertebrates have adapted a more complex system of protection which responds specifically to a foreign molecule and remains in a primed state throughout the host’s lifetime to provide a rapid response if the pathogen is reintroduced into the host. This specific response is carried out chiefly by a population of cells called lymphocytes which are morphologically similar to one another but vary in their function, development and physiology.
B lymphocytes are responsible for the humoral arm of immunity which is effective in the elimination of extracellular pathogens. These cells which are derived from bone marrow, will go on to differentiate into plasma cells which in turn secrete antibodies in response to the introduction of a foreign material into the host. Antibodies are glycoproteins found either bound to cell membranes or freely circulating in plasma which will bind specifically to an antigen or foreign molecule. The formation of an antigen-antibody complex thus brings about elimination of the pathogen through opsonization which enhances uptake by phagocytic cells or by activation of lytic proteins collectively known as complement. Antibody structure is characterized by the presence of a Fab fragment which recognizes and binds to the antigen, while the other end of the molecule called the Fc portion functions in the binding of the antibody to the immune cells.

The cell-mediated immune system is effective in reactions to intracellular pathogens, tumor cells or transplanted tissue. These functions are carried out by T lymphocytes which mature in the thymus and subsequently migrate to the peripheral lymphoid organs.

T cells also recognize foreign antigens in a specific manner, but only when presented in association with the host's self major histocompatibility complex (MHC) antigens. These molecules are encoded by a set of closely linked loci located on chromosome 6 in man and chromosome 17 in mice. MHC antigens are essential in the presentation of foreign antigen to the T lymphocytes and subsequent processes such as antibody production, graft rejection, and graft versus host disease. MHC restriction dictates that a host will only respond to an antigen when it is associated with a marker which distinguishes self from non-self.

Antigen recognition and the response to that antigen is also dependent upon the presence of a receptor located on the T cell. This polypeptide TCR is the actual recognition site located on the immune effector cells (Strominger, 1989). Antigen-specific T cell recognition will result in cell proliferation and differentiation of the cells.
The T cell subsets can be generally grouped into three distinct functional populations. These populations can be identified phenotypically by the presence of surface antigens which play a role in T cell recognition and activation.

The presence of the T helper cell population was demonstrated in a series of experiments by Claman (1978) in which lethally irradiated mice were unable to produce antibody in response to antigen unless reconstituted with both T cells and B cells. These cells which are phenotypically characterized by the presence of the CD4 surface antigen, were later shown to carry out several other types of helper functions.

Helper T cells when activated by foreign antigen in association with class II MHC molecules produce growth and differentiation factors which stimulate their own proliferation as well as affecting other immune cells. This interaction between CD4 cells and other cells occurs either through cell to cell interaction or through the production of chemical mediators called cytokines. T cell derived cytokines include interleukin-2 (IL-2) or T cell growth factor and IL-4 which stimulates B cell growth and differentiation. The end result of the activation of the T helper cell is a heightened immune response to antigen.

Another functional population of T lymphocytes includes the cytotoxic T lymphocytes or CTL's which bear the CD8 cell surface antigen. These cells are able to recognize antigen when presented in association with Class I MHC molecules and are then able to directly lyse the antigen-bearing cells. The most important role of the CTL is to eliminate intracellular pathogens such as viruses. A virally infected cell is recognized by the presence of viral antigens located in the cell membrane and lysis is carried out through the release of lymphotoxins and perforins. (Barrett, 1988).
The third population of T lymphocytes is made up of suppressor T cells (Ts) which act to downregulate the immune response allowing the components of the immune system to return to normal conditions. The presence of the Ts was demonstrated in a series of experiments in which the removal of the thymus in mice enhanced the B cell response to T cell independent antigens. Cell kinetics show that T suppressor cell response is low when an antigen is introduced, while Th activity is quite high. Ts activity will then increase and will remain high for a period of about two months. (Barrett, 1988). Like CTLs, T suppressor cells are phenotypically characterized often by the presence of the CD8 surface antigen, but can be shown to be functionally distinct from these cells.

In order for the T lymphocyte to recognize an antigen, it must first be presented in an appropriate manner. This function is carried out by the antigen presenting cell (APC). The most common type of APC is the monocyte-derived macrophage, but other cells such as dendritic cells or Langerhans cells can also present antigen. Macrophages are large, irregularly shaped phagocytic cells morphologically distinct from the other group of cells which carry out phagocytosis such as the neutrophils (Kimball, 1983).

Macrophages engulf foreign particles which are then packaged into vacuoles. These vacuoles will fuse with lysosomes containing hydrolytic enzymes thus digesting and breaking down the engulfed particle. In a process which is not well understood, the antigen must then associate with either Class I or Class II MHC molecules on the cell membrane, and finally the complex will be recognized by the lymphocyte.

Macrophages also affect immune functions by secreting monokines such as IL-1 which stimulates T lymphocyte production and CSF which stimulates the proliferation and maturation of additional macrophages (Barrett, 1989).
Natural killer (NK) cells are cytotoxic effector cells which do not have the characteristics of macrophages, granulocytes or CTLs (Otter, 1986). These cells are not MHC restricted, but are actively induced by interferon, and are effective in the elimination of viruses and tumor cells.

The immune response involves a complex and dynamic set of interactions. Any perturbations involving the cells or pathways can lead to invasion of pathogens, tumor growth or in some cases autoimmune disease.

1.2 Tumor Immunology

The theory of immunosurveillance was proposed by Burnet and describes the ability of the cell mediated immune system to eliminate tumor cells. This theory is supported by the following observations: Tumor incidence is higher when the immune system is not fully functional (in the young and very old); treatment with immunosuppressive drugs may cause the appearance of tumors; and finally the increased incidence of Kaposi’s sarcoma in Acquired Immunodeficiency Syndrome (Roitt, 1989).

Tumor growth may result from the transformation of a single cell. Initiation of neoplastic transformation is brought about by changes in DNA. These mutations may involve entire chromosomal segments or, in some cases single base mutations caused by exposure to a chemical, virus or some other factor. The susceptibility of DNA to mutation varies and can be related to faulty DNA repair mechanisms, expression of oncogenes or the state of the host’s immune system (Haskell, 1985).
Growth of a transformed cell may continue and subsequently lead to the development of subclones with genetic variability. This results in cellular heterogeneity within the tumor and thus makes treatment difficult. The neoplasm may remain localized or spread through various routes to other locations in the body (Haskell, 1985).

Cell transformation can affect the body's immunosurveillance ability in several ways. Alterations can cause membrane changes that affect cell to cell interactions, loss of normal cellular antigens, or the appearance of new antigens (Roitt, 1989).

Foley (1953) and Prehn (1957) demonstrated the presence of tumor specific antigens in mice with chemically induced tumors, but it took many years to demonstrate TSAs in humans. Experiments with human hepatomas and carcinomas have now defined a class of molecules known as oncofetal antigens. During embryonic development, these antigens will be expressed, and then soon after birth will disappear and will not be expressed in normal adult tissue (Morton et. al., 1985). In some individuals, defective genetic regulatory mechanisms may allow for the expression of these antigens; a process known as retrogenic expression (Roitt, 1989).

Two of these fetal antigens have been well characterized. Alpha-fetoprotein (AFP), when found in high levels in an adult is associated with liver and germ line neoplasms. Carcinoembryonic antigens (CEA) can be isolated in those with tumors derived from ectodermal tissue; that is breast, intestinal, pulmonary, gastric and pancreatic cancers. Levels of these antigens are used clinically to measure disease progression (Roitt, 1989).

The body's response to tumor specific antigens can range from an allogeneic like reaction to no reaction at all. The concept of immunological escape describes the ability of a tumor to grow within a host due to a malfunction in the immune system's ability to recognize and destroy neoplastic tissue. Several mechanisms have been proposed which might explain this

1.0 GENERAL INTRODUCTION AND REVIEW OF LITERATURE
occurrence. Firstly, the kinetics of tumor growth may be so low at the outset that the tissue may not be recognized until growth is well established. Secondly, the tumor may be undergoing antigenic modulation during which surface antigens may be shed and redistributed inside the cell. Another method of immunological escape may be due to genetic factors; that is, certain haplotypes are unable to mount a normal CTL response. This may be caused by the inability of the MHC molecules to bind properly to antigen. Finally, some tumors are able to secrete immunoregulatory molecules which can effect the immune system in a negative manner. Prostaglandins have such an effect on NK cells.

In a normal host there are several immune mechanisms by which tumor cells are destroyed. CTLs are thought to play a role in the regression of strongly antigenic tumors, such as in Burkitt's lymphoma. These cells are also found in weakly antigenic spontaneous tumors, but in low concentrations (Otter, 1986).

There is some evidence that macrophages can control tumor metastases. Macrophages have no direct effect on tumor cells unless they are activated. The structure on the tumor cell which is recognized by the macrophage is unknown, but they are highly efficient in distinguishing tumor cells from normal cells (Roitt, 1989).

Tumor antigens have been proven to induce antibody production. The titers will vary according to the type of tumor and the progression of the disease. The mechanism of tumor destruction may be through the blockage of a cell receptor essential in cell proliferation or direct cell lysis through the activation of complement. The generation of antibodies may allow cells such as lymphocytes or neutrophils to carry out antibody dependent cell-mediated cytotoxicity. Studies have demonstrated that NK cells are effective against lymphoproliferative tumor cells. NK activity is low in solid tumors or those which are well established, but seem functional in first line defense against neoplastic cells prior to the occurrence of a specific response (Otter, 1986). Lymphokine activated killer cells (LAK cells)
display a broad specificity against several solid tumors which are resistant to NK cells. LAK cells also show very little cytotoxicity towards normal cells (Roitt, 1989).

As we attain more understanding of the complex mechanisms of host-tumor interaction, we may be better able to effectively intervene in the progression and possibly the initiation of neoplastic diseases. Current areas of research are focusing in on pharmacologic and immunologic manipulation of immune functions as possible modes of therapy.

1.3 Nitrosoureas

Early treatment of cancer was limited to surgical removal of cancerous tissue. Metastases were common and therapy included only measures to reduce and relieve symptoms. In the late 1950's research demonstrated that some chemotherapeutic agents were able to prevent widespread metastases of choriocarcinoma and Burkitt's lymphoma (Lessner, 1978). Today chemotherapy in combination with surgery and radiation therapy is effective in treating many types of cancers.

In 1955 the U.S. government began a collaborative effort with the National Cancer Institute to screen anti-cancer drugs. The alkylating agents known collectively as nitrosoureas have now become the most highly researched chemotherapeutic agents in the U.S. (Montgomery, 1981). Two nitrosoureas (CCNU and BCNU) are in use for the treatment of several types of cancers, while several other of these drugs are undergoing clinical trials (Montgomery, 1981).

These drugs were developed after research involving animal models demonstrated that methylnitrosoguanidine and methylnitrosourea were effective anti-tumor agents. Further
studies carried out proved that two chloroethyl derivitives CCNU and BCNU were highly effective against tumors of the central nervous system due to their ability to cross the blood-brain barrier (Chabner, 1982).

The alkylating properties of these drugs is essential in tumor cell destruction for it has been demonstrated that nitrosoureas which lack this property are inactive. Alkylation is dependent upon the presence of the N-nitroso group, which when decomposed gives rise to a molecule known as diazotate. This compound in turn, is capable of the alkylation of DNA under physiological conditions (Johnson, 1986).

Tumor cell transformation may occur due to lesions in the DNA. While a normal human cell would contain highly efficient repair mechanisms to correct the damage, tumor cells would not exhibit the same repair capability. Nitrosoureas carry out alkylation at the DNA guanine-O6 position to produce interstrand cross-links in cellular DNA. The aforementioned repair process in normal cells would remove this lesion, while the repair deficient tumor cells could not and would be eliminated by the drug (Chabner, 1982).

Carbomoylation of nitrosoureas yields highly reactive compounds called isocyanates. This activity is responsible for the harmful side effects associated with the drugs by causing the inhibition of macromolecular synthesis, RNA processing, and DNA repair (Kann, 1986). As mentioned previously, the tumoricidal effect of the drugs are attributed to the process of alkylation.

The most dramatic side effect of nitrosoureas is cumulative myelosuppression. Research has shown that the presence of a glucose group alters the site of drug interaction with the chromatin of bone marrow cells, which in turn, reduces the toxicity of the drug (Chabner, 1986). Chlorozotocin, a glucose containing nitrosourea with low carbomoylating activity is highly tumoricidal and produces little to no side effects. Streptozotocin, a naturally occurring
antibiotic is another nitrosourea which contains a glucose moiety. This drug also has low carbomoylating activity, but has been found to be toxic to pancreatic beta cells (Mitchell and Schein, 1986). Despite this, streptozoticin has been found to be effective in the treatment of some types of tumors. A great deal of current research is being done to isolate or produce similar types of drugs.

Other toxic side effects caused by the carbomoylating property of the drugs are renal toxicity, pulmonary toxicity and the development of secondary tumors due to the myelosuppression. A current method of cancer therapy includes high doses of BCNU in conjunction with bone marrow transplantation to offset the myelosuppressive effects of the drug. Nitrosoureas are now being utilized alone or in combination with other drugs to treat several types of cancers. These include Hodgkin’s disease, malignant lymphoma, small cell lung cancer, brain tumors and malignant melanoma.

1.4 Specific Aims

Recent studies have demonstrated that nitrosoureas such as BCNU and CLZ are effective in curing mice bearing syngeneic tumors while, STZ failed to cure the tumor-bearing mice. The fact that nitrosoureas are effective only in normal but not in immunodeficient mice suggested that these drugs may also have the capability of modulating the host’s anti-tumor immunity to bring about a cure.

This study was therefore aimed at determining the effects of three nitrosoureas on the immune system of normal and tumor-bearing mice and will specifically address the following:
1. A better understanding of the effects of BCNU, CLZ and STZ on normal and tumor-bearing mice; specifically on the T cell phenotype in the thymus, spleen, and peritoneal exudate.

2. A study of the response of purified T cells from untreated and drug-treated normal mice to T cell activation signals.

3. A study of the the effect of drug treatment on the B cells and accessory functions of macrophages of normal mice.
2.0 EFFECT OF NITROSOUREAS ON THYMOCYTE
DIFFERENTIATION AND T CELL ACTIVATION

2.1 Introduction

Nitrosoureas (NUs) have been used extensively as antineoplastic agents in the treatment of a variety of human cancers and experimental tumors (Carter et al., 1972; Schabel, 1976; Suami et al., 1981; Montgomery, 1981; Weinkam and Huey-Shin Lin, 1982). The tumoricidal activity of the nitrosoureas is believed to be due to the alkylating property, whereas, the severe toxic effects such as myelosuppression seen is due to the carbamoylating property (Kann, 1981).

1, 3-bis (2-chloroethyl)-1-nitrosourea (BCNU) is one of the most widely used NUs. Since its original introduction, several different analogs of NUs have been developed and investigated for more efficient antitumor activity and lesser toxicity. Streptozocin (STZ) and
chlorozolocia (CLZ) are two NU analogues having low carbamoylating activity (Byrne et al., 1981; Hoth and Duque-Hammershaimb, 1981; Kann, 1981) and are therefore believed to be less toxic to the bone marrow when compared to BCNU while retaining similar anti-tumor activity.

In spite of having similar activities in cell culture, NUs display a wide range of activities in vivo and therefore vary in their tumoricidal and toxic effects in vivo (Weinkam and Huey-Shin Lin, 1982). Our laboratory has been investigating the tumoricidal activity of various NUs and correlating this with their action on the immune system of the host. Earlier studies demonstrated that treatment of C57BL/6 mice bearing a syngeneic tumor, LSA, with 20 mg/kg body weight of BCNU or CLZ resulted in 90-100% survival of the mice and, more interestingly, 100% of the cured mice rejected secondary rechallenge with the homologous tumor but not with a heterologous syngeneic tumor such as EL-4 (Nagarkatti and Kaplan, 1985; Feola and Maruyama, 1986; Nagarkatti et al., 1988a, 1988b; Nagarkatti et al., 1989a). Interestingly, STZ totally failed to cure LSA tumor-bearing mice, even at higher doses when the tumoricidal properties were comparable to those obtained with BCNU and CLZ (Nagarkatti et al., 1989a).

Further studies showed that BCNU and CLZ can eliminate tumor-specific T suppressor cells and enhance the host’s T cell mediated immunity whereas, STZ fails to act on T suppressor cells (Nagarkatti et al., 1989a).

Additional work suggests that BCNU through its interactions with the host’s immune system may further bring about tumor rejection by enhancing the infiltration of CD4+ cells into the peritoneum. This is the primary site of LSA tumor cell growth.

These studies together suggested that the outcome of chemotherapy with nitrosoureas depends, in addition to the tumoricidal activity of the drug, on the modulating action on the immune mechanism of the host. In the present study attempts were made to investigate directly the immunomodulating properties of various nitrosoureas in normal and tumor-bearing mice. The data suggested that BCNU and CLZ were highly immunosuppressive in normal mice, and altered the thymocyte differentiation, whereas, STZ failed to cause significant immunosuppression.

2.0 EFFECT OF NITROSOUREAS ON THYMOCYTE DIFFERENTIATION AND T CELL ACTIVATION
2.2 Experimental Procedures

2.2.1 Animals:

Adult, female C57BL/6 mice were obtained from National Cancer Institute, Bethesda, MD.

2.2.2 Drugs:

BCNU, CLZ and STZ were obtained from the National Cancer Institute, Bethesda, MD, and stored at -20°C. BCNU was dissolved in absolute ethanol and diluted further with PBS, pH 7.2. whereas CLZ and STZ were dissolved directly in PBS (Nagarkatti et al., 1989a).

2.2.3 In Vivo drug treatment:

It was observed earlier that 20 mg/Kg bodyweight of BCNU or CLZ can cure 100% of LSA tumor-bearing mice whereas 20, 100 or 200 mg/kg body weight of STZ fails to cure any mice (Nagarkatti et al; 1989a). Also, 200 mg/kg body weight of STZ is the maximum tolerated dose in C57BL/6 mice. To study the immunomodulating properties of nitrosoureas, therefore, BCNU and CLZ were injected intraperitoneally (i.p.) at 20 mg/kg body weight for 5 days. STZ was used at two concentrations, 20 and 100 mg/kg body weight and was similarly injected for 5
days. Groups of 5 mice were usually used in each experiment. Control groups received only the vehicle.

2.2.4 Cell separation:

Single cell suspensions from the thymus, lymph nodes and spleens were prepared by using a laboratory tissue homogenizer (Stomacher, Tekmar Co., Cincinnati, OH). The erythrocytes were removed by lysis with 0.83% solution of ammonium chloride. The medium used throughout these studies consisted of RPMI-1640 supplemented with 10% fetal calf serum (GIBCO Lab., Grand Island, NY), 10mM HEPES, 1mM glutamine, 40μg/ml of gentamicin sulfate and 50mM 2-mercaptoethanol.

2.2.5 B and T cell proliferation assay:

Spleen cells were plated in triplicate at a concentration of 2×10⁴ cells/well in 96-well flat bottomed plates in 200μl of cultured medium. These cultures were stimulated with 2μg/ml of ConA, 1:100 final dilution of anti-CD3 mAb or with phorbol myristate acetate (PMA) plus calcium ionophore (Kakkanaiah et al. 1990; Nagarkatti and Nagarkatti, 1989). PMA and calcium ionophore (A23187) were purchased from Sigma Chemical Co., St. Louis, MO. They were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. PMA was used at 10ng/ml and calcium ionophore was used at 0.5μM. The hybridoma 145.2C11 secreting anti-CD3 mAbs was kindly provided by Dr. J.A. Bluestone, Univ. of Chicago, IL. It was grown as ascites in nude mice. To study B cell responsiveness, spleen cells were incubated in a similar fashion with lipopolysaccharide (LPS) (5μg/ml). All cultures were incubated at 37°C for 48 hours. The cells
were pulsed with 2μCi of [³H] thymidine 18 hr. prior to cell harvesting. The cells were harvested onto glass fiber filter paper discs and counted with scintillation fluid in a liquid scintillation counter. The results were expressed as mean counts per minute (CPM) ± S.E.M. of triplicate cultures.

2.2.6 Purification of T cells and macrophages from the spleen:

In some assays purified T cells were used in mitogen assays. The T cells were purified from the spleens by first passing them over nylon wool. The non-adherent cells were treated with J11d mAb and anti-la plus complement to deplete further any contaminating B cells (Selvan et. al., 1990). The T cells thus purified were greater than 95% Thy 1.2+. The macrophages were purified from the spleens by adherence on plastic tissue culture plates for 2 hrs. at 37°C (Nagarkatti et. al., 1988a).

2.2.7 Two color fluorescent staining for CD4 and CD8 markers:

One million cells were incubated with FITC-conjugated anti-CD8 mAb and phycoerythrin (PE)-conjugated anti-CD4 mAb for 30 min. at 4°C. The cells were washed twice and finally resuspended in 0.5 ml medium. During the entire staining procedure, the medium consisted of cold PBS containing 0.02% sodium azide (Seth et al., 1988).
2.2.8 Flow cytometry:

Fluorescence of individual cells was measured by flow cytometry. (Seth et al., 1988). The analysis was performed on an Epics V, model 752 (Coulter Electronics, Hialeah, Fla). The four parameters per cell that were studied included forward angle and 90° light scatter, green fluorescence and red fluorescence. A total number of 10,000 cells were studied in each sample.

2.2.9 Interleukins:

Recombinant murine interleukin-2 (rIL-2), recombinant interleukin-4 (rIL-4) and recombinant interleukin-6 (rIL-6) were purchased from Genzyme Corporation, Boston, Mass. The activity of rIL-2 was expressed in Biological Response Modifiers Program (BRMP) units, with one unit equivalent to the amount of IL-2 required to support half maximal proliferation of indicator cells used by the National Institute of Health. We observed that 25 units/ml of rIL-2 induced optimum proliferation of HT-2 cell line and IL-2 dependent autoreactive T cell clones (Nagarkatti et al., 1989b). The activity of rIL-4 was expressed in proliferation units based on a coproliferation assay using splenic B cells and anti-lg. It was observed that 50 units/ml of rIL-4 could support optimal proliferation of HT-2 cell line and induced hyper-Ia expression by B cells (Nagarkatti et al, 1989b). The activity of IL-6 was expressed in units, with one unit equivalent to the amount required to augment the production of Ig by CESS cell line to the half maximal level and was further observed at 100 units/ml to induce 151% increase in lymph node T cell proliferation to Con A as described by Garman et al. (1987). The rIL-1 was obtained from the National Institute of Health, Bethesda, Md. Based on thymocyte costimulation
assay, 5 units/ml of rIL-1 was found to be optimal concentration for the activation of T cells (Dean et al., 1990).

### 2.2.10 Statistical Analysis:

All experiments were repeated at least 3 times with consistent results. The statistical analysis of the data was performed using Student’s ‘t’ test and p values less than 0.05 between two groups were considered to be statistically significant.
2.3 Results

2.3.1 BCNU and CLZ but not STZ alter the T cell subsets in the thymus of normal mice:

Normal C57BL/6 mice were treated with 20mg/kg bodyweight of BCNU, CLZ or with 20 or 100mg/kg of STZ for five days and on day 6, the thymocytes were examined for T cell subsets bearing CD4 and CD8 antigens. A representative experiment of the two-color flow cytometric analysis of different cell populations has been shown in Figs. 1 and 2. The first figure panel depicts a representative negative control in which unstained cells were analyzed and gated. The second panel (normal) demonstrates vehicle-treated control mice showing 15% CD4+ CD8- (CD4+), 73% CD4+ CD8+ (double-positive), 7% CD4- CD8- (double-negative) and 5% CD4- CD8+ (CD8+) T cells in the thymus. These data were consistent with the proportion of different subsets of thymocytes studied by us and other investigators in the normal thymus (Scollay et al., 1984; Nagarkatti et. al., 1990). As shown in Fig. 2, treatment with BCNU led to a significant decrease in the percentage of double-positive T cells and an increase in the percentage of double-negative and CD4+ T cells. Similar results were obtained following CLZ-treatment. Interestingly, treatment with 20 or 100 mg/kg of STZ failed to induce any significant alterations in the thymocyte subsets.

Attempts were also made to study the effect of nitrosoureas on the total cellularity of the thymus. The data shown in Table 1 suggested that BCNU and CLZ caused ~ 50% decrease in the cellularity of the thymus. Based on these values and the percentages of different T cell subsets shown in Figs. 1 and 2 when the total number of T cell subsets were enumerated, the most dramatic effect of BCNU and CLZ-treatment was found to be restricted only to the CD4+CD8+ T cells whose number was significantly decreased (Table 1). In contrast, STZ failed
Figure 1. T cell subsets in the thymus of normal C57BL/6 mice. Thymocytes from mice were stained with FITC-conjugated anti-CD8 mAb and PE-conjugated anti-CD4 mAb and analyzed using the flow cytometer. (Mice injected with only the vehicle were used as controls depicted as normal). The negative controls included thymocytes which were not stained with the above mAbs depicted in the first panel as control. In each panel, quadrant 1 depicts CD4+CD8- cells; quadrant 2, CD4+CD8+ cells; quadrant 3, CD4+CD8- cells and quadrant 4 shows CD4+CD8+ T cells. The fluorescence intensity was depicted on log scale.
Figure 2. Effect of nitrosourea treatment on T cell subsets in the thymus: C57BL/6 mice were treated with 20mg/kg body weight of BCNU, CLZ, or with 20 or 100 mg/kg body weight of STZ for 5 days by the i.p. route. Twenty four hours later the thymocytes from mice were stained with FITC-conjugated anti-CD8 mAb and PE-conjugated anti-CD4 mAb and analyzed using the flow cytometer. In each panel, quadrant 1 depicts CD4⁺CD8⁺ cells; quadrant 2, CD4⁺CD8⁺ cells; quadrant 3, CD4⁻CD8⁻ cells and quadrant 4 shows CD4⁻CD8⁺ T cells. The fluorescence intensity was depicted on log scale.
to significantly alter the total cellularity of the thymus and the absolute number of various T cell subsets (Table 1).

2.3.2 Effect of BCNU, CLZ and STZ treatment of normal mice on T cell subsets in the spleen:

When spleen cells were analyzed for CD4 and CD8 markers, it was observed that normal untreated mice had ~ 34% T cells of which 22% were CD4+ and 12% were CD8+ (Fig. 3). Interestingly, treatment with BCNU, CLZ or STZ did not significantly alter the percentages of CD4+ and CD8+ T cells (Fig. 4). However, as seen from Table 1, treatment with BCNU and CLZ induced a significant decrease in the total cellularity of the spleen which therefore resulted in a significant reduction in the absolute numbers of CD4+ and CD8+ T cells. Treatment with STZ, however failed to induce significant alterations in the total cellularity and the number of CD4+ and CD8+ T cells in the spleen.

Thus far the current studies demonstrated the immunomodulatory properties of BCNU and CLZ on normal T cell subpopulations whereas STZ failed to induce any changes. Further studies were carried out to delineate whether these nitrosoureas have similar effects on tumor-bearing mice which will be discussed later on.
### Table 1. Total thymus and spleen cellularity in C57BL/6 mice treated with various nitrosoureas

<table>
<thead>
<tr>
<th>Organs</th>
<th>Nitrosourea treatment</th>
<th>Total cells x 10^6 (p value)</th>
<th>Total T cells x 10^6/organ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CD4⁺</td>
</tr>
<tr>
<td>Thymus</td>
<td>Vehicle</td>
<td>43.7 ± 1.9</td>
<td>6.55</td>
</tr>
<tr>
<td>Thymus</td>
<td>BCNU</td>
<td>15.8 ± 1.32 (&lt;.005)</td>
<td>7.42</td>
</tr>
<tr>
<td>Thymus</td>
<td>CLZ</td>
<td>22.7 ± 2.6 (&lt;.025)</td>
<td>7.03</td>
</tr>
<tr>
<td>Thymus</td>
<td>STZ 20</td>
<td>47.7 ± 1.8 (&gt;0.50)</td>
<td>8.58</td>
</tr>
<tr>
<td>Thymus</td>
<td>STZ 100</td>
<td>39.3 ± 1.4 (&gt;0.50)</td>
<td>5.89</td>
</tr>
<tr>
<td>Spleen</td>
<td>Vehicle</td>
<td>34.55 ± 2.4</td>
<td>7.60</td>
</tr>
<tr>
<td>Spleen</td>
<td>BCNU</td>
<td>15.35 ± 1.5 (&lt;.001)</td>
<td>3.33</td>
</tr>
<tr>
<td>Spleen</td>
<td>CLZ</td>
<td>12.6 ± 0.5 (&lt;.001)</td>
<td>2.52</td>
</tr>
<tr>
<td>Spleen</td>
<td>STZ 20</td>
<td>32.3 ± 2.1 (&gt;0.50)</td>
<td>5.16</td>
</tr>
<tr>
<td>Spleen</td>
<td>STZ 100</td>
<td>27.8 ± 2.5 (&lt;.050)</td>
<td>5.28</td>
</tr>
</tbody>
</table>

C57BL/6 mice were treated with the vehicle (control) or with various nitrosoureas. Twenty four hours after the last treatment, thymus and spleens were harvested from individual mice and single cell suspensions were made. The total number of cells were enumerated using trypan blue dye exclusion test. The total number of various subsets of T cells/organ were next calculated using the percentages obtained from Figs. 1 and 2.
Figure 3. T cell subsets in the spleen of normal C57BL/6 mice: Splenocytes from mice were stained with FITC-conjugated anti-CD8 mAb and PE-conjugated anti-CD4 mAb and analyzed using the flow cytometer. (Mice injected with only the vehicle were used as controls depicted as normal). The negative controls included splenocytes which were not stained with the above mAbs, depicted in the first panel as control. In each panel, quadrant 1 depicts CD4⁺CD8⁺ cells; quadrant 2, CD4⁻CD8⁺ cells; quadrant 3, CD4⁺CD8⁻ cells; and quadrant 4 shows CD4⁻CD8⁻ T cells. The fluorescence intensity was depicted on log scale.
Figure 4. Effect of nitrosourea treatment on T cell subsets in the spleen: C57Bl/6 mice were treated with 20mg/kg body weight of BCNU, CLZ or with 20 or 100 mg/kg body weight of STZ for 5 days by the i.p. route. Twenty-four hours later the splenocytes from these mice were stained with FITC-conjugated anti-CD8 mAb and PE-conjugated anti-CD4 mAb and analyzed using the flow cytometer. In each panel, quadrant 1 depicts CD4+CD8- cells; quadrant 2, CD4+CD8- cells; quadrant 3, CD4+CD8+ cells and quadrant 4 shows CD4+CD8+ T cells. The fluorescence intensity was depicted on log scale.
2.3.3. BCNU and CLZ but not STZ, suppress the T cell activation induced by mitogens, PHA and Con A.

To study the effect of nitrosoureas on normal T cell activation, a variety of activation signals were tried since it was possible that these may have varying effects on different sub-populations of T cells.

To study the T cell activation, spleen cells from various nitrosourea-treated normal mice were cultured with Con A and T cell proliferation was measured using \(^{3}\text{H}\)-thymidine incorporation assay. The data shown in Fig. 5 clearly suggested that BCNU and CLZ treatment suppressed the T cell activation by Con A, whereas 20 or 100 mg/kg body weight of STZ failed to cause any T cell suppression. Similar results were obtained when other mitogens such as PHA were used (Fig. 6).

2.3.4 BCNU and CLZ suppress the accessory functions of macrophages:

T cell responsiveness to Con A requires accessory cells such as macrophages. Thus, to address whether BCNU and CLZ inhibited the T cell functions directly or whether these drugs also had an effect on macrophages, T cells from normal mice were stimulated with Con A in the presence of irradiated macrophages from vehicle-treated (control) or nitrosourea-treated mice. The data shown in Fig. 7 suggested that macrophages from BCNU and CLZ-treated mice provided decreased accessory help to Con A activated T cells. In contrast, macrophages from STZ-treated mice exhibited normal accessory functions.
Figure 5. Effect of treatment with nitrosoureas on the responsiveness of spleen cells to Con A: C57BL/6 mice were treated with BCNU, CLZ (20mg/kg) or with STZ (20 or 100 mg/kg) as described in Fig. 2. Twenty-four hours later, 2x10⁵ spleen cells were analyzed for their responsiveness to Con A (2μg/ml) in 96-well tissue culture plate. The cultures were incubated at 37°C for 48 hours and pulsed with 2μCi of [³H]-thymidine 18 hours prior to cell harvesting. The controls included mice treated with vehicle alone. Spleen cells incubated with medium alone usually incorporated less than 5000 cpm. The vertical bars represent mean cpm of triplicate cultures ± S.E.M.
Figure 6. Effect of treatment with nitrosoureas on the responsiveness of spleen cells to PHA: C57BL/6 mice were treated with BCNU, CLZ (20mg/kg) or with STZ (20 or 100 mg/kg) as described in Fig. 2. Twenty four hours later, 2x10⁷ spleen cells were analyzed for their responsiveness to PHA(2μg/ml) in 96-well tissue culture plate. The cultures were incubated at 37°C for 48 hours and pulsed with 2μCl of ³H-thymidine 18 hours prior to cell harvesting. The controls included mice treated with vehicle alone. Spleen cells incubated with medium alone usually incorporated less than 5000cpm. The vertical bars represent mean cpm of triplicate cultures ± S.E.M.
Figure 7. BCNU and CLZ suppress the accessory functions of macrophages: T cells were purified from the spleens of normal C57BL/6 mice and 2x10^5 T cells were incubated with Con A (2μg/ml) in the presence of 4x10^5 irradiated (2000 rads) macrophages purified from various nitrosourea-treated mice as described in Fig. 1 or with macrophages from vehicle-treated mice (depicted as control). Purified T cells or macrophages incubated with medium alone incorporated less than 3000 cpm. The vertical bars represent mean cpm of triplicate cultures ± S.E.M.
2.3.5 Effect of BCNU, CLZ and STZ on T cell responsiveness to anti-CD3 mAbs.

Antibodies directed against CD3 antigens on T cells have been shown to activate the T cells (Leo et al., 1987). Although soluble anti-CD3 mAbs do not by themselves stimulate the T cells, cross-linking the CD3 molecules as achieved by use of Fc receptor-bearing macrophages, can activate the T cells. To study the anti-CD3 response, whole spleen cells from various nitrosourea-treated mice were stimulated with anti-CD3 mAbs and T cell proliferation was measured. It was observed that BCNU and CLZ treatment induced a dramatic decrease in the T cell proliferation (Fig. 8). These responses were 95% and 92% lower than that of the control T cells. In contrast, treatment with 20 or 100 mg/kg of STZ lowered the T cell responses by only 32% and 24% respectively.

2.3.6 Responsiveness of T cells from nitrosourea-treated mice to PMA and calcium ionophore.

Purified T cells have been shown to proliferate directly in response to stimulation with PMA and calcium ionophore in the absence of additional signals from the accessory cells. In the present study, therefore, the effect of nitrosourea-treatment on T cell activation was also investigated using PMA and calcium ionophore. As seen from Fig. 9, treatment with BCNU or CLZ caused ~65% decrease in the T cell response when compared to the controls. However, STZ at both doses failed to inhibit the T cell proliferation significantly.
Figure 8. Effect of nitrosourea-treatment on T cell responsiveness to anti-CD3 mAbs: C57BL/6 mice were treated with various nitrosoureas as described in Fig 2 and the spleen cells were incubated with 1:100 final dilution of anti-CD3 mAb for 48 hours. The cultures were pulsed with 2μCi of 3H-thymidine 18 hours prior to cell harvesting.
Figure 9. Responsiveness of T cells from nitrosourea-treated mice to PMA and calcium ionophore: T cells were purified from the spleens of various nitrosourea-treated mice as described in Fig. 1. The T cells ($2\times10^6$) were incubated with PMA (10ng/ml) and calcium ionophore (0.5μM). The cell proliferation assay was carried out and depicted as described earlier in Fig. 8.
2.3.7 Responsiveness of B cells to LPS in nitrosourea-treated mice.

To investigate whether BCNU and CLZ would also affect the B cell responsiveness, spleen cells from various nitrosourea-treated mice were stimulated with a B cell mitogen, LPS. The data shown in Fig. 10 suggested that BCNU and CLZ decreased the B cell response to LPS by 80% and 82% respectively when compared to the controls. Once again, STZ was unable to significantly suppress the B cell response.

2.3.8 T cell growth factors fail to reconstitute the defective response of T cells from BCNU and CLZ-treated mice.

To investigate whether the decreased T cell responsiveness seen following BCNU- and CLZ-treatment was due to decreased production of T cell growth factors, exogenous rIL-2, rIL-4 and rIL-6 were added to the cultures, stimulated with Con A. As seen from Fig. 11, BCNU and CLZ-treatment caused significant suppression of the responsiveness to Con A as seen before. Addition of exogenous rIL-2, rIL-4 and rIL-6 caused >100% increase in control T cell responsiveness to Con A. In contrast, addition of similar amounts of interleukins completely failed to reconstitute the defective response induced by BCNU and CLZ.
Figure 10. Effect of nitroso compounds on B cell responsiveness to LPS: C57BL/6 mice were treated with nitroso compounds or vehicle alone (control) as depicted in Fig. 2. The spleen cells (2x10^6) were next incubated with LPS (5μg/ml) and the cell proliferation was measured and depicted as before (Fig. 6).
Figure 11. Effect of exogenous rIL-2, rIL-4 and rIL-6 on T cell responsiveness: T cell responsiveness to Con A was studied as described in Fig. 5 from vehicle-treated (control) or from BCNU or CLZ-treated mice. The cultures were incubated in the absence or presence of 25 units/ml of rIL-2, 50 units/ml of rIL-4 or 100 units/ml of rIL-6. The cell proliferation was performed as described in Fig. 5.
2.3.9 Effect of addition of exogenous rIL-1 and rIL-6 on the accessory cell functions of the macrophage.

Recent studies have suggested that IL-1 and IL-6 share similar functions and can substitute for the accessory cells in T cell responsiveness to mitogens (Wong and Clark, 1988). Attempts were therefore made to investigate whether the defect in the accessory cell functions of the macrophages induced by BCNU and CLZ can be reconstituted by addition of exogenous rIL-1 and rIL-6. To address this, T cells were purified from the spleens of C57BL/6 mice. These T cells were next incubated with Con A in the presence or absence of irradiated macrophages purified from the spleens of control, BCNU- or CLZ-treated mice. The data shown in Table 2 suggested that T cells incubated with Con A alone failed to respond as expected. In the presence of control accessory cells, the T cells responded significantly to Con A, whereas in the presence of BCNU-treated or CLZ-treated macrophages, no significant T cell proliferation was seen. When rIL-1 or rIL-6 were added to the control cultures, the T cell response increased markedly. However, when similar concentrations of IL-1 and IL-6 were added to BCNU or CLZ-treated cultures, although the T cell response increased significantly, they were markedly decreased when compared to the controls.

These data suggested that IL-1 and IL-6, at concentrations used in this study fail to reconstitute the accessory cell defect of the macrophages.
### Table 2. Effect of IL-1 and IL-6 on the accessory cell functions of the macrophage from BCNU and CLZ-treated mice.

<table>
<thead>
<tr>
<th>Accessory cells (macrophages)(^a)</th>
<th>Con A(^b)</th>
<th>Interleukins(^c)</th>
<th>T cell proliferation (cpm ± S.E.M.)(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>554 ± 44</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>215 ± 13</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>-</td>
<td>25,617 ± 2,832</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>IL-1</td>
<td>91,928 ± 1,998</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>IL-6</td>
<td>163,864 ± 1,835</td>
</tr>
<tr>
<td>BCNU-treated</td>
<td>+</td>
<td>-</td>
<td>391 ± 113</td>
</tr>
<tr>
<td>BCNU-treated</td>
<td>+</td>
<td>IL-1</td>
<td>3,710 ± 811</td>
</tr>
<tr>
<td>BCNU-treated</td>
<td>+</td>
<td>IL-6</td>
<td>7,587 ± 1,222</td>
</tr>
<tr>
<td>CLZ-treated</td>
<td>+</td>
<td>-</td>
<td>421 ± 25</td>
</tr>
<tr>
<td>CLZ-treated</td>
<td>+</td>
<td>IL-1</td>
<td>7,810 ± 1,102</td>
</tr>
<tr>
<td>CLZ-treated</td>
<td>+</td>
<td>IL-6</td>
<td>14,325 ± 2,686</td>
</tr>
</tbody>
</table>

\(^a\) Macrophages were purified from the spleens of vehicle-treated (control), BCNU-treated, or CLZ-treated mice, irradiated at 2000 rads and used at 4 x 10\(^6\) cells/well. The irradiated control, BCNU and CLZ-treated macrophages when incubated alone incorporated 364 ± 142, 504 ± 176 and 374 ± 6 cpm.

\(^b\) Con A was used at 2μg/ml.

\(^c\) IL-1 was used at 5 units/ml and IL-6 at 100 units/ml.

\(^d\) T cell proliferation was studied after 48 hours incubation. \(^3\)H-Thymidine was added during the last 18 hours of culture.
2.3.10 Analysis of T cells in the thymus, spleen and peritoneum of BCNU-treated or untreated LSA tumor-bearing mice.

Phenotypic studies were undertaken to investigate the nature of changes occurring in the lymphoid organs and at the site of tumor growth following BCNU-treatment. For this purpose, thymocytes, spleen cells and PEC were collected from BCNU-treated or untreated normal or tumor-bearing mice and stained simultaneously for CD4 and CD8 antigens. The data obtained from flow cytometric analysis has been depicted in Figs. 12, 13 and 14. In these experiments the negative controls consisted of unstained cells and since this was similar in all groups, only one representative control from normal mice was depicted. Normal thymocytes demonstrated ~11% CD4+CD8- (CD4+) cells, ~77% CD4+ CD8+ cells, ~7% CD4- CD8- and ~5% CD4- CD8+ (CD8+) cells (Fig. 12). These proportions of various subsets were similar to those reported by other investigators for normal mice. The thymocytes from tumor-bearing mice and BCNU-treated tumor-bearing mice exhibited identical proportions of T cell subsets thereby suggesting that tumor growth and BCNU treatment had no significant effect on thymocyte subsets (Fig. 12). Normal spleens contained ~20% CD4+ T cells and ~7% CD8+ cells with the CD4+:CD8+ cell ratio of ~2:1. The spleens of tumor-bearing mice demonstrated similar percentages of CD4+ (~16%) and CD8+ T cells (~9%) (Fig. 13). Following BCNU treatment, the tumor-bearing mouse spleens had similar percentages of CD4+ T cells (~18%). However, the percentage of CD8+ T cells increased to ~14% (Fig. 13). The PEC from normal mice had ~10% CD4+ T cells and ~3% CD8+ cells and tumor-bearing mice had ~4% CD4+ T cells and ~5% CD8+ cells. Interestingly, however, following BCNU-treatment there was a heightened influx of CD4+ T cells (~41%) and also a significant increase in CD8+ T cells (~9%) (Fig. 14).
Figure 12. Phenotypic analysis of T cell subsets in the thymus of tumor-bearing mice. Sub-populations of T cells based on the expression of CD4 and CD8 antigens in the thymus using two-color flow cytometry in untreated or BCNU-treated normal or LSA tumor-bearing mice. Cells were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 mAbs. The negative control depicts auto fluorescence and only one representative experiment using normal mice has been depicted.
Figure 13. Phenotypic analysis of T cell subsets in the spleens of tumor-bearing mice: Subpopulations of T cells based on the expression of CD4 and CD8 antigens in the spleen using two-color flow cytometry in normal or LSA tumor-bearing mice, untreated or treated with BCNU. Cells were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 mAbs. The negative control depicts auto fluorescence and only one representative experiment using normal mice has been depicted.
These experiments were repeated 3 times with consistent results. It should be noted that in addition to the increase in the percentages of CD4+ and CD8+ T cells, the total number of such cells found in the PEC were also increased two-fold in BCNU-treated tumor-bearing mice. Also, a single injection of BCNU in normal mice did not affect the T cell subsets in any lymphoid organs or in the PEC, unlike earlier studies wherein multiple doses of BCNU altered the thymocyte subsets. These data also suggested that the infiltration of CD4+ T cells in BCNU-treated tumor-bearing mice was probably not caused by the direct action of the drug.
Figure 14. Phenotypic analysis of T cell subsets in the PEC of tumor-bearing mice: Subpopulations of T cells based on the expression of CD4 and CD8 antigens in the PEC using two-color flow cytometry in normal or LSA tumor-bearing mice, untreated or treated with BCNU. Cells were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 mAbs. The negative control depicts auto fluorescence and only one representative experiment using normal mice has been depicted.
Nitrosoureas have been used for several years in the treatment of experimental tumors and on several types of human cancers. BCNU has been found to be effective in the treatment of central nervous system tumors because of its ability to cross the blood-brain barrier.

Several studies have indicated that treatment of tumor-bearing mice with BCNU cures 90-100% of mice bearing a syngeneic tumor (Feola and Maruyama, 1986; Nagarkatti and Kaplan, 1985). Normally a single dose treatment with an anti-cancer drug at an advanced stage of tumor growth is not effective because such a treatment fails to kill all tumor cells and therefore any tumor cells spared from the action of the drug will eventually proliferate and kill the host. However, earlier studies demonstrated that BCNU-treatment was very effective even when given in a single dose and at an advanced stage of tumor growth (Nagarkatti and Kaplan, 1985). Several hypotheses were proposed to explain the mode of action of nitrosoureas, but recent data suggested that nitrosoureas may inhibit tumor-specific T suppressor cells. This inhibition consequently allows CTLs and T helper cells to operate at maximum efficiency and destroy any tumor cells not affected by the direct tumoricidal action of the drug. These studies further suggested that the efficacy of nitrosoureas may depend on their immunomodulating properties. (Nagarkatti and Kaplan, 1985).

When C57BL/6 mice were injected with LSA, which is a syngeneic thymic lymphoma, all the mice died within 9 days. When these mice were are treated with 20mg/kg body weight of
BCNU or CLZ, 90-100% of these mice survive up 120 days. When these cured mice were subsequently rechallenged with LSA, they resisted tumor growth. On the other hand, when these cured mice were rechallenged with a heterologous tumor (EL-4) they failed to resist the rechallenge. These data suggest that BCNU and CLZ treatment may modulate the host's anti-tumor immunity in such a manner to eliminate the tumor cells which are not destroyed by the drug. (Nagarkatti et al., 1989a).

Positive modulation of the host's anti-tumor immunity brought about by nitrosoureas may affect many cell types. Earlier studies demonstrated that treatment of tumor target cells with BCNU increased the lytic activity of macrophages but had no effect on cytotoxic T lymphocytes (CTLs), natural killer (NK) cells or lymphokine activated killer (LAK) cells (Nagarkatti et al., 1988a). On the other hand, the results of BCNU-treatment on effector cell functions include enhanced tumor-specific CTL and LAK cell activity, while having no effect on macrophage or NK cell activity. Prior evidence suggested that CTLs have a recognition, binding and lytic mechanism which may be different from that of activated macrophages. These data together with the fact that BCNU-treated tumor targets are not more immunogenic than untreated targets, suggests that on the level of the macrophage, BCNU has no effect on target-effector cell recognition or cell binding, but rather, enhances macrophage mediators of target cell lysis (Nagarkatti, 1988a).

Original studies suggested that LAK cells and NK cells were two distinct populations (Herberman, 1987), but at the present time, LAK cell activity is believed to be generated from IL-2 stimulation of NK cells. BCNU-treatment has been shown to enhance LAK activity, while having no effect on NK cells. These results may be explained in one of two possible ways. The LAK activity induced by drug treatment may be mediated by a population which is distinct from NK cells or it may be possible that although there is an increase in the number of IL-2 receptors on the NK cells following treatment, this increase may not be enough to sufficiently stimulate the NK cells.

Tumor-bearing mice which have been treated with BCNU generate higher levels of tumor-specific CTL and T helper cell activity (Nagarkatti et al., 1988b). This may continue for
as long as 2-3 months after treatment. To investigate the effect of BCNU-treatment on the 
ability of LSA tumor-bearing mice to generate tumor-specific CTL activity, spleen cells from 
BCNU-treated or untreated tumor-bearing mice were stimulated in vitro with irradiated LSA 
tumor cells. After 5 days of culture, the CTL activity generated was tested using \(^{51}\)Cr-labelled 
LSA targets. The data suggested that LSA tumor-bearing mice failed to generate CTL activity, 
while the BCNU-treated mice demonstrated strong CTL activity. This high CTL activity ob-
served in BCNU-treated mice was comparable to that seen in mice which had previously been 
immunized repeatedly with irradiated LSA cells and were therefore tumor-immune (Nagarkatti 
and Kaplan, 1985).

CTL-generated tumor cell cytotoxicity has been shown to be effective in mediating tumor 
rejection. A decrease in CTL activity due to the presence of suppressor cells has been char-
acterized in many tumor systems (Hellstrom, 1978). Rous virus-induced sarcoma is an ex-
ample of a tumor which is allowed to grow because of the presence of tumor-specific T 
suppressor cells (Hellstrom, 1978).

BCNU-treatment has also been shown to affect the host’s tumor-specific T helper cell 
activity. Following treatment with the drug, tumor-bearing mice exhibited a strong tumor-
specific delayed-type-hypersensitivity (DTH) reaction. For example, when normal, tumor-
bearing and BCNU-treated were challenged in the footpad with irradiated LSA cells, it was 
observed that normal and tumor-bearing failed to elicit a LSA-specific DTH response. BCNU-
cured mice elicited a strong DTH response to LSA cells, but not to a heterologous tumor (EL-4) 
(Nagarkatti, et.al., 1985).

Evidence suggests that any agent which can prevent the production or the activity of T 
suppressor cells should be effective in optimizing the host’s anti-tumor immunity. 
Cyclophosphamide is capable of inhibiting Ts function and subsequently allowing adoptively 
transferred T cells to mediate tumor rejection (North, 1982).

Using this data, it may be reasoned that BCNU, which is quite effective against the LSA 
tumor, may indeed be preventing the production or the function of tumor-specific T 
suppressor cells. This inhibition consequently allows CTLs and T helper cells to operate at
maximum efficiency and destroy any tumor cells not affected by the direct tumoricidal action of the drug. If this conclusion is correct, then the transfer of T suppressor cells into BCNU-cured mice will then make these mice susceptible to rechallenge with LSA tumor cells. When T suppressor cells from the spleens of normal mice were adoptively transferred into BCNU-cured mice and were then rechallenged with LSA, it resulted in a low survival rate. BCNU-cured mice that did not receive spleen cells were able to resist rechallenge even with high numbers of tumor cells. (Nagarkatti and Kaplan, 1985).

It has also been observed that BCNU-treatment was not effective at early stages (1-3 days) of tumor growth. In contrast, treatment was highly effective later, correlating with heightened T suppressor cell activity generated by LSA tumor-bearing mice.

The exact mechanism by which BCNU downregulates T suppressor cells is unclear, but evidence suggests that activation of Ts cells prior to treatment is important. This is supported by the fact that drug-treated mice demonstrate lower Ts activity in response to the syngeneic LSA tumor, but not to the non-syngeneic EL-4 tumor. Earlier data from our lab also showed that mice which were first treated with BCNU and then injected with LSA tumor cells failed to survive the challenge.

If the hypothesis that BCNU, in addition to being tumoricidal, positively modulates the host’s anti-tumor activity is correct, then BCNU-treatment should have varying effects in normal versus in nude or irradiated mice. To address this, the efficacy of BCNU-treatment on LSA tumor survival in normal, nude or irradiated mice was compared. All of the untreated tumor-bearing mice died in approximately 11 days, while 100% of the BCNU-treated mice survived for more than 80 days. On the other hand, BCNU-treated LSA tumor-bearing nude or irradiated mice all died by day 17. These data suggested that an intact immune system particularly T cells, is important in the efficacy of BCNU treatment of tumor-bearing mice (Nagarkatti et al., 1988b).

In order to determine the phenotype of the cells which mediate tumor rejection adoptive transfer experiments were carried out in normal, nude and irradiated mice. It was demonstrated that both CD4+ and CD8+ cells could mediate rejection. Furthermore, T cells cells
were not effective in tumor rejection when they were isolated from normal or tumor-bearing mice, while these cells were quite effective when isolated from BCNU-treated mice. It was also demonstrated that tumor-specific T cells were effective when they were transferred into nude or irradiated mice, but were not effective in tumor rejection in normal mice probably due to the presence of T suppressor cells (Nagarkatti et al., 1988b).

Earlier studies have suggested that administration of 20mg/kg body weight of BCNU and CLZ cured almost 100% of C57BL/6 mice bearing syngeneic LSA tumor, whereas STZ failed to cure any tumor-bearing mice even at a high dose of 200mg/kg body weight (Nagarkatti et al., 1989a; Feola and Maruyama, 1986). The failure of STZ to cure LSA tumor-bearing mice may be due to three possible mechanisms. First, the drug may have lower tumoricidal activity than BCNU or CLZ, but prior studies have demonstrated that this is not the case. In fact, STZ at 100mg/kg body weight had comparable tumoricidal activity to 20 mg/kg body weight of BCNU and CLZ. The second possibility is that STZ may in some way be suppressing the host’s anti-tumor immunity. This possibility was also ruled out by studies which demonstrated using various mitogens such as anti-CD3 monoclonal antibodies or PMA plus calcium ionophore that STZ is actually much less immunosuppressive than BCNU or CLZ. The elimination of these two possibilities further indicates that the inability of STZ to cure the tumor-bearing mice was probably due to the failure of STZ to deplete tumor-specific Ts (Nagarkatti et al., 1989a). These data also suggested that BCNU, CLZ and STZ may have differential effects on the T cells and therefore in the current study, a detailed analysis of the T cell subsets and their activation in various nitrosourea-treated mice was conducted. The data is in agreement with the notion that STZ fails to suppress the T cell function and therefore in a tumor-bearing host may be unable to inhibit the Ts function which is so critical for the effector cells to become activated and kill the tumor cells spared from the action of the drug.

The results presented in the current study demonstrated that nitrosoureas such as BCNU and CLZ but not STZ, caused significant alterations in the normal thymocyte subpopulations. Interestingly both BCNU and CLZ caused a decrease in the percentage of CD4+ CD8+ T cells and an increase in the percentage of CD4− CD3+ T cells. Furthermore, these nitrosoureas
induced a decrease in total cellularity of the thymus and in the absolute numbers of CD4⁺CD8⁺ T cells. Also, BCNU and CLZ caused a significant decrease in the total number of CD4⁺ and CD8⁺ T cells in the spleen without affecting the ratios of these subsets. We also observed that in tumor-bearing mice that BCNU treatment was followed by a dramatic infiltration of CD4⁺ cells into the peritoneal cavity which is the site of tumor growth. In contrast to BCNU and CLZ, STZ-treatment failed to bring about any changes in the T cell subsets in both the thymus and spleen. When the splenic T cells were tested for their responsiveness to a variety of mitogens, it was observed that BCNU and CLZ caused marked suppression of the T cell responses whereas similar or higher concentrations of STZ failed to induce significant immunosuppression. Also, addition of exogenous T cell growth factors such as rIL-2, rIL-4 or rIL-6, failed to reconstitute the decreased T cell responses seen in BCNU or CLZ- treated mice. Additional experiments carried out with tumor-bearing mice showed that drug treatment resulted no significant decreases in T cell responses.

The thymus is a primary lymphoid organ in which all T cells differentiate and mature. In a normal thymus, the majority (~80%) of the cells are CD4⁺ CD8⁺ (double-positive). These are believed to arise from a small number (~5%) of CD4⁻ CD8⁻ (double-negative) T cells (Scollay et al., 1985; Fowlkes et al., 1985; Kingston et al., 1985). The single positive T cells namely CD4⁺ and CD8⁺ T cells constitute ~10% and 5% respectively. The double-positive subset is believed to represent an intermediate stage in the differentiation pathway giving rise to the mature CD4⁺ and CD8⁺ T cells and furthermore it is likely that the T cell tolerance to self antigens occurs at the double-positive stage of T cell development (Smith, 1987; Kappler et al., 1987; MacDonald, et al., 1988). Recently cyclosporin A (CsA) which acts as a powerful immunosuppressive agent was reported to exhibit striking effect on the thymus. CsA blocked the differentiation of immature CD4⁺ CD8⁺ thymocytes into mature single positive T cells (Gao et al., 1988). The effect of nitrosoureas on thymocyte-differentiation has not been studied thus far. In the current study it was observed that BCNU and CLZ significantly decreased the total number of double-positive cells in the thymus. This suggested that BCNU and CLZ may se-
lectively act on immature cortical cells or alternately may inhibit the differentiation of double-negative T cells into double-positive T cells.

When the effect of nitrosoureas on the spleen was investigated, BCNU and CLZ-treatment decreased the absolute number of mature CD4+ and CD8+ T cells in the spleen. Also, when functionally analyzed, the T cells from the spleen were markedly inhibited in their responsiveness to a variety of T cell mitogens. Interestingly, STZ in most experiments failed to cause significant alterations in the T cell functions.

The biological effects of nitrosoureas include alkylation, carboxamoylation, inhibition of repair of induced DNA strand breaks, interference with synthesis and processing of ribosomal and nucleoplasmic RNA, and inhibition of DNA polymerase II. These drugs are highly reactive with short half-lives due to spontaneous decomposition, with the generation of highly active products including those capable of alkylation and carboxamoylation (Kohn, 1977).

These decomposition products include a chlorehthyl dihydroxide and an isocyanate. The chlorehthyl molecule in turn, decomposes further to produce a chlorehthyl carbonium ion which is responsible for the alkylation of DNA by producing interstrand cross-links and breaks. The isocyanate reacts with intracellular proteins via the carboxamoylation of the isocyanate molecule.

The tumorcidal properties of nitrosoureas are due to alkylation. The specific mechanism involves the chlorehthylation of a nucleophilic site on one strand of a DNA molecule. A chloride ion is then cleaved off the opposite strand generating a reactive carbonium ion and an ethyl bridge forms between the two strands (Kohn, 1977). Cross-linking may also occur between DNA and nuclear proteins.

The organic isocyanates produced by the decomposition of nitrosoureas undergo carboxamoylation reactions. This activity varies among the various drugs, with BCNU and CCNU being strong carboxamoylators and CLZ and STZ having only weak activity (Wheeler, et. al., 1974). The repair of DNA damage is impaired by nitrosoureas which have high carboxamoylating activity. Also, carboxamoylation has little if any effect on the anti-tumor activity of the drugs. CLZ which is a weak carboxamoylator has significant anti-tumor activity.
The structure of the individual nitrosourea determines the site of binding to specific areas of nuclear chromatin. There is preferential binding to either transcriptional regions, nucleosomes or non-transcriptional regions. CCNU, which is a highly myelosuppressive drug, binds to the nucleosome regions of marrow cell chromatin. On the other hand, CLZ which has low myelosuppressive activity, binds at internucleosomal linker regions. STZ is also minimally myelosuppressive. Both of these nitrosoureas possess within their structure a glucose moiety which causes a difference in the nuclear binding site of the drug and therefore reduces the toxic side effects of the drug. Nitrosoureas with glucose moiety such as STZ and CLZ have therefore been tried clinically because they are believed to be less myelosuppressive but having similar or more alkylating activity (Anderson et al., 1975). Earlier studies have shown that BCNU and CLZ had comparable toxicity in vitro and in vivo against LSA tumor. However, at similar drug concentrations, STZ was less effective (Nagarkatti et al., 1989a). This suggests that STZ may have lesser alkylating activity when compared to BCNU and CLZ and this property may be related to its inability to act on T cells is not clear and needs further studies.

Although the exact mechanism by which BCNU and CLZ suppress the T cell activation and the macrophage accessory functions is not clear, our studies suggest that it is probably not due to inhibition of interleukin secretion as seen using certain drugs such as CsA inasmuch as, addition of IL-1, IL-2, IL-4 and IL-6 failed to reconstitute their defect. Alternately, the defect in T cells may be related to unresponsiveness to the interleukins.

Although BCNU was found to be immunosuppressive in normal mice, its effect in tumor-bearing mice on anti-tumor immunity was not clear. In the current study when this question was addressed, we made an interesting observation that immediately following BCNU injection there was a significant infiltration of T cells, particularly CD4+ T helper cells at the site of tumor-growth. This infiltration was not due to the direct action of BCNU, inasmuch as, in normal mice BCNU failed to induce a similar infiltration.

Further studies demonstrated that the CD4+ T helper cells secrete IL-2 and IFN-γ but not IL-4. Also, functionally they could activate only the macrophages to become tumoricidal, but failed to activate the B cells. These data clearly suggested that the tumor-infiltrating CD4+ T
cells belong to the Th1 subset. Such cells could also induce tumor rejection in nude mice, independent of CD8+ T cells, by activating the macrophages (Nagarkatti et al., 1990).
References


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