# The Immunomodulatory Properties of AZT Used In The Treatment of AIDS

by

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# Abstract

AZT (3'-azido-2', 3'-dideoxythymidine) has been shown to prolong the survival of patients infected with human immunodeficiency virus (HIV) and decrease the severity of opportunistic infections. Such studies have prompted the use of AZT to treat symptomless individuals infected with HIV in the hope of delaying or even preventing the progression to acquired immunodeficiency syndrome (AIDS). However, before chronic use of AZT in symptomless individuals is initiated, it is important to establish whether this anti-viral drug would directly alter the phenotype and functions of the cells involved in the immune system. In the current study we observed that AZT when administered orally for 7 -14 days into DBA/2 mice at 500 - 1000 mg/kg body weight induced a dose dependent decrease in cellularity of the thymus. AZT caused significant alterations in the thymus resulting from a significant decrease in the number of double-positive ($CD4^+CD8^+$) cells and increase in the number of double-negative ($CD4^-CD8^-$) cells. Interestingly, after the i.p. administration of interleukin-2 (IL-2) simultaneously with AZT, the total cellularity of the thymus was completely reconstituted. We also observed that AZT effectively suppressed the *in vivo* T cell response to conalbumin and gp120 of HIV. Furthermore, addition of AZT to *in vitro* cultures caused a dose dependent decrease in T and B cell proliferative responses to mitogens at 50μM or greater concentrations. Also, AZT inhibited the generation of cytotoxic T lymphocytes when added in culture and this inhibition was reconstituted by the addition of exogenous IL-2. Together, our studies demonstrate that AZT modulates the phenotype and function of cells of the immune system which, in turn, could have marked repercussions on immune responses of the host toward infections and cancers. Also, our data demonstrating that AZT can suppress T cell responsiveness against HIV antigens caution against chronic use of AZT in asymptomatic HIV-infected individuals.

**Abstract**

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# 1.0 General Introduction and Review of Literature

## 1.1 Immune System:

The exact time in which the concept of immunity was first described is not known. Some of the earliest cases come from ancient African tribes. It is reported that these people mixed dried and pulverized poison glands from snakes and then injected them under their skin, producing a resistance to snake bite venom. As early as 1000 A.D. the Chinese, noticing that it provided resistance, inoculated healthy people with material from pustules of a person suffering from smallpox (Klein, 1982).

One of the first recorded immunological discoveries was in 1798 when Edward Jenner devised a vaccination for smallpox. Almost a century later, in 1879, Pasteur injected an altered strain of chicken cholera (non virulent) into chickens causing them to be resistant to reinfection by the virulent strain. This discovery was said to be the beginning of the modern field of immunology. The golden era for microbiology and immunology came at the turn of the century when Pasteur, Koch, and their colleagues and students, did work to establish the germ theory of disease and to isolate many disease causing agents (Klein,1982).

Soon it was discovered that specific immunity to infectious diseases could be developed. However, the mechanism by which the immune system worked was still a mystery. This question has challenged man for centuries. Much has been discovered; however, much still needs to be learned.

A primitive system of defense can be found in all animals. This system consists of natural barriers against pathogenic invasions and includes the skin, mucosal surfaces and other factors such as lysozymes and lactic acid. Phagocytic cells with their ability to non-specifically engulf and destroy foreign substances provide another primitive means of defense (Roitt, 1989).

The vertebrate population has developed a more complex and unique form of immunity. This immune system is composed of several lymphoid organs and cellular populations. The primary organs in the immune system are the thymus and the bone marrow. Both of these entities play distinct and vital roles in the immune system (Roitt 1989).

The bone marrow can be separated into two distinct areas, the red marrow and the yellow marrow. The red marrow gets its color from the abundance of erythrocytes. The yellow marrow is generally hematopoietically inactive giving it a relatively colorless appearance. The bone marrow is the area in which pluripotent cells originate. Pluripotent cells are cells that can differentiate into more than one type of cell. This differentiation is limited to certain types of cells. For example, hematopoietic cell can only differentiate into erythrocytes or monocytes, but not into neurons or epidermal cells. The cells within the bone marrow develop and eventually become the mature cells of the immune system (Klein, 1982).

The thymus also plays an important role in the immune system. The thymus consists of two elongated pyramidal lobes which are superficially joined. Each lobe is enclosed into a membraneous capsule. Within each lobe there are two distinct regions. The peripheral region, or cortex, is primarily composed of lymphocytes and the inner region, or medulla is primarily epithelial cells. The organ itself reaches its greatest relative rate at birth, and the absolute weight increases until puberty. From puberty on, the thymus becomes reduced until it reaches about 50% of its maximum weight. The decrease in size is attributed to its loss in function as the individual ages. The main function of the thymus is to participate in the education and maturation of the T lymphocytes. Lymphocytes as well as other leucocytes express a large number of molecules on their surface. Many of these molecules are present on the cell for a short period of time related to a particular stage of cell differentiation or maturation, while others are specific for different cell lineages. These molecules can serve as markers and many can be identified by specific monoclonal antibodies. A systematic nomenclature has been developed for these molecules. This system is referred to as the CD (cluster designation) system. In many cases the function of these molecules is known. For example, the CD4 molecule is present on $T_h$ cells and functions in the recognition of MHC class II.

Stem cells from the bone marrow enter the thymus and begin their journey to becoming mature T cells. These cells go through a series of developmental stages. In the first stage, the cells express adhesion molecules, CD7 along with CD2 and CD5 all of which do not distinguish the T cell lineage. The cells then progress to a stage in which they express CD1, as well as the coexpression of the CD4 and CD8 molecule. Finally the cells lose the CD1 molecule and gain the expression of CD3 and lose either the CD4 or the CD8 marker. Throughout this process, the cells go through a series of negative and positive selection ensuring that the cells allowed to mature will function properly in the immune system. An important characteristic for a mature T cell to possess is the ability to discriminate between self and nonself. If cells are allowed to leave the thymus without this ability, it is possible that the host will develop autoimmune diseases, in which the T cells would attack and destroys its own tissue (Klein, 1982).

Lymph nodes act as major secondary organs in the immune system. The lymph nodes are spherical or bean shaped bodies that range from the size of a pin head to as large as a walnut. Lymph nodes are located strategically throughout the body. The areas in which they are located drain different parts of the body, enabling them to filter antigens from tissue fluids and lymph. The lymph nodes are composed of T and B cell areas. The T cells are located in the paracortex and the B cells are located in the cortex. In addition there is a central area that contains both T and B cells. When an antigen is drained into the appropriate area of the lymph node, the appropriate lymphocyte interacts with it and initiates an immune response. The lymph nodes ultimately provide an environment in which the chances of an antigen interacting with a lymphocyte are greatly increased, making the immune system more efficient (Roitt, 1989).

Another important secondary organ in the immune system is the spleen. Although the presence of the spleen is not essential for life, it does play a significant role. Basically the spleen acts as a filtering unit for blood cells and fluids and as an organ of immunity. As a filterer, the spleen sorts out blood cells and examines them for imperfections. Macrophages play a vital role in this process by stopping and engulfing any cell with a defect. As an immune organ, the spleen traps and processes particles that are able to elicit an immune response. In addition, the spleen provides a home for lymphocytes and macrophages, enabling the interactions between the antigen and immune cells to take place. As seen with the lymph nodes, the spleen also creates a situation in which the immune system can function more efficiently (Klein, 1982).

There are a number of responses which the immune system can exhibit to eliminate foreign invaders from the immune system. The responses can be divided into two major categories, specific and nonspecific. The specific responses can be further subdivided into two separate but related systems, humoral and cell-mediated immunity.

Humoral immunity works through the actions of antibodies which are secreted into the body's fluids (humors) by B cells. B cells represent approximately 5 to 15% of circulating lymphoid cells. A distinguishing characteristic of B cells is the presence of endogenously produced immunoglobulins (antibodies) on their surface. These antibodies act as receptors for the specific antigen. After initial interaction with the antigen, the B cells become activated, producing a high number of endogenous as well as exogenous antibodies. There are 5 distinct classes of immunoglobulins; IgG, IgA, IgM, IgD, and IgE. The majority of B cells express IgM or IgD. Antibodies express 2 distinct areas; one region facilitates the binding to the antigen and the second is involved with binding the immunoglobulin to the host cells of the immune system (Roitt, 1989).

Once released into the bloodstream, the antibodies can act in a number of ways. First, antibodies can act by directly combining and neutralizing bacterial toxins. Secondly they can prevent virion attachment to target cells. Finally, antibodies can coat bacteria leading to an increased chance of phagocytosis. Protection of this type can be transferred from one individual to another by taking the cell-free serum from an individual producing the antibody and injecting it into another individual. This type of immunity includes both classical antibody-mediated immunity combating viruses and bacteria, and immediate hypersensitivity reactions better known as allergies (Roitt, 1989).

Cell-mediated immunity is a specific response which is dependent on the presence of sensitized T cells. The main action of cell-mediated immunity is to protect the host against intracellular infectious agents. T cells facilitate immunity in a variety of ways. Some populations of T cells synthesize and secrete lymphokines that stimulate macrophage or additional T cell growth, others produce lymphokines that attract immune cells to the area of infection and other T cells become cytotoxic, with the ability to directly kill target cells.

T cells differentiate and mature in the thymus and can recognize an antigen only in the association with cell surface molecules encoded by the major histocompatibilty complex (MHC). This phenomena, known as MHC- restriction, helps the T cells to distinguish self from non-self cells (Roitt, 1989).

It is possible to distinguish T cells from other lymphocytes by the presence of the T cell receptor molecule (TCR). There are two distinct types of TCRs: TCR-1 and TCR-2. TCR-1 consists of a heterodimer of $\gamma$ and $\delta$ polypeptides. TCR-2 is a heterodimer of two disulfide linked polypeptides, $\alpha$ and $\beta$. Both receptors are associated with a complex known as CD3. Approximately 95% of the T cells within the blood have TCR-2. TCR-2 can divided into two distinct populations. The first group is known as the T helper cells ($T_H$), which express CD4 molecules, important for MHC class II restricted immune recognition and a second group known as Cytotoxic T cells which express the CD8 molecules important in MHC class I restricted immune recognition (Roitt, 1989).

$T_H$ cells recognize antigens in association with molecules encoded by class II region of the Major Histocompatibility Complex (MHC). A major role of the $T_H$ cells is to control and modulate the development of the many immune responses. These cells upon stimulation, produce growth and differentiation factors which can stimulate their own growth as well as affect other immune cells. The interactions between the helper T cell and other immune cells occur through cell to cell interactions or through the production of cytokines (chemical mediators). Recent studies have suggested that the $T_H$ cells can be functionally divided into two subgroups. $T_H1$ cells, which produce IL-2, an important T, B and NK growth factor and IFN-γ, which is important in the activation of numerous lymphocytes and induces the expression of MHC class II molecules, and provide help to the macrophages. In contrast to $T_H2$ cells, which secrete IL-4 and IL-5 and provide help to T and B cells (Mosmann and Coffman, 1989).

In order for cells to be stimulated by the interleukins (IL) they must express the proper receptors. The receptor for IL-2 consists of two chains; α and β. Resting T cells have small numbers of the α chain and essentially no β chains. After activation, the β chains are actively produced while the α chains are produced to a lesser extent, resulting in approximately 5,000 α chains and 50,000 β chains being expressed on each activated T cell. The high affinity receptor for

IL-2 are the $\alpha-\beta$ heterodimers which are capable of driving T cell growth. The low affinity receptors are the $\beta$ chains which are unable to activate the cells. The $\alpha$ chain is an intermediate receptor which can activate T cells when it is present in high concentrations. Once induced IL-2 receptors remain on the cell surface for about one week, unless reinduced. The action of IL-2 on the IL-2 receptor can be autocrine or paracrine but no evidence for endocrine action has been shown.

Cytotoxic T lymphocytes (CTL) express CD8 adhesion molecule and recognize antigens in association with the class I molecules encoded by the Major Histocompatibility Complex (MHC). CTLs are able to directly lyse virally infected or transformed target cells thereby playing an important role in protection against viral infections and cancer. Infected cells are recognized by the presence of viral or tumor-specific antigens in association with class I MHC molecules on the cell membrane. The CTL, after recognizing the antigens, release lymphotoxins and perforins which are capable of killing the infected cell (Roitt, 1989).

In order for $T_H$ lymphocytes to recognize an antigen, it must first be presented in an appropriate manner. This task is taken care of by a specialized group of cells known as antigen presenting cells (APC). Macrophages are the most common APCs. Other APCs include dendritic cells, B cells, and NK cells. Macrophages have the ability to engulf particles which are then packaged into vacuoles. These vacuoles then fuse with lysosomes containing hydrolytic enzymes which break down the engulfed particles into an antigenic form. The antigen then associates with Class II MHC molecule on the membrane. This association allows $T_H$ cells to recognize the antigen and mount an appropriate immune response. Macrophages also, upon activation, secrete monokines such as IL-1 and colony stimulating factor (CSF). IL-1 acts by stimulating T cells and CSF has the ability to stimulate proliferation and maturation of additional macrophages (Roitt, 1989).

Another cell population involved in the overall immune response is the natural killer (NK) cells. These cells are derived from large granular lymphocytes and comprise about 5% of lymphoid cells. The majority are $CD16^+$ and $CD3^-$. A distinguishing function of NK cells is their ability to lyse target cells without any MHC restrictions. They are effective in eliminating virus infected and tumor targets (Roitt, 1989). This function could play an important role in eliminating tumor cells that have escaped recognition by the CTL by down regulating their MHC class I molecule.

## 1.2 Immunotoxicology

With the recent advent of the appropriate immunodiagnostic tests it is possible to assess the number and function of the various components of the immune system. With this ability, it is also possible to determine the presence of any immunodeficiency. The immune system is a complex arrangement of cells that work in cooperation to protect the host from infectious agents. During their interactions, a number of molecular communications occur. These communications ultimately result in the modulation and exhibition of the proper immune response. For example, many cells release various molecular regulators, including complement, prostoglandins, lymphokines and other allergic and inflammatory mediators. Any modification of this process could lead to the malfunctioning of the immune system. Many secondary immune deficiencies involve defects in cellular response mediated by T cells, macrophage and NK cells. In most cases, modifications of the immune system by xenobiotics, either immunotherapeutic agents or immunotoxins, are not completely selective for a single cell and no defect involving one cell population occurs without affecting another population. These conditions, therefore, make the determination of immunotoxic effects difficult.

Because of location and distribution, mononuclear phagocytes and lymphocytes must deal with many xenobiotics entering the body. These interactions could ultimately lead to immune suppression, immune dysregulation (autoimmunity) or response of immunologic defense mechanism to xenobiotics (hypersensitivity). Immunosuppressive agents frequently cause serious secondary complications, resulting in an increase in bacterial, viral, fungal and parasitic infections in addition to increased frequency of cancers.

Both acute and chronic exposures to xenobiotics can alter a specific immune response.

Toxicological manifestations in the immune system can appear in many ways. Alterations in lymphoid organs, weights and histology, quantitative or qualitative changes in cellularity of lymphoid tissues, along with impairment of cell function at effector and regulating level and increased susceptibility to infectious agents are all signs of immunotoxicity. Immunotoxic effects can occur at exposure levels below the levels where other toxic effects are recognized. Ultimately any agent that alters the delicate balance of the immune system can lead to an immunotoxic state (Dean and Ward, 1983).

Many immunosuppressive agents have been characterized. For example, Azathioprine is an immunosuppressive drug used in organ transplantations. Cyclosporin A is another immunosuppressive agent used in treating allograft rejection and graft- versus-host disease. In addition to these drugs, several environmental immunotoxicants have been identified. One such agent is benzene. Benzene is associated with suppression of all marrow derived elements. Exposure results in the death of the animal due to opportunistic infections (Dean, et al., 1985).

It is critical to study immunotoxicology for a number of reasons. There a number of different substances to which we are exposed to daily, from environmental pollutants to drugs taken therapeutically. Throughout history and especially since the industrial revolution, numerous potential toxicants are being introduced into the environment at increasingly high concentrations. Along with the classical toxicological studies, it is important to study the effects on the immune system but do not have other toxic effects. In many cases substances adversely affects the immune system. With a down-regulated immune system individuals could succumb to infections and diseases that would not originally be tolerated, significantly decreasing overall health. Secondly, many individuals are presently taking drugs to treat or cure an undesirable health condition. It is important to determine the immunotoxic effects of these therapeutic agents. Individuals taking these drugs are using them in the hopes of treating a particular condition, however, if these drugs simultaneously cause immunosuppressive effects, the individual under treatment may become susceptible to other problems. Some of these could be more serious than the original condition.

## 1.3 Acquired Immunodeficiency Syndrome (AIDS):

The human immunodeficiency virus (HIV) is a retrovirus that causes AIDS (Barre-Sinoussi, et al., 1983). HIV was first discovered in 1981 and has since developed into a world wide epidemic. The disease is progressive and irreversible, with a mortality rate reaching 100 percent (Fauci, 1988). The main target of HIV is the immune system. Without an intact immune system, individual's defenses are severely compromised, leading to increased susceptibility to opportunistic infections and cancers. Individuals with AIDS do not die directly from the human immunodeficiency virus but from opportunistic infections that take advantage of the individual's suppressed immune system. Untreated, AIDS will lead to the death of the infected individual in 2 to 6 years after clinical diagnosis (Georgiev and McGowan, 1990).

An intricate part of the immune system is the $CD4^+$ T helper subset of cells. T helper cells are important in both humoral immunity to extracellular pathogens and cell mediated immune responses. Without the $CD4^+$ subset the immune system would become severely compromised. The surface of HIV contains an envelope glycoprotein known as gp120. Gp120 has a high affinity for the CD4 molecule present on the T helper cells. Shortly after the virus binds to its host it is able to enter the cell and begin replication.

The process of replication used by HIV is unique to retroviruses in that they contain RNA as their genomic material and use the enzyme reverse transcriptase to transcribe RNA to DNA. The information gathered on HIV has allowed investigators to devise different approaches for treatments or cures for the disease. Two of the major areas of research involve the blocking of HIV from binding to the host cell and secondly, preventing the virus from replicating by blocking the activity of reverse transcriptase. Significant success has been accomplished in in trying to inhibit reverse transcriptase by treatment with nucleoside analogs.

## 1.4 NUCLEOSIDE ANALOGS USED IN THE TREATMENT OF AIDS:

The development of rapid assay systems for the testing of compounds for anti-HIV activity has led to the discovery of numerous drugs with potential for the treatment of AIDS. The most promising of these drugs were found to be deoxynucleoside analogs that contained an azido group in substitution of the 3'-hydroxyl group. These drugs have been shown to inhibit the activity of reverse transcriptase by acting as a chain terminator or by acting as a competitive inhibitor of the physiologic deoxynucleoside 5'-triphosphates. In addition to true dideoxynucleosides, a number of other nucleoside analogues have been found to have anti-HIV activity. For example, acyclic compounds may structurally resemble dideoxynucleosides and act in a similar way. The first drug to undergo testing was 3'-azido 2'-3-dideoxythymidine (AZT). This drug was selected because it already had a corporate sponsor and had undergone animal toxicology testing. At the same time other similar drugs, such as dideoxycytidine (ddC), dideoxyinosine (ddI), and dideoxyadenosine (ddA), were being considered for entry into clinical trial.

## 1.5 3'-azido 2'-3'-dideoxythymidine (AZT):

AZT is a dideoxynucleoside that is now being used in the treatment of AIDS. Although there are differences among the dideoxynucleosides, most act in a similar way. These drugs are effective in inhibiting reverse transcriptase (Furman et al., 1986; Hoa et al., 1987; Mitsuya et al., 1988.). In order to carry out this function, each must be converted into its 5'-triphosphate form by the target cell. This activation process called anabolic phosphorylation, is carried out by a series of kinases (Cheng, et al., 1987; Starnes and Chang,1987; Vrang, et al., 1987). Once in their active form, the dideoxynucleosides are able to inhibit HIV replication. AZT and other dideoxynucleosides are able to do this by interfering with the viral DNA polymerase (reverse transcriptase). Reverse transcriptase is essential for viral replication. After entering the cell, the virus, utilizing reverse transcriptase, makes a complementary strand of DNA copy from the viral genomic RNA and then initiates the the production of a second copy, making a double stranded copy of its genomic information. There are two proposed mechanisms for AZT's anti-retroviral action. First as a triphosphate, it competes with cellular deoxynucleoside-5'-triphosphates that are essential substrates for the formation of proviral DNA by reverse transcriptase (Furman et al., 1986; Mitsuya et al., 1984). Second, they act as chain terminators. AZT differs from deoxythymidine by the substitution of a hydroxyl group on the 3' carbon of the sugar complex. This substitution prevents any further 5' to 3' phosphodiester links within the DNA complex, ultimately terminating DNA synthesis by reverse transcriptase (Furman et al., 1986; Waquar et al., 1984).

HIV reverse transcriptase is approximately 100 times more susceptible to this type of inhibition than mammalian DNA polymerase alpha making it a reasonable target for retroviral control . However, it has been shown that mammalian DNA polymerase gamma, found in mitochondria, and DNA polymerase beta are sensitive to dideoxynucleosides, leading to possible toxic effects from AZT exposure (Furman et al., 1986; Waquar et al., 1984).

The most effective route for administration of AZT is by intermittent high dose administration by gavage, as opposed to continuous low dose infusions. Researchers suggest that this may be due to AZT pharmokinetics. It has been shown that mice implanted with ALZET miniosmotic pumps loaded with 25mg/ml of AZT had a plasma level of AZT of approximately 1μM. However, if the mice were treated with 50 mg/kg body weight AZT by gavage, the peak plasma level would reach approximately 150μM within 5 minutes of administration (Bilello et al., 1990). Studies have shown that AZT efficacy is greater when given in intermittent high doses by oral gavage suggesting that reaching high peak levels may enhance the chemotherapeutic applications of AZT as opposed to maintaining a constant plasma level (Bilello et al., 1990)

AZT enters cells by passive, nonfacilitated diffusion (Zimmerman et al., 1987). Once inside the cell, AZT is transformed into its activated form by a series of kinases. In the first step, thymidine kinase produces 3'-azido 2'-3'-dideoxythymidine-5'-monophosphate (AZT-MP). Next two additional phosphates are added by thymidylate kinase and nucleotide diphosphate kinase to form 3'-azido-2'-3'-dideoxythymidine-5'-triphosphate (AZT-TP), its active form (Chang et al., 1987; Starnes et al., 1987; Vrang et al., 1987). Once in this form, AZT has potent activity against a wide spectrum of retroviruses, including human immunodeficiency virus type 2, human T-cell lymphotrophic virus Type I, animal lentiviruses and murine retroviruses (Dahlberg et al., 1987; Waquar et al., 1984).

The use of AZT in treatment of HIV may be limited. There is growing evidence that cells belonging to the monocyte-macrophage lineage may be a primary target for HIV infection. These nonproliferating cells have lower levels of kinases and may not be able to convert AZT into its active form. One study suggested that AZT is poorly phosphorylated in peripheral monocytes and macrophage and does not protect against infection by HIV (Richman et al., 1987). However, two additional studies showed that AZT at low concentrations was able to significantly inhibit the replication of HIV in monocytes and macrophage (Perno et al., 1988; Skinner et al., 1988). It has been suggested that the reason for these results can be explained by the fact that these cells possess very low levels of thymidine-5'-triphosphates creating a situation in which even small concentrations of activated AZT could effectively compete with the thymidine-5'-triphosphates necessary for HIV replication (Perno et al., 1988).

A little over two years after AZT was shown to inhibit HIV replication *in vitro* , its use was approved by the Food and Drug Administration for the treatment of

AIDS. Because the drug was approved so rapidly, it is possible that many possible traits of this drug were not determined. Currently, preclinical toxicity testing does not include examining the effects exhibited upon the immune system, but is mainly concerned with determining the target organs/systems and endpoints of the drug. In the case of HIV infection it is important to assess the effects of drugs being used in its treatment on the immune system because HIV replicates in the cells of the immune system and AIDS patients represent an immunologically compromised population. Most of the drugs being used are nucleoside inhibitors that target rapidly cycling cells, which are characteristic of cells involved in the immune system. It is possible, therefore, that AZT could have adverse effects on these cells.

Preliminary studies have shown some drawbacks to treatment of AIDS with AZT. Some negative side effects have been noted with the use of AZT. These include malaise or fatigue, dyspepsia, nausea, vomiting, and bloating (Fischl et al., 1990). Prolonged use in humans has been linked to the development of bone marrow toxicity, characterized by apparent megablastic anemia and granulocytopenia. AZT at concentrations of 0.5 - 50$\mu$M resulted in the induction of hematopoietic suppression manifested by anemia, neutropenia, and overall bone marrow failure (Ruprecht et al., 1986). One study suggests that AZT may selectively inhibit human lymphocyte functions. This may have implications for long term therapeutic use of AZT (Shaw, et al. 1991). These studies have created concern over the possible uses of AZT and reaffirmed the need for additional studies.

## 1.4 Specific Aims:

With the discovery that AZT can inhibit HIV replication, it has been suggested that chronic use and early treatment should be initiated in symptomless individuals infected with HIV, with the hope of delaying or even preventing progression to AIDS. However recent studies have shown some drawbacks in the chronic use of AZT, such as bone marrow suppression characterized by apparent megoblastic anemia and granulocytopemia. Inasmuch as HIV replicates in the $T_H$ cells, macrophages and thymocytes, it is important to know whether AZT-treatment would alter phenotype and functions of the cells of the immune system. Any alteration in the immune function mediated by AZT in turn could affect not only the replication of HIV but also susceptibility to other infections or development of cancer. With this view in mind, we initiated the studies to test the hypothesis of whether AZT being a nucleoside analog was able to alter the differentiation or functions of immune cells in the normal murine host.

The specific aims of the research were to determine:

1. The *in vivo* effects of AZT on T cell differentiation in the thymus and spleen.

2. The *in vivo* effects of administration of AZT on T cell-mediated immune response to foreign antigens such as conalbumin and gp120 of HIV.

3. The *in vitro* and *in vivo* modulation of polyclonal T and B cell activation by AZT.

# 2.0 IMMUNOMODULATORY PROPERTIES OF AZT

## 2.1 Introduction:

AZT (3' azido 2' 3' dideoxythymidine) was one of the first drugs to demonstrate anti-HIV activity (Yarchoan et al., 1986). AZT shows potent activity against a broad spectrum of retroviruses: HIV type 2, human lymphotrophic virus type 1, animal lentivirus and murine retrovirus (Yarchoan, 1986).

AZT enters the cell by passive, non facilitated diffusion (Zimmerman, 1987). Once inside the cell, AZT can inhibit HIV replication in two ways. First, AZT can act as an inhibitor by interfering with the synthesis of new DNA during reverse transcription. Inside the cell AZT is phosphorylated into a triphosphate. Once in its active form ( 3' azido 2' 3' dideoxythymidine), AZT binds to the RNA dependent DNA polymerase (reverse transcriptase) so that incoming molecules have no attachment site. AZT differs from deoxythymidine by the presence of azido group in place of an hydroxyl group at the 3' carbon of the sugar complex. This 3' substitution prevents further 5' to 3' phosphodiester linkages, resulting in the termination of DNA synthesis. Reverse transcriptase is approximately 100 times more susceptible to inhibition by AZT than mammalian DNA polymerase, making it a reasonable treatment against HIV. Secondly, as a triphosphate, AZT can compete with cellular deoxynucleoside 5' triphosphates essential for formation of proviral DNA by reverse transcriptase (Waqar, 1984).

Recently AZT was shown to prolong the survival of patients with AIDS. This has led to the debate on whether AZT should be offered to symptomless individuals infected with HIV with the hope of delaying or even preventing the progression to AIDS. Since the immune system is the primary target of HIV, it is critical to know if the drugs used in the treatment of AIDS alter the functions of the immune system. With initial studies showing the possibility of AZT causing toxicity to cells, it is of great importance to investigate the properties of AZT more closely. If symptomless individuals take these drugs for extended periods of time, it is possible that AZT could alter the immune system in a way making the host more accessible for HIV and other opportunistic infectious agents, creating additional problems instead of treating them.

## 2.2 Experimental Procedures

### 2.2.1 Mice

Adult, female DBA/2 were used in the majority of the experiments. Spleen cells from C57BL/6 mice were used as allogeneic stimulator cells. All the mice were obtained from National Cancer Institute, Bethesda, MD. The weight of the mice ranged from 25 to 30 grams. The mice were housed in polyethylene cages (Animal Storage Isolators, Nu Aire Inc., Plymouth, MN) in groups of five mice per cage. The cages contained wood shavings and were shelved in a laminar flow cabinet, providing a sterile environment. The rooms were kept on a twelve hour light/dark (6 a.m. to 6 p.m. light) cycle at a temperature of $74 \pm 2\ ^{0}F$.

### 2.2.2 Drugs

AZT was kindly provided by Burroughs Wellcome Pharmaceuticals, Research Triangle Park, NC and stored at $-20^{0}C$. AZT was dissolved in PBS for *in vivo* administration and in media for *in vitro* studies. IL-2 was obtained from Hoffman LaRoche and stored at $-80^{0}C$

## 2.2.3 In Vivo drug treatment:

To study the immunomodulating properties of AZT, the drug was administered orally by gavage. AZT was dissolved in PBS and was administered in 0.2 ml volume. In this study 0, 100, 500 and 1000 mg/kg body weight were used. The length of administration ranged from 7 to 14 days. Groups of 3 to 5 mice were used.

## 2.2.4 Cell separation:

After the appropriate time period the mice were sacrificed by chloroform asphyxiation. The thymus, lymph nodes and spleen were then surgically removed placed in stomacher bags, containing 10 ml of medium, and kept on ice. The cells from the thymus, lymph nodes and spleen were made into single cell suspensions by a laboratory homogenizer (Stomacher, Tekmar Co., Cincinnati, OH). The production of single cell suspension was confirmed by microscope observation. The cells were then placed into 12 ml culture tubes and spun down for 10 minutes at 1000 rpm. The erythrocytes were removed by lysis with 4 ml of 0.83% ammonium chloride in solution, after which the cells were washed two additional times. The medium for homogenization and resuspension consisted of RPMI-1640 supplemented with 10% fetal calf serum (GIBCO Lab., Grand Island, NY), 10 mM HEPES, 1mM glutamine, 40 mM/ml of gentamicin sulfate and 50mM 2-mercaptoethanol.

### 2.2.5 Two color fluorescent staining for CD4 and CD8 markers:

Pelleted cells (2 x $10^6$) were incubated with 1μl of FITC-conjugated anti-CD8 (Pharmigen) and 1 μl of phycoerythrin (PE) conjugated anti-CD4 mAb (Pharmigen) in 5 ml culture tubes for 30 minutes at 4 °C. The cells were washed twice with medium and finally suspended in 0.3 ml medium. The medium used throughout this procedure was PBS containing 0.02% sodium azide.

### 2.2.6 Flow cytometry:

Fluorescence of the cell populations was measured by flow cytometric analysis (Seth et al., 1988). The analysis was performed by an Epics V, model 752 (Counter Electronics, Hialeah, Fla.). The four parameters per cell that were studied included forward angle and 90 degree light scatter, green and red fluorescence. Ten thousand cells were analyzed in each sample.

## 2.2.7 Determination of total cellularity:

The thymus, spleen or lymph nodes were surgically removed and transformed into single cell suspensions. The cells were washed three times and resuspended in medium. One-hundredth or .05 ml of trypan blue dye was added to .0025 ml of the cell samples. The suspension was then placed on a hemocytometer microscope slide. The number of cells within the grid was used to determine the total cellularity. Cells able to exclude the dye from their interior were determined to be viable by observing the cells under an inverted phase contrast microscope (Olympus model T041).

## 2.2.8 Direct determination of cell viability exposed to AZT:

4 x $10^6$ splenic cells per well were cultured in the presence or absence of various concentrations of AZT (0, 1, 10, 100, 1000, or 2000 $\mu$M) for 48 hours in 24 well plates. The number of viable cells was then determined by trypan blue exclusion.

## 2.2.9 Thymidine uptake assay

Single cell suspensions containing both T and B cells were plated in triplicates, at a concentration of $4x10^5$ to $5x10^5$ cells per well, in 96 well flat bottomed plates and were stimulated by various mitogens. T cells were stimulated with Con A (5 mg/ml), PHA (50 mg/ml), anti-CD3 mAb (1:25), or anti-$\alpha\beta$ mAb (1:50). B cells were stimulated with lipopolysaccharide (LPS) (50 $\mu$g/ml), or anti-IgM (20 $\mu$g/ml). LPS is a product of some gram-negative bacterial cell walls which acts as a B cell mitogen. Cells were cultured in the presence of the various mitogens for 48 hours in the presence or absence of AZT for 48 hours. The samples were pulsed with 2 $\mu$Ci of ($^3$H) thymidine during the last 18 hours of culture. The cells were harvested onto glass fiber filter discs and the incorporated radioactivity was counted by a scintillation counter. The controls in each experiment were cells cultured in the absence of the mitogens, as well as groups set up in the absence of AZT.

## 2.2.10 Colorimetric MTT (tetrazolium) assay:

Single cell suspensions containing both T and B cells were plated in triplicates, at concentrations of 4 x $10^5$ cells per well in 96 well flat bottomed plates and the cells were stimulated with various mitogens. MTT (3-(4,5-dimethylthiazol 2-yl)-2,5 diphenyl tetrazolium bromide) was dissolved in PBS at 5 mg/ml and filtered to sterilize and remove any small amount of insoluble residue present in some batches of MTT. Five-hundredth ml of stock MTT solution (10 ml per 100 ml medium) was added to all wells of the assay, and plates were incubated at 37$^o$C for 4 hours. The supernatant (.01 ml) was removed from each well and 0.1 ml of Acid-isopropanol (100 ml of .04 NHCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After 5 minutes at room temperature, ensuring that all crystals had dissolved, the plates were read by an ELISA reader using the test wavelength of 570nm (Mosmann T, 1983). The controls for each experiment were cells cultured in the absence of any mitogen, as well as cells not exposed to AZT.

## 2.2.11 In Vivo T cell stimulation:

Mice were orally treated with various concentrations of AZT for 14 days. Seven days prior to the last AZT treatment, 100μg of conalbumin mixed with complete Freund's adjuvant (1:1), in the volume of 50μl, was injected into the rear footpads of the mice in each dosage group. At the end of the treatment period, the lymph nodes draining the rear footpads were removed and single cell suspension were prepared. The cells were then cultured with antigen presenting cells (APC) and conalbumin *in vitro* to stimulate the antigen-specific T cells. The proliferative response was then determined by a thymidine uptake assay after 48-72 hours of culture. T cell proliferative response to gp120 of HIV was studied in a similar way as described above by injecting mice with 25μg of gp120 in their rear footpads followed by culturing the lymph node T cells with gp120 *in vitro.*

## 2.2.12 Chromium release assay:

The Chromium release assay was used in this study to determine the cytotoxic ability of AZT treated T cells. Splenic cells (4 x $10^6$) from DBA/2 mice (H-$2^d$) were cultured with X-irradiated allogeneic spleen cells (4 x $10^6$) from C57BL6 (H-$2^b$) mice in the presence or absence of various concentrations of AZT (0, 1, 5, 50, 100, 500 or 1000μM) in 24 well plates for 4 days. Contact between the DBA cytotoxic T lymphocytes and the X-irradiated splenic cells led to the activation of CTLs, specific for cells expressing the same MHC haplotype as the C57BL6 mice. The cells were next harvested and washed three times. The harvested cells were then incubated with $^{51}$Cr labeled EL-4 tumor targets (H-$2^b$) for 4 hours in 96 well plates. The supernatants were then collected and the $^{51}$Cr released was measured by a gamma counter. The percent lysis was determined as follows: [(experimental release - control release) / (total release - control release)] x 100. The control release was measured by incubating $^{51}Cr$-labeled targets alone while, the total release was measure by incubating labeled tumor cells with SDS.

### 2.2.13 Statistical Analysis:

In all *in vivo* experiments, lymphocytes were pooled from groups of 2 to 5 mice. All assays were performed in triplicate and each assay was done at least two times. The standard errors (S.E.) were calculated from the data obtained. The various experimental groups were compared to the control groups, using Student's t-test. Differences with p values less than 0.05 were considered statistically significant.

## 2.3 Results:

### 2.3.1 The effects of AZT on T cell differentiation in the thymus and the spleen

The spleen and the thymus play an important role as a secondary lymphoid organ. . It is therefore of great importance to determine if AZT could play any significant role in altering the normal functions or the phenotypes of T cells in these organs. It is possible that AZT could disrupt the thymic activity in a way leading to it's inability to properly replenish the immune system with mature T cells or affect splenic activity thereby disrupting it's normal functions. If this were to occur, the drug could enhance the immunosuppressive state being caused by HIV instead of delaying it.

The first goal of this research was to determine if AZT could affect the ratio of the different T cells in the thymus or spleen. To accomplish this, various concentrations of AZT (0, 100, 500, 1000 mg/kg) were administered into DBA/2 mice by gavage for 7 or 14 days. After the designated time periods the thymus and spleen were removed and the ratios of T cell subsets were determined by flow cytometric analysis. The data from a representative experiment depicted in Fig. 1 demonstrated that control mice had 74.8% $CD4^{+}CD8^{+}$, 17.3% $CD4^{+}$, 2.1% $CD8^{+}$ and 5.8% $CD4^{-}CD8^{-}$ T cells in the thymus, which is consistent with earlier studies (Scollay, et. al. 1984). Upon treatment with AZT at both 500 or

1000 mg/kg for 14 days, there was a marked decrease in $CD4^+CD8^+$ T cells, a increase in $CD4^-CD8^-$, and a increase in $CD4^+$ and $CD8^+$ T cells in the thymus. These data suggested that AZT altered the T cell phenotype or differentiation in the thymus. When similar studies were conducted using the spleen, AZT did not significantly alter T cell subsets as compared to the controls except 14 days after treatment with 1000 mg/kg (Table 1). Note that the spleen have only mature $CD4^+$ or $CD8^+$ T cells and this percentage increased marginally upon AZT-treatment. Experiments depicted in Table 1, further demonstrated that AZT-treatment for 7 days at any concentration tested failed to induce alterations in the T cell subsets in the thymus and spleen. However, upon 14 days treatment, there were marked alterations in the % of T cell subsets in the thymus.

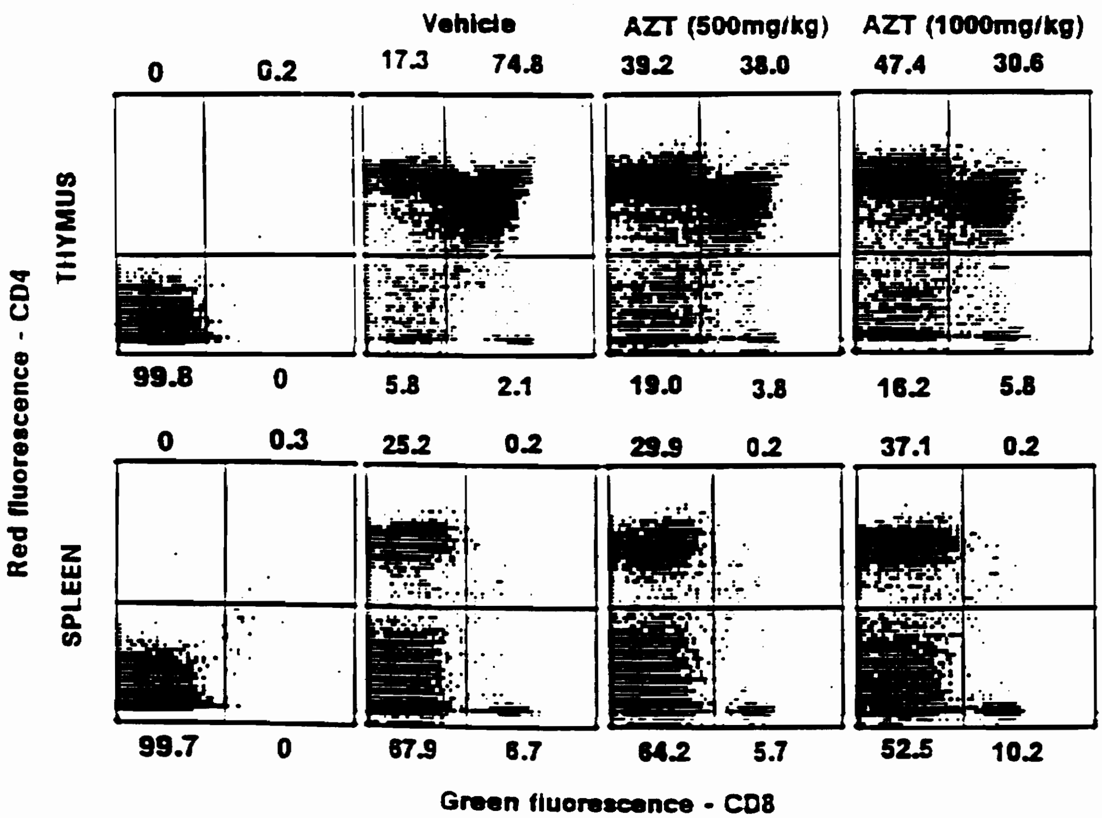


**Fig. 1. Treatment with AZT causes alterations in the ratios of T cell subset within the thymus.** DBA/2 mice were administered with AZT (0, 100, 500, or 1000 mg/kg) for 14 days. Next, the cells were stained with PE-conjugated anti CD8 and FITC-conjugated anti-CD4 antibodies. The populations were then analyzed by flow cytometry and the various ratios of T cell subsets were determined.

*Table 1*

**Percentage of T cell subsets in the thymus and spleen of DBA/2 mice treated with AZT.**

| Expt. | Organs | AZT dose (mg/Kg) | Days of treatment | Percentages of T cell subsets | | | |
|---|---|---|---|---|---|---|---|
| | | | | $CD4^+$ | $CD8^+$ | $CD4^+ CD8^+$ | $CD4^- CD8^-$ |
| 1. | Thymus | vehicle | 7 | 20.6±1.0 | 2.7±0.7 | 67.3±2.8 | 9.4±1.3 |
| | Thymus | 100 | 7 | 21.8±0.1 | 2.5±0.1 | 65.0±2.2 | 10.8±1.6 |
| | Thymus | 500 | 7 | 23.3±1.3 | 4.3±1.3 | 58.7±9.0 | 13.7±7 |
| | Thymus | 1000 | 7 | 23.3±0.1 | 4.5±0.3 | 60.6±1.8 | 11.6±1.7 |
| | Spleen | vehicle | 7 | 34.1±2.8 | 7.7±0.4 | — | — |
| | Spleen | 100 | 7 | 32.4±1.6 | 7.0±0.7 | — | — |
| | Spleen | 500 | 7 | 31.8±4.5 | 8.1±1.1 | — | — |
| | Spleen | 1000 | 7 | 32.1±0.3 | 7.7±0.4 | — | — |
| 2. | Thymus | vehicle | 14 | 20.9±1.8 | 2.4±0.2 | 69.4±2.7 | 7.3±0.9 |
| | Thymus | 500 | 14 | 35.9±2.3 | 4.1±0.2 | 42.4±4.4 | 17.5±1.5 |
| | Thymus | 1000 | 14 | 35.4±5.9 | 7.2±0.8 | 39.6±4.6 | 17.8±0.8 |
| | Spleen | vehicle | 14 | 25.4±0.9 | 6.3±0.3 | — | — |
| | Spleen | 500 | 14 | 28.3±1.9 | 6.2±0.3 | — | — |
| | Spleen | 1000 | 14 | 35.4±1.1 | 8.7±0.8 | — | — |

Mice were treated with various concentrations of AZT (0, 100. 500, or 1000mg/kg body weight) by gavage for 7 or 14 days. The thymuses and spleens were removed and the total cellularity was determined by trypan blue exclusion. Results suggesting significant changes in total cellularity are denoted by underline ($p \leq .05$).

### 2.3.2 The effect of AZT on the total cellularity of the thymus and the spleen:

The second objective of this study was to determine if AZT could significantly affect the total cellularity of the thymus or the spleen. If AZT is able to significantly decrease the total number of lymphoid cells in these organs, it could lead to a reduction in the ability of the immune system to function correctly. To determine this, DBA/2 mice were administered 0, 100, 500, 1000 mg/kg body weight AZT for 7 or 14 days. At the end of administration, the thymus and spleen from each mouse were removed and total cellularity was determined by trypan blue exclusion. The results shown in Table 2 indicated that the total cellularity in the thymus of mice treated with 500 and 1000 but not 100 mg/kg body weight of AZT for 7 days decreased dramatically, whereas, the cellularity in the spleen was not altered significantly. Similar results were obtained using 14 day treatment with AZT. Furthermore, when total number of T cells were enumerated based on the percentage of T cells shown in Fig 1 and Table 1, it was observed that AZT-treatment at 500 or 1000 mg/kg body weight induced marked decrease in the total number of all subsets of T cells in the thymus at both 7 and 14 days treatment, resulting from the overall decrease in thymic cellularity.

*Table 2*

**Total cellularity of thymus and spleen and T cell subsets in these organs of DBA/2 mice treated with AZT.**

| Expt. | Organs | AZT dose (mg/Kg) | Days of treatment | Total cells x $10^6$ | Total T cells x $10^6$/organ | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | CD4$^+$ | CD8$^+$ | CD4$^+$ CD8$^+$ | CD4$^-$ CD8$^-$ |
| 1. | Thymus | vehicle | 7 | 18.8±2.5 | 3.87 | 0.50 | 12.65 | 1.76 |
| | Thymus | 100 | 7 | 17.1±1.6 | 3.72 | 0.42 | 11.11 | 1.84 |
| | Thymus | 500 | 7 | 6.9±2.7(64) | 1.60(59) | 0.29(42) | 4.05(68) | 0.94(47) |
| | Thymus | 1000 | 7 | 2.6±1.5(87) | 0.63(84) | 0.11(78) | 1.57(88) | 0.30(83) |
| | Spleen | vehicle | 7 | 113.5±6.1 | 38.7 | 8.8 | — | — |
| | Spleen | 100 | 7 | 125±12.9 | 40.7 | 8.8 | — | — |
| | Spleen | 500 | 7 | 109±15.7 | 34.6 | 8.8 | — | — |
| | Spleen | 1000 | 7 | 92±20 | 29.5 | 7.0 | — | — |
| 2. | Thymus | vehicle | 14 | 19.5±3.2 | 4.07 | 0.46 | 13.53 | 1.42 |
| | Thymus | 500 | 14 | 8.2±2.6(58) | 2.94(28) | 0.33(29) | 3.47(75) | 1.43(0) |
| | Thymus | 1000 | 14 | 3.5±1.2(83) | 1.23(70) | 0.25(46) | 1.38(90) | 0.62(57) |
| | Spleen | vehicle | 14 | 85.6±17.4 | 21.7 | 5.4 | — | — |
| | Spleen | 500 | 14 | 75.0±8.1 | 21.2 | 4.7 | — | — |
| | Spleen | 1000 | 14 | 77.0±14.6 | 27.2 | 6.7 | — | — |

Mice were treated with various concentrations of AZT (0, 100, 500, or 1000 mg/kg body weight) by gavage for 7 or 14 days. The thymuses and spleens were removed and the cells were stained with fluorescently tagged mAb specific for CD4 and CD8 molecules. The various ratios of T cell subsets were determined by flow cytometric analysis. Results suggesting significant changes are denoted by underline ($p \leq .05$). The numbers in parenthesis represents the percentage decrease.

### 2.3.3 Administration of Interleukin-2 (IL-2) reconstitutes thymic cellularity:

IL-2 is a growth factor produced by T cells which has been shown to play an important role in T cell differentiation in the thymus. We therefore wanted to determine if the toxicity of AZT on the thymus was in some way related to decreased IL-2 production and, therefore, whether administration of IL-2 into AZT-treated mice could reconstitute the total cellularity of the thymus. In this experiment, mice were given AZT at doses of 0 or 500 mg/kg body weight for 14 days. In addition, these mice were administered with IL-2 (i.p.) at 500 or 5000 units/mouse, daily. After 14 days, the total cellularity was determined by trypan blue exclusion. The results showed that IL-2 was able to completely reverse the toxic effects of AZT by reconstituting the total cellularity of the thymus (Fig. 2). It should be noted that injection of IL-2 alone into normal DBA/2 mice did not have any significant effect on total cellularity in the thymus.

### 2.3.4 The effects of AZT on the activation of T and B cells to polyclonal mitogens:

In addition to determining the effects of AZT on the ratios of T cells in the

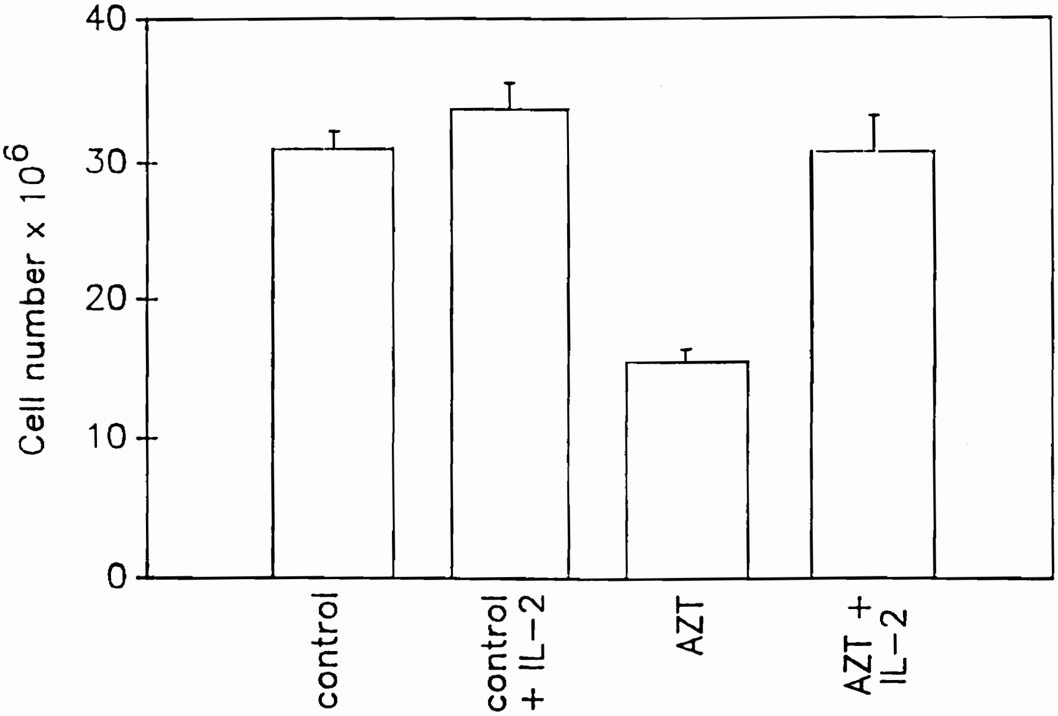


**Fig. 2. Administration of IL-2 reverses the toxicity induced by AZT and reconstitutes the total cellularity of the thymus.** DBA/2 mice were administered, by gavage, with PBS (control) or 500 mg/kg of AZT for 14 days. In addition, the control and AZT-treated mice were injected i.p. with 500 units of IL-2 simultaneously with AZT for 14 days. After 14 days of treatment, the thymuses were removed and the the total cellularity was determined by trypan blue exclusion. Significant decrease in cellularity was observed at 500 mg/kg bodyweight when compared to the controls ($p \leq .003$). Injection of IL-2 significantly reconstituted the total cellularity of the thymus.

lymphoid organs and total cellularity, it is important to determine the effects of AZT on the activation of T and B cells. In order for T and B cells to function properly in the immune system, these cells must be able to become activated when challenged with an antigen. If AZT is able to interfere with the activation of T or B cells, these cells could become nonfunctional. In this set of experiments, spleen cells from DBA/2 mice administered for 14 days with AZT (0, or 100, mg/kg) were cultured with various polyclonal mitogens such as Con A and anti-CD3, to stimulate the T cells, or LPS, to stimulate the B cells. The results from this experiment (Fig. 3) indicated that AZT at concentrations of 100 mg/kg body weight administered for 14 days did not have any significant ability to alter T and B cell activation. However, when the concentration of AZT was increased to 500 or 1000 mg/kg body weight, there was a moderate decrease in T cell ability to respond to mitogens particularly to Con A (Fig. 4). These results were consistent with earlier data which indicated that AZT may affect T cells at the time of differentiation rather than on naive or mature T cells (Table 1 and 2).

### 2.3.5 The effects of AZT on T cells response to stimulation by conalbumin (in vivo):

In a normal situation, the immune system is not activated by a polyclonal

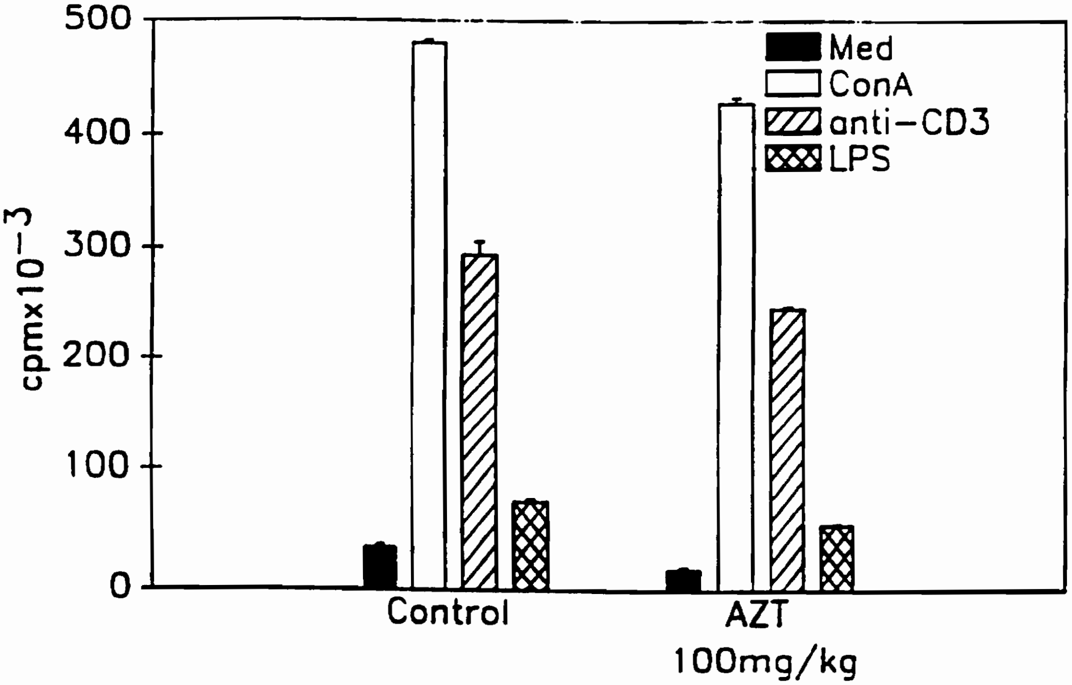


**Fig. 3. Effects of AZT treatment in *vivo* on T cells ability to respond to stimulation by polyclonal mitogens *in vitro*.** DBA/2 mice were administered by gavage, with PBS (control) or with 100 mg/kg body weight of AZT for 14 days. The splenic cells were then stimulated with various mitogens for 40 hours and the proliferation was then determined by thymidine uptake assay. Significant suppression was not observed at concentration of 100 mg/kg body weight or greater when compared to the controls for LPS, anti-CD3 and Con A mitogens ( $p \geq .05$).

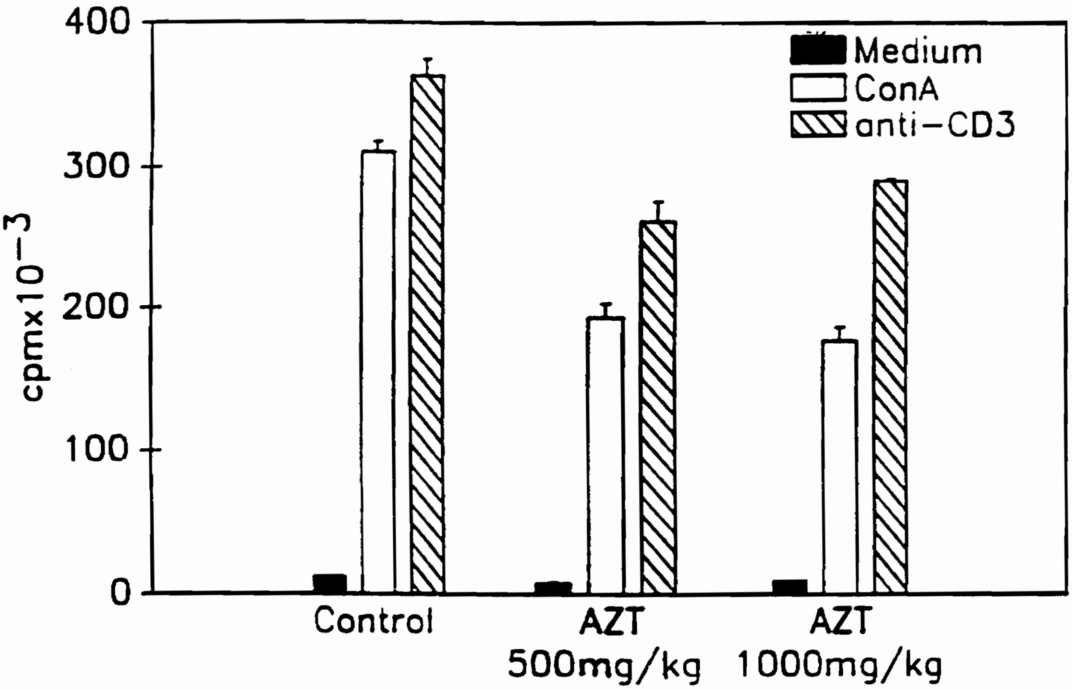


**Fig. 4. AZT in *vivo* suppresses T cells ability to respond to stimulation by polyclonal antigens *in vitro*.** DBA/2 mice were administered, by gavage with PBS (control) or with various concentrations of AZT (500 or 1000 mg/kg body weight) for 14 days. The splenic cells were then stimulated with various mitogens *in vitro* for 40 hours and the proliferation was then determined by thymidine uptake assay. Significant suppression was observed at concentration of 500 mg/kg body weight or greater when compared to the controls for both anti-CD3 and Con A ( $p \leq .005$).

antigen but is challenged by a specific antigen recognized by only a small subset of T and B cells. Also, in the earlier experiments described above, spleen cells from AZT-treated mice were cultured *in vitro* with polyclonal mitogens and the immune response was studied. These experiments did not provide information on how T cells would respond to antigens *in vivo* when simultaneously encountered with AZT. To address this, we decided to immunize mice undergoing AZT-treatment with foreign antigens such as conalbumin and study the T cell response to this antigen. To this end, DBA/2 mice were gavaged with various concentrations of AZT for 14 days. On the 7th day of AZT administration, the mice were injected with conalbumin/Fruend's adjuvant into their rear foot pads. On day 14, the lymph nodes draining the rear footpads were removed. The T cells were then cultured with conalbumin (*in vitro*) for 48 hours. The T cell proliferation was then measured by $^{3}$H-thymidine uptake assay. The results from this experiment showed that AZT at concentrations of 100 mg/kg body weight or greater, administered for 14 days, significantly decreased, in a dose dependent fashion, the T cell ability to respond to stimulation by conalbumin (Fig. 5). These data suggested that AZT can suppress the ability of T cells to respond to foreign antigens *in vivo.*

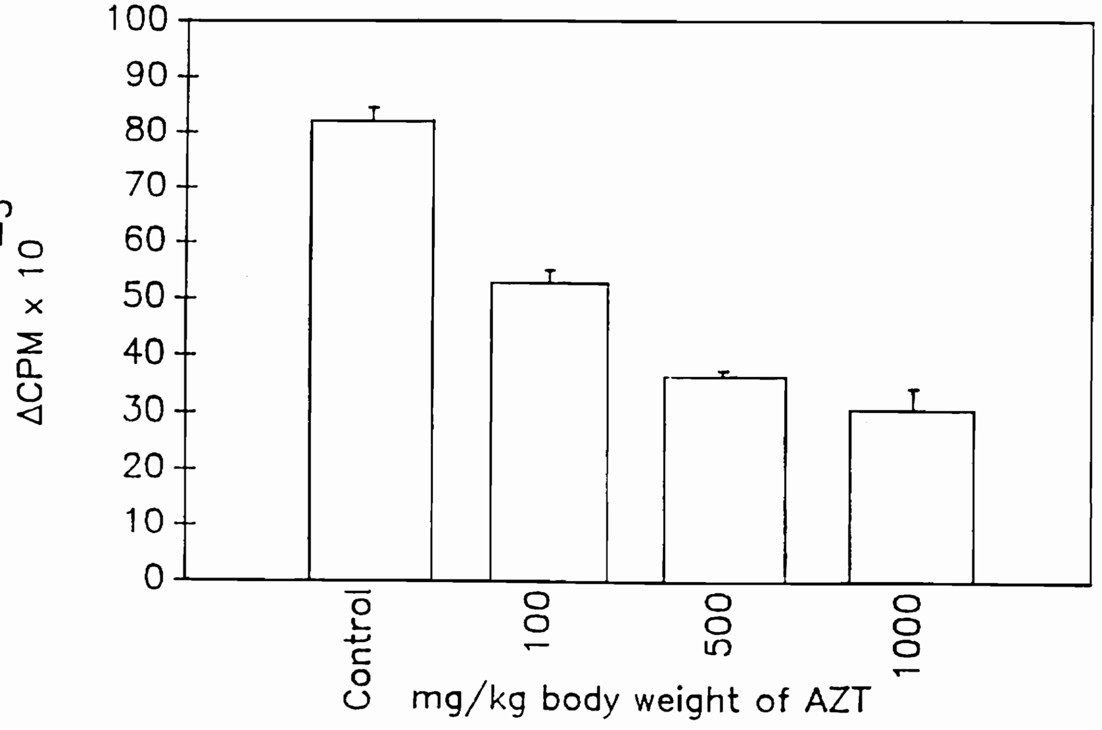


**Fig. 5. Effects of AZT on T cell response to conalbumin.** Mice treated for 14 days with PBS (control) or with 100, 500, or 1000 mg/kg body weight of AZT were immunized with conalbumin in their rear footpads on day 7. Following AZT treatment the lymph nodes draining the rear footpads were removed and the T cells were restimulated with conalbumin *in vitro*. The proliferative response was determined by $^3H$ uptake assay. Δcpm represents cpm in cultures incubated with conalbumin minus cpm in cultures incubated without conalbumin. Significant suppression was observed at concentrations of 100 mg/kg body weight or greater when compared to the controls ($p \leq .0005$).

## 2.3.6 AZT-treatment decreases the ability of T cells to respond to gp120 of HIV:

Next, we wished to delineate whether AZT-treatment would alter the T cell responsiveness to HIV antigens. It was important to know this, if AZT-treatment were to be recommended for use by asymptomatic HIV-infected individuals at an early stage of the disease. To this end, mice treated for 14 days with AZT were injected with gp120 of HIV into the rear footpads on day 7. A week later, the draining lymph node T cells were studied for their ability to respond to gp120 *in vitro* . The study was carried out in a similar way as studying the response to conalbumin, described earlier. The data shown in Fig. 6 suggested that the T cell-mediated immune response to gp120 in AZT-treated mice decreased significantly at all AZT concentrations tested. The data confirms the above studies that AZT when present at the time of T cell responsiveness to foreign antigens *in vivo* , could suppress their ability to respond to the specific antigen.

## 2.3.7 The direct effects of AZT on cell viability:

Splenic cells cultured in the presence of AZT at concentrations of 1000 μM or greater showed a significant reduction in the total number of viable cells after 48

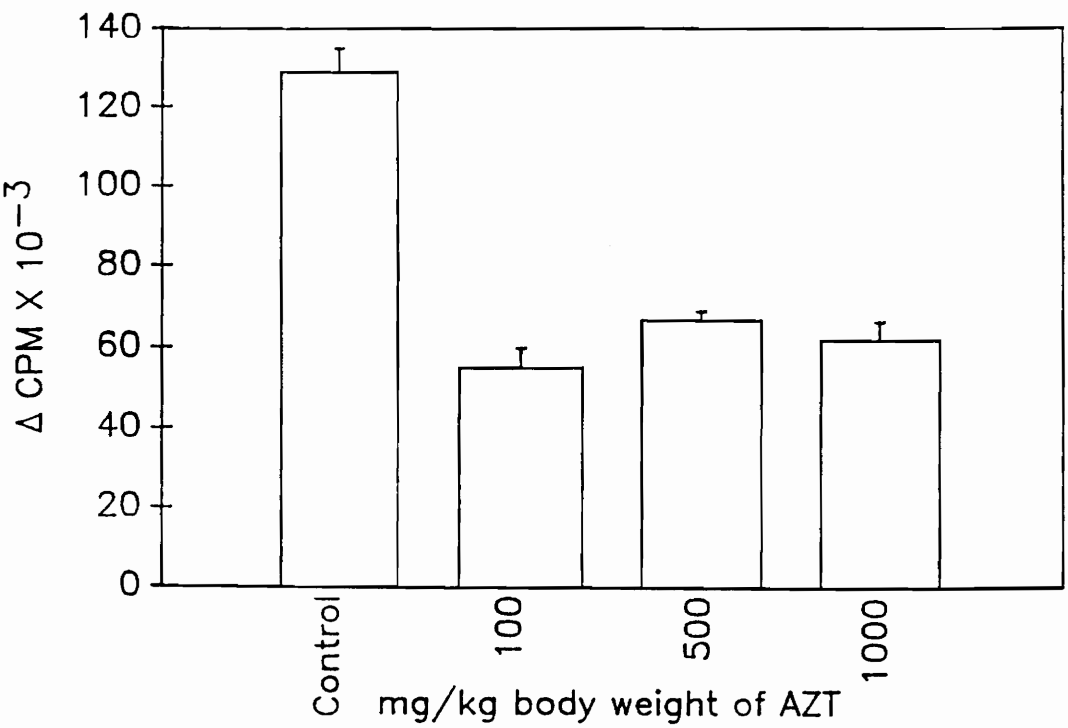


**Fig. 6. Effects of AZT on T cell response to gp120 *in vivo*.** Mice administered for 14 days with PBS (control) or with 100, 500, or 1000 mg/kg body weight of AZT were immunized with gp120 in their rear foot pads on day 7. Following AZT treatment, the lymph nodes draining the rear footpads were removed and the T cells were restimulated with gp120 *in vitro*. The proliferative response was determined by $^{3}H$ thymidine uptake assay. Δcpm represents cpm in cultures incubated with gp120 minus cpm in cultures incubated without gp120. Significant suppression was observed at concentrations of 100 mg/kg body weight or greater when compared to controls ($p \leq .0005$).

hours of exposure. This data suggest that at the higher concentrations, any effects caused by AZT may be due to the direct toxic effects of the drug resulting in the death of the cell, however at the lower concentrations, AZT does not seem to directly kill the cells, suggesting that AZT alters cellular functions involving other mechanisms (fig. 7).

### 2.3.7 The effects of AZT on T and B cell activation (in vitro ):

While the data presented thus far demonstrated clearly that AZT altered the T cell differentiation and function, it was not clear whether AZT directly or indirectly affected the T cells. Previous studies using immunotoxic drugs have shown that sometimes the immuonmodulators can bring about alterations by indirectly affecting the nutritional status, or by triggering stress hormones or other endocrine responses. To address this, we decided to directly test *in vitro* whether AZT would alter the ability of B and T cells to respond to mitogens. To determine the effects of AZT on T and B cells *in vitro*, whole spleen cells from DBA/2 mice were cultured with various mitogens in the presence of various concentrations of AZT (0,1,5,50,100,500,1000μM) for 48 hours. Con-A, PHA, anti-CD3 mAbs or anti-TCR were used to stimulate T cells and LPS or anti-IgM were used to stimulate the B cells.

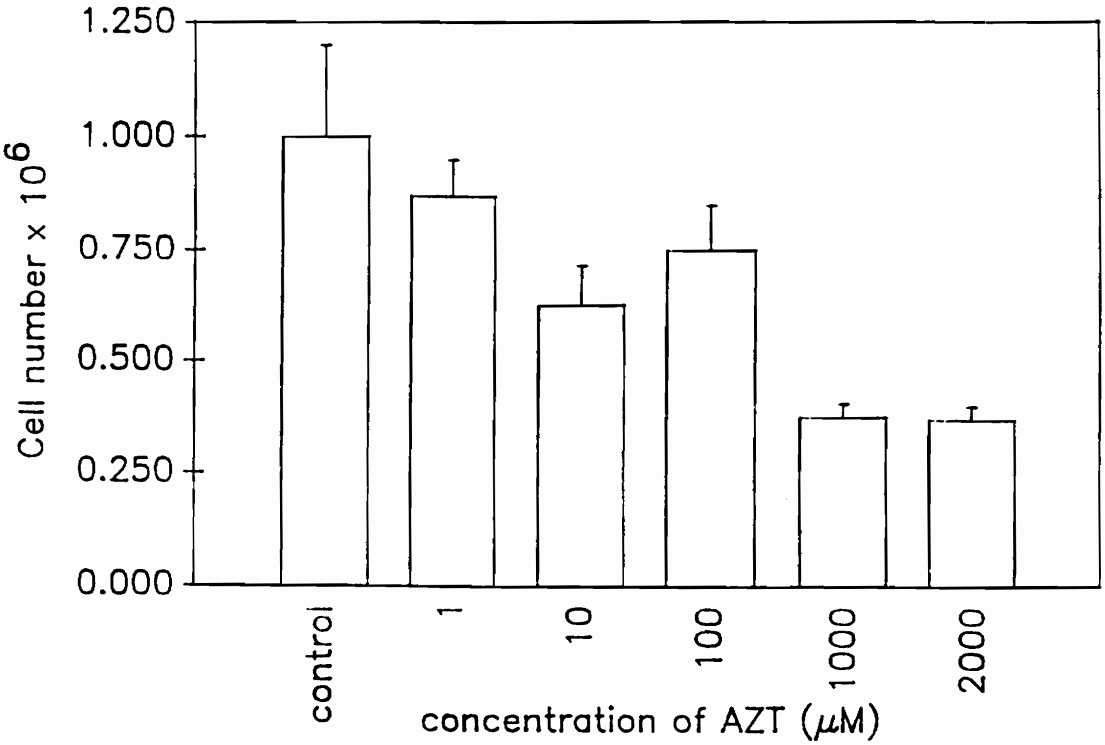


**Fig. 7. Effects of AZT on cell viability.** Splenic cells from DBA/2 mice were cultured *in vitro* with various concentrations of AZT for 48 hours. The number of viable cells was then determined by trypan blue exclusion. Significant reduction in cell viability was observed at concentrations of 1000 μM or greater when compared to controls ($p \leq .05$).

A common test used to determine proliferation is the thymidine uptake assay, in which the $^3H$ thymidine is incorporated into newly synthesized DNA. The measurement of the amount of $^3H$ thymidine is then used to determine the amount of cell proliferation. This test had been used earlier to study the effect of AZT on T and B cell proliferation (Daniak, et al. 1988). However, we reasoned that inasmuch as AZT is a thymidine analog, there is a possibility that it might compete with $^3H$ thymidine for uptake during DNA synthesis. In such an instance, the thymidine uptake assay would not be valid in testing the effects of AZT on cell proliferation. To determine this, two separate groups were set up. In the first group, whole spleen cells from normal mice were simultaneously cultured with mitogens and various concentrations of AZT and 40 hours later, the proliferation was measured by adding $^3H$-thymidine to the cell cultures and incubating the cells for an additional 8 hours. A second group was set up in a similar way. However AZT was not added until 40 hours after the cells had been exposed to the mitogens. Proliferation of this group of cells was then determined by the thymidine uptake assay. If there was no competition between AZT and the $^3H$ thymidine during DNA synthesis, one would expect no significant effect on DNA synthesis in lymphocytes stimulated with mitogens.. The data presented in Figs.7-9 suggested that when AZT was added at the beginning of culture, it induced a dose dependent inhibition of proliferation of T and B cells to various mitogens. Interestingly, at higher concentrations of AZT, such as 50 $\mu$M or greater, addition of AZT even at 40 hours after the initiation of cultures caused marked inhibition of T and B cell proliferative responses to mitogens (Figs. 8-10), thereby suggesting that AZT does compete with $^3$H-thymidine in the proliferation assay and therefore this assay may not represent an appropriate assay to study the immunotoxic effects of AZT *in vitro.*

To avoid this problem, a colorimetric assay using MTT was standardized and used to measure the cell proliferation. At first, the MTT assay and $^3$H thymidine assay were set up simultaneously to determine if results from both assays were comparable. The results from these tests showed that the MTT assay was suitable for measuring proliferation in the presence of AZT (Fig. 11-13 ).

After standardizing the MTT assay, further studies were conducted to determine the effects of AZT on the activation of T and B cells *in vitro.* Spleen cells from normal DBA/2 mice, were cultured with various mitogens in the presence of various concentrations of AZT (0, 1, 5, 50, 100, 500, and 1000$\mu$M) for 48 hours. The proliferative response was then measured by MTT assay. The results revealed that AZT at concentrations of 50$\mu$M or greater, decreased the ability of both T and B cells to respond to activation by mAbs against $\alpha\beta$ TCR and LPS (Fig. 14), and concentrations of 100 $\mu$M or greater decreased the ability of both T and B cells to respond to Con A and anti-IgM (Fig. 14).


**Fig. 8. AZT competes with $^3H$ thymidine for incorporation during the cell proliferation assay.** Splenic cells were cultured in the presence or absence of various concentrations of AZT for 40 hours and stimulated with anti-IgM (αμ) Abs. Groups treated with AZT from the initial time of culture were labeled the time 0 group. Groups treated with AZT at the time of $^3H$ thymidine addition were labeled time 40. After 40 hours tritiated thymidine was added to the cultures to determine the relative proliferation. Any inhibition in the time 0 group could be attributed to suppression by AZT. However any inhibition seen in the time 40 group would be attributed to competition instead of suppression by AZT, showing that AZT does compete with thymidine for incorporation. Significant suppression was observed at concentrations of 5μM or greater in the time 0 group when compared to the controls ($p \leq .005$). Significant suppression was observed at concentrations of 5μM or greater in the time 40 group when compared to controls ($p \leq .03$).

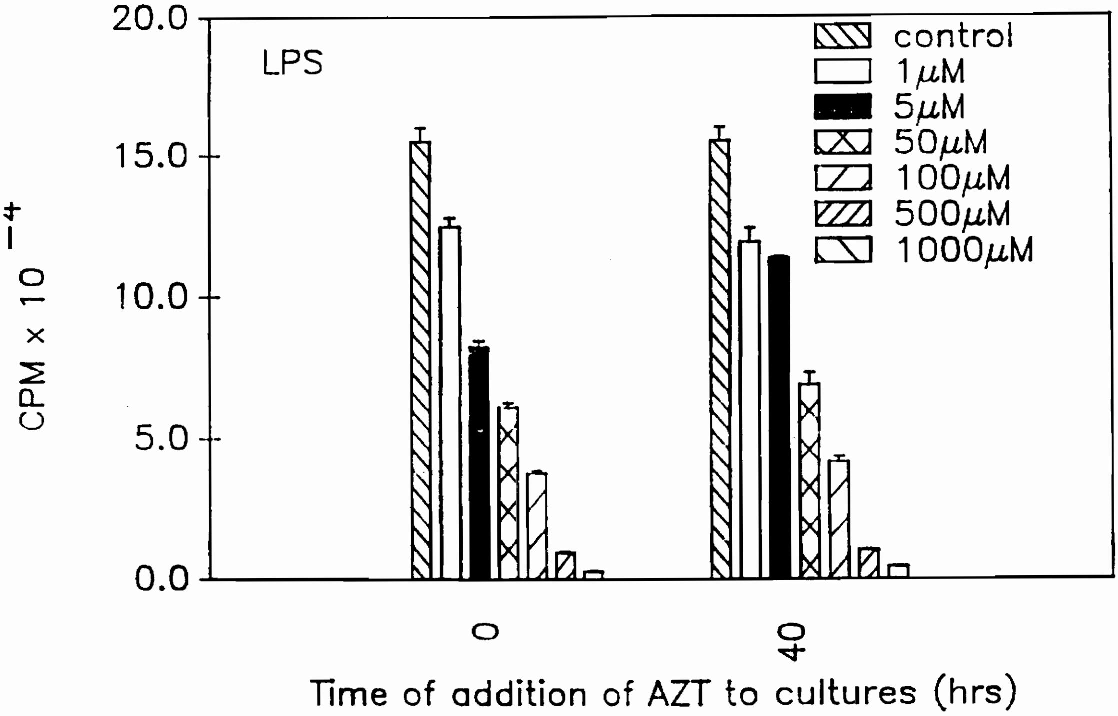


**Fig. 9. AZT competes with $^3$H thymidine for incorporation during the thymidine uptake assay.** Splenic cells were cultured in the presence or absence of various concentrations of AZT for 40 hours and stimulated with LPS. Groups treated with AZT from the initial time of culture were labeled the time 0 group. Groups treated with AZT at the time of thymidine addition were labeled time 40. After 40 hours tritiated thymidine was added to the cultures to determine the relative proliferation. Any inhibition in the time 0 group could be attributed to suppression by AZT. However if any inhibition was seen in the time 40 group it would be attributed to competition instead of suppression by AZT, showing that AZT does compete with thymidine for incorporation. Significant suppression was observed at concentrations of 1μM or greater in the time 0 group ($p \leq .004$) when compared to controls. Significant suppression was observed at concentrations of 5μM or greater in the time 40 group when compared to controls ($p \leq .001$).

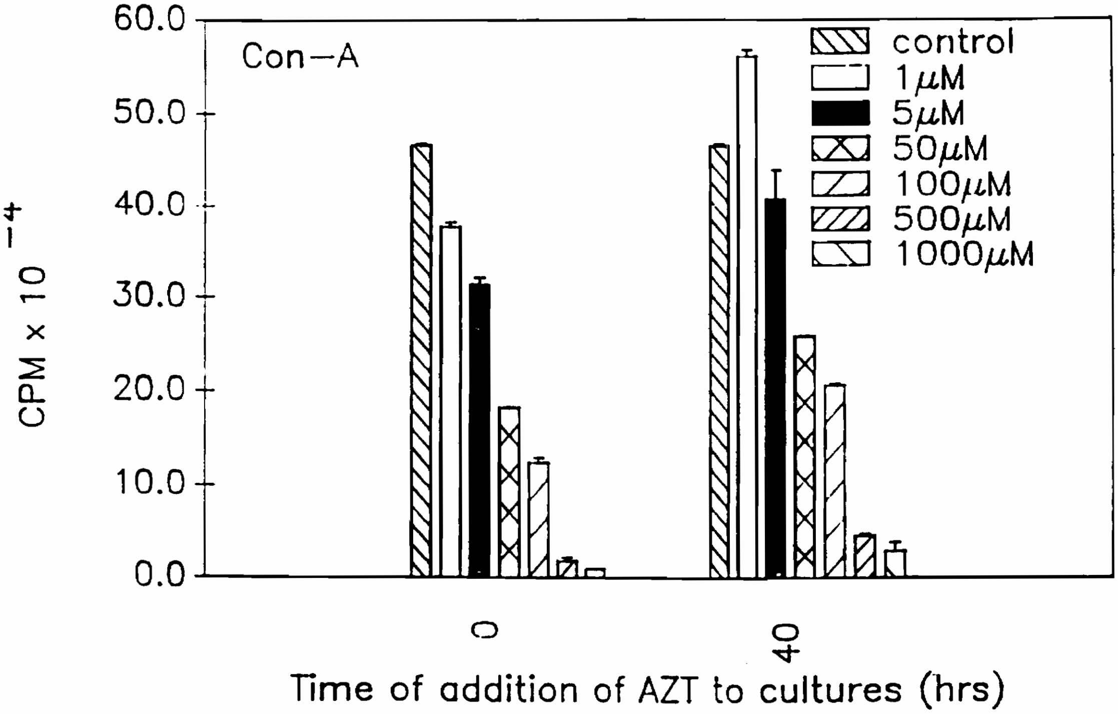


**Fig. 10. AZT competes with $^3H$ thymidine for incorporation during the thymidine uptake assay.** Splenic cells were cultured in the presence or absence of various concentrations of AZT for 40 hours and stimulated with Con A. Groups treated with AZT from the initial time of culture were labeled the time 0 group. Groups treated with AZT at the time of thymidine addition were labeled time 40. After 40 hours tritiated thymidine was added to the cultures to determine the relative proliferation. Any inhibition in the time 0 group could be attributed to suppression by AZT, However if any inhibition was seen in the time 40 group can be attributed to competition instead of suppression by AZT, showing that AZT does compete with thymidine for incorporation. Significant suppression was observed at concentrations of 1μM or greater in the time 0 group when compared to controls ($p \leq .007$). Significant suppression was observed at concentrations of 5μM or greater in the time 40 group when compared to controls ($p \leq .001$).

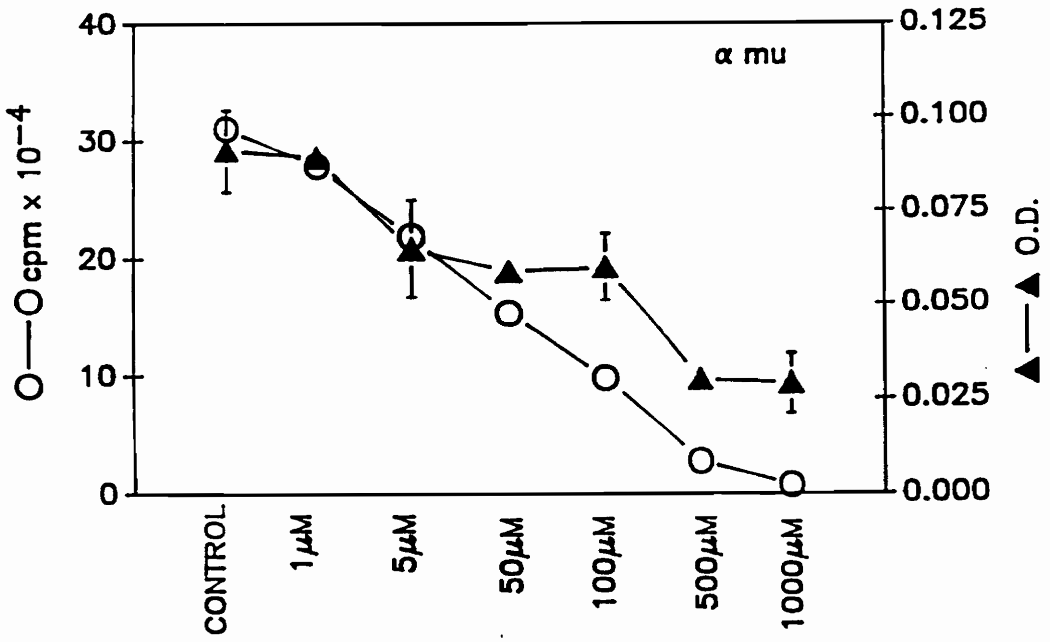


**Fig. 11. Comparison of MTT assay and $^3H$ thymidine incorporation assay using anti-IgM Abs as a mitogen.** A MTT assay and thymidine uptake assay were set up simultaneously to measure spleen cell proliferation using various concentrations of AZT and anti-IgM Abs (20µg/ml). The results show that the MTT assay is an appropriate and comparable assay to determine proliferation in the presence of AZT. Significant suppression was observed at concentrations of 5µM or greater in the $^3H$ thymidine uptake assay when compared to controls ($p \leq .005$). Significant suppression was observed at concentrations of 100µM or greater using MTT assay, when compared to controls ($p \leq .006$).

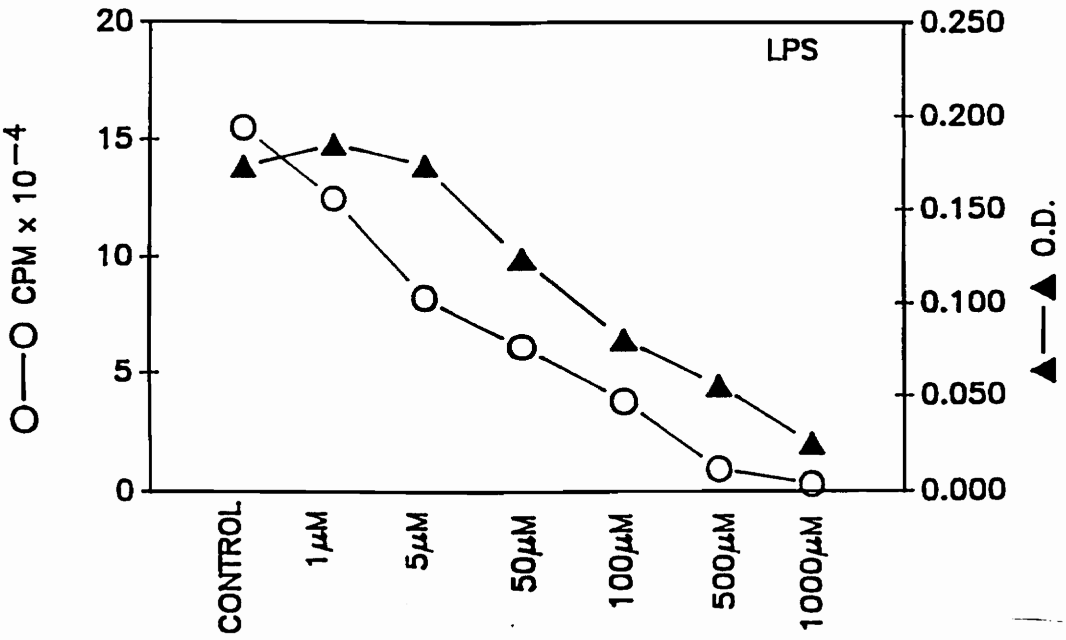


**Fig. 12. Comparison of MTT assay and $^3$H thymidine incorporation assay using LPS as a mitogen.** A MTT assay and thymidine uptake assay were set up simultaneously using various concentrations of AZT and LPS (20µg/ml). The results show that the MTT assay is an appropriate and comparable assay to determine proliferation in the presence of AZT. Significant suppression was observed at concentrations of 1µM or greater in the $^3$H thymidine uptake assay when compared to controls ($p \leq .007$). Significant suppression was observed at concentrations of 5µM or greater in the MTT assay when compared to controls ($p \leq .001$).

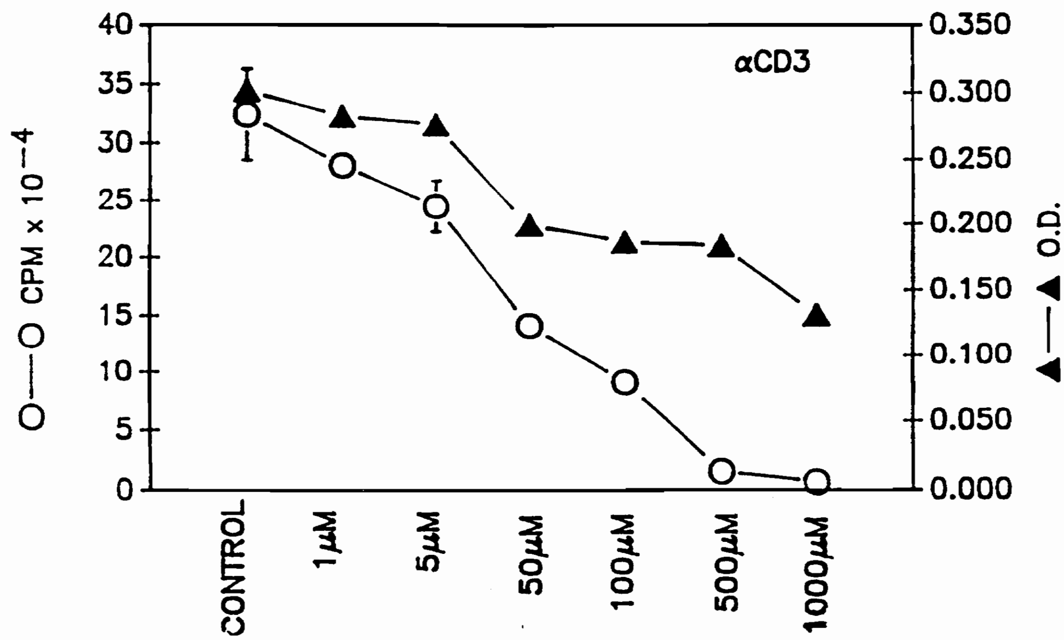


**Fig.** 13. **Comparison of MTT assay and $^{3}H$ thymidine incorporation assay using anti-CD3 as a mitogen.** A MTT assay and thymidine uptake assay were set up simultaneously using various concentrations of AZT and anti-CD3 Abs (2μg/ml). The results show that the MTT assay is an appropriate and comparable assay to determine proliferation in the presence of AZT. Significant suppression was observed at concentrations of 5μM or greater in the $^{3}H$ thymidine uptake assay when compared to controls ($p \leq .004$). Significant suppression was observed at concentrations of 50μM or greater in the MTT assay when compared to controls ($p \leq .002$).

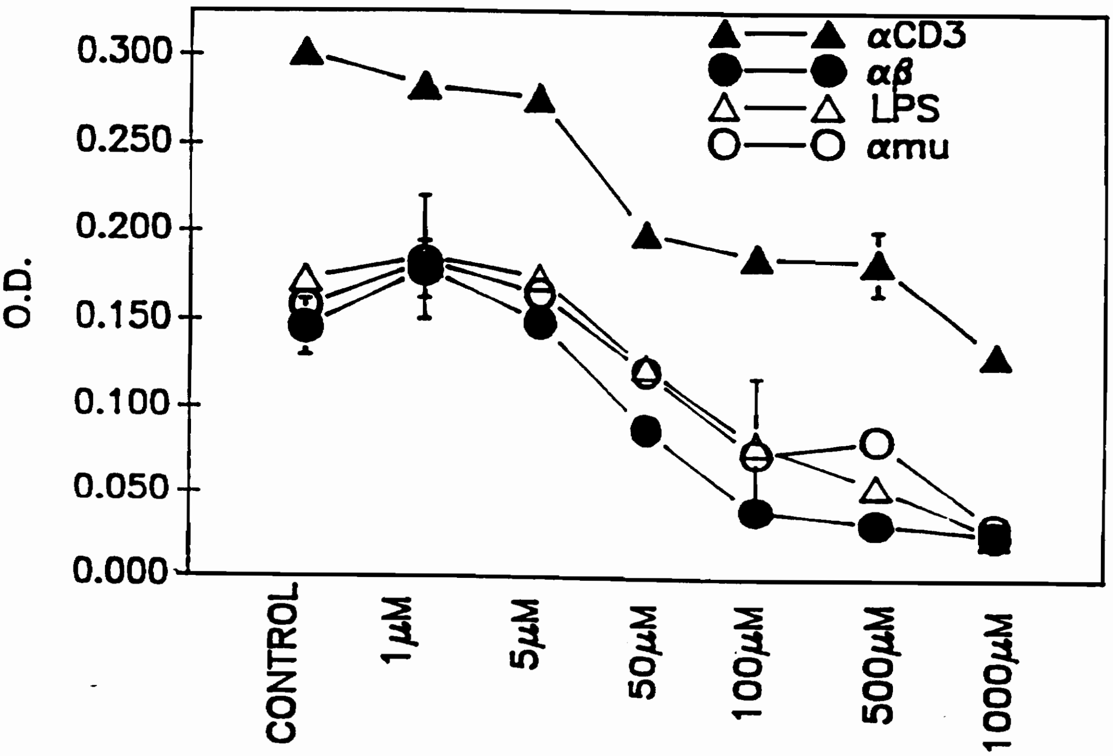


**Fig. 14. AZT suppresses T and B cell proliferation in response to polyclonal mitogens *in vitro* .** Splenic cells from DBA/2 mice were cultured in the presence and absence of various concentrations of AZT for 48 hours and stimulated with various polyclonal mitogens. The proliferation was then measured by MTT assay. Significant suppression was observed at concentrations of 100 uM or greater for anti-CD3 and anti-IgM Abs ($p \leq .05$) and at concentrations of 50 uM or greater for αβ and LPS when compared to controls ($p \leq .05$).

### 2.3.9 The effects of AZT on T cell activation in response to Con A *in vitro.*

In the previous experiments a MTT assay was used to rapidly determine the proliferative response. To further demonstrate that the MTT assay is an appropriate assay for proliferation, an assay directly measuring the proliferative response was set up and the results were compared to the results obtained from the MTT assay. Spleen cells were cultured in 24 well plates in the presence or absence of various concentrations of AZT and stimulated with Con A for 48 hours. The proliferative response was then determined directly by trypan blue exclusion. The results suggested that AZT inhibits the proliferation in response to Con A at AZT concentrations of 50 μM or greater (Fig. 15). These results were comparable to the results obtained by the MTT assay, thus reaffirming that the MTT assay is an appropriate means for measuring proliferation in the presence of AZT.

### 2.3.10 The effects of AZT on the activation and generation of Cytotoxic T Lymphocytes (CTL):

In the previous assay, the amount of new DNA synthesized was measured

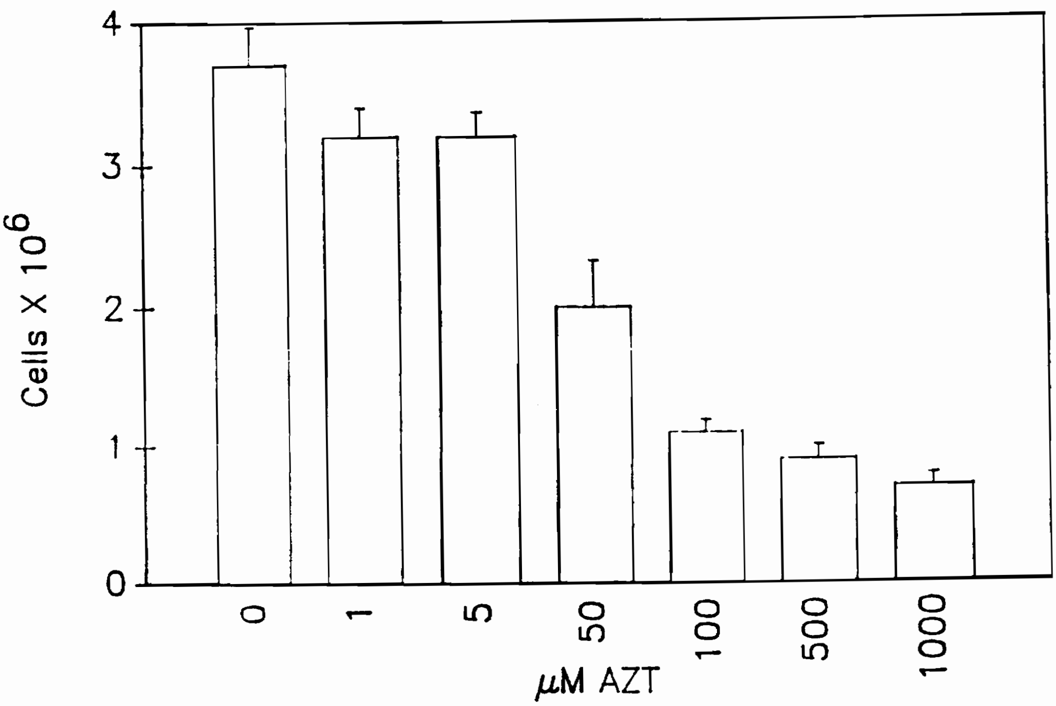


**Fig. 15. Effects of AZT on T cell response to *in vitro* stimulation by Con A.** Spleen cells were cultured in 24 well plates for 48 hours in the presence or absence of various concentrations of AZT (0, 1, 5, 50 100, 500, or 1000μM) and stimulated with Con A. The proliferative response was then determined by trypan blue exclusion. Significant suppression was observed at concentrations of 50μM or greater when compared to controls ($p \leq .007$).

which can be correlated with the proliferation and production of new cells. An additional important factor is the ability of the CTL to differentiate upon antigenic challenge and produce certain lytic molecules ultimately leading to the destruction of foreign cells. To determine the ability of AZT to affect this process, spleen cells from DBA/2 (H-$2^d$) mice were cultured in the presence of various concentrations of AZT, along with X-irradiated allogeneic spleen cells (C57BL/6, H-$2^b$) for 5 days. The addition of allogeneic spleen cells would stimulate the production of cytotoxic T cells specific for the H-$2^b$ bearing tumor targets. The cells were then harvested and tested for their ability to kill $^{51}$Chromium labeled H-$2^b$ tumor targets at various effector:target (E:T) ratios. The results revealed that AZT at concentrations of 100 μM weight or greater significantly inhibited the generation of CTLs *in vitro* (Fig. 16). These data suggested that AZT affects the cytotoxic T cell differentiation as well as the T cell proliferative responsiveness to mitogens.

### 2.3.11 Ability of IL-2 to reconstitute cytotoxic T lymphocyte (CTL) activity:

Interleukin-2 (IL-2) is an important lymphokine involved in T cell differentiation. To investigate whether AZT was inhibiting the IL-2 production, we tested

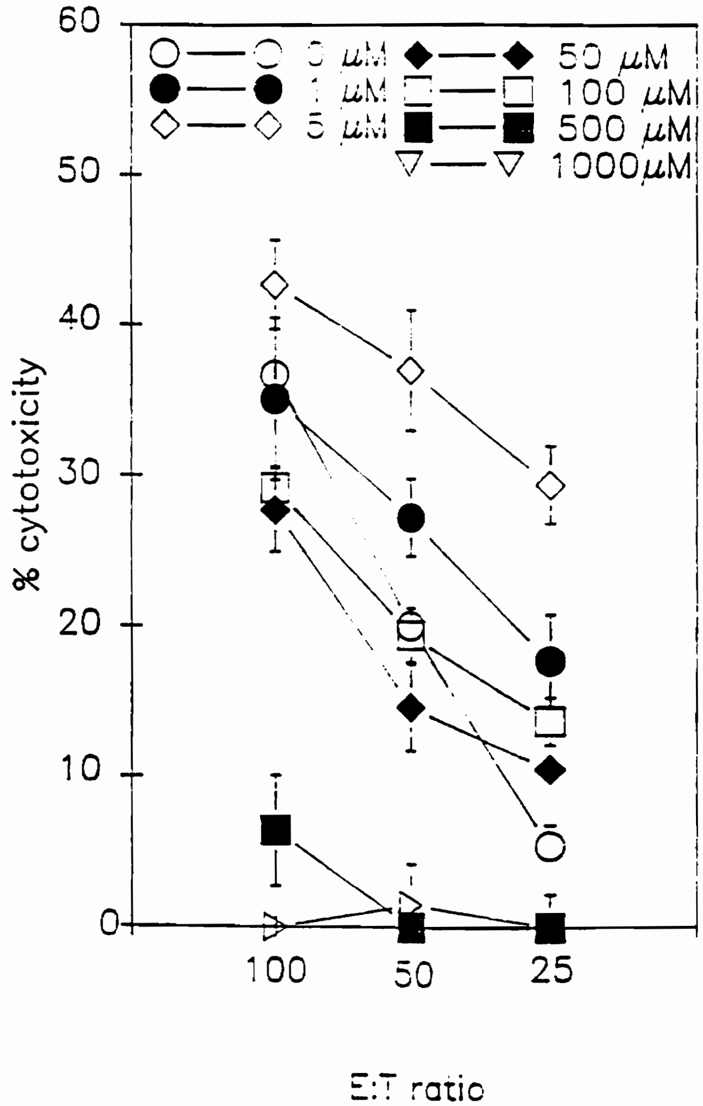


**Fig. 16. AZT suppresses cytotoxic T lymphocyte activation *in vitro*.** Splenic cells from DBA/2 mice were cultured in the presence of various concentrations of AZT (0, 1, 5, 50, 100, 500, or 1000μM) and stimulated with X-irradiated allogeneic splenic cells for 4 days. The CTLs were then harvested and tested for cytotoxicity against $^{51}Cr$ labeled specific tumor targets. The cytotoxicity was determined by the amount of $^{51}Cr$ released into the supernatant. Significant suppression was observed at concentrations of 500 μM or greater at each E:T ratio when compared to controls ($p \leq .04$).

end, spleen cells were cultured with various concentrations of AZT for 5 days. In addition, groups treated with AZT were exposed to exogenous rIL-2 to determine if it could reconstitute the the differentiation and generation of cytotoxic T lymphocytes in response to specific stimulation. The results clearly showed that the addition of IL-2 could reverse the suppressive effects caused by AZT treatment (Fig 17). These data suggested that the mechanism by which AZT inhibits the CTL differentiation may involve IL-2 production by the CTL upon activation.

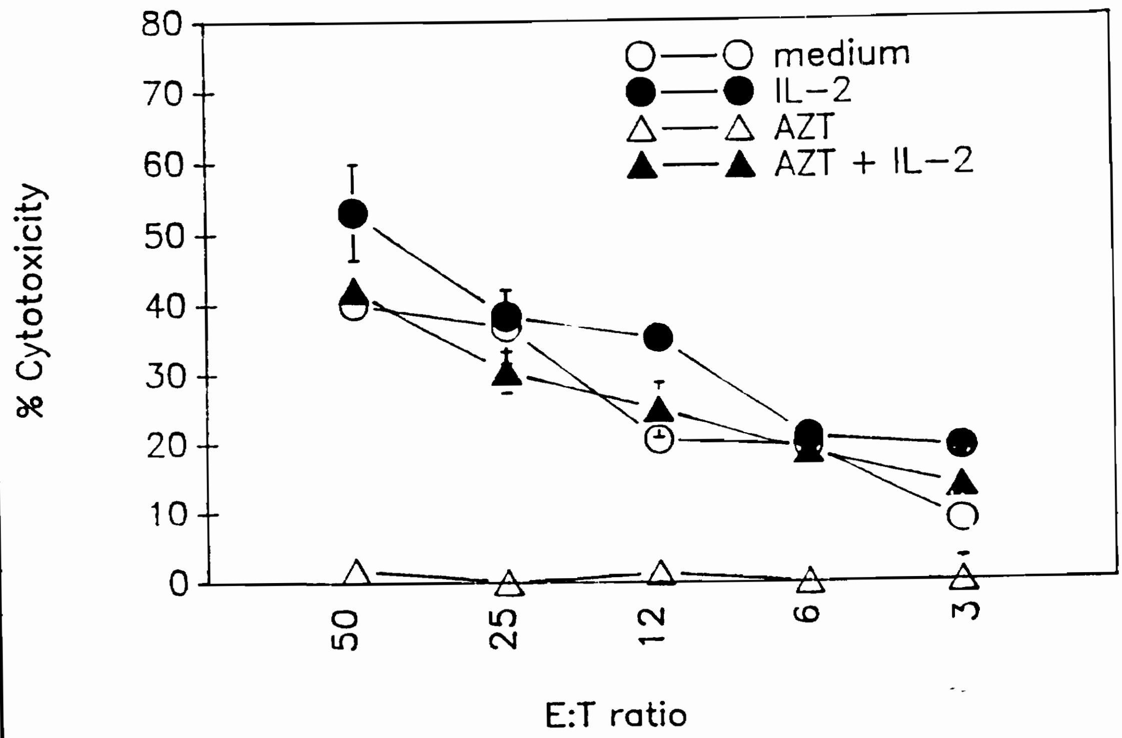


**Fig. 17. Addition of exogenous IL-2 reconstitutes Cytotoxic T lymphocyte function *in vitro*.** Splenic cells from DBA/2 mice were cultured with various concentrations of AZT (0 or 500 µM) in the presence or absence of exogenous IL-2 and stimulated with allogeneic splenic cells for 4 days. The CTLs were then harvested and tested for cytotoxicity against $^{51}Cr$ labeled specific tumor targets. The cytotoxicity was determined by the amount of $^{51}Cr$ released into the supernatant. Significant reduction of CTL activity was observed in the presence of 500 µM of AZT when compared to controls ($p \leq .001$), however CTL activity was reconstituted with the addition of exogenous IL-2.

## 2.4 DISCUSSION:

The current study was initiated with the aim of finding out whether AZT exhibited immunomodulatory properties in the normal murine host. Such an investigation is critical because HIV infection affects T cells, and secondly, if AZT is to be recommended for use in asymptomatic HIV infected individuals, it is important to know whether AZT has any effect on normal T cell differentiation and function. Previous work done by Sommadossi et al, (1989) reported that AZT directly suppressed human bone marrow progenitor colony growth in a dose dependent manner. These results were consistent with the clinical observations that neutropenia and anemia are the major adverse effects of AZT treatment (Balzarini et. al., 1988). These adverse effects caused by AZT treatment resulted in significant limitations in the use of AZT, causing a dosage reduction or discontinuation of use in approximately 40% of patients (Hirsch, 1988). The bone marrow serves as the primary source for progenitor cells for the immune system. In addition, other important functions of the host rely on the bone marrow. The possible implications of AZT toxicity on the bone marrow are great. Suppression of the bone marrow could potentially lead to the disruption of other essential functions within an individual. Recently, Mansuri et al. (1990) reported that administration of 100, 250, 500, and 1000 mg/kg of AZT for 30 days ,caused lymphoid depletion, reticuloendothelial hyperplasia in spleen and thymus, and bone marrow hypocellularity. There was also lymphopenia, neutropenia and anemia in peripheral blood (Mansuri et al. 1990). These, as well as other reports, support the importance of further research on the effects of AZT on the immune system.

The current study has identified several important immunomodulatory properties of AZT. It was observed that the thymus was highly sensitive to AZT treatment. Also, AZT when present at the time of T cell differentiation was found to play a critical role in inducing immunosuppression. AZT was less inhibitory or toxic to naive or resting mature T cells. The thymus is the primary lymphoid organ in which the T cells develop and mature. Stem cells from the bone marrow migrate into the thymus. Once inside, the stem cells begin to differentiate and become thymic lymphocytes. Within this specialized microenvironment the cells go through a series of stages before becoming mature T cells. In the first stage, the immature thymocytes express CD7 along with CD2 and CD5, all of which are not specific for the T cell lineage. In the second stage, there is the appearance of the CD1, as well as the coexpression of CD4 and CD8 markers. In the final stage, the cells lose the CD1 marker gain the expression of CD3 on the cell surface and finally become either $CD4^+$ or $CD8^+$ mature T cells. When mice were orally treated with AZT, an alteration was seen in the ratio of the various T cell subsets in the thymus. We noticed a significant decrease in the percentage of $CD4^+CD8^+$ cells and a significant increase in the percentage of the $CD4^-CD8^-$, $CD4^+$ and $CD8^+$ T cells. These data clearly showed that AZT can alter the normal ratios of the various T cell subsets within the thymus and possibly disrupting the normal maturation process within the thymus. Because it is believed that negative and positive selection of T cells takes place at $CD4^+CD8^+$ stage, such cells are actively differentiating in the thymus and AZT may have a direct effect on these cells. This may explain a decreased percentage of $CD4^+CD8^+$ T cells in the thymus. Such a decrease in turn can offset the percentage of other T cell subsets thereby explaining an increase in the percentage of other subsets.

To further understand the effects of AZT, we examined whether treatment with AZT would alter the total cellularity of the thymus. In this case, mice were orally treated with AZT for 7 or 14 days. After the designated time period, the thymuses were removed and the total cellularity was determined by trypan blue exclusion. It was observed that AZT at concentrations of 500 mg/kg body weight or greater could significantly decrease the total cellularity of the thymus in both the 7 and 14 day groups. In the groups treated with 1000 mg/kg, the thymus was almost completely atrophied. These results demonstrated that AZT can significantly decrease the total cellularity of the thymus. It is not clear as to how AZT may cause thymic atrophy. We suggest that this may result from the direct effect of AZT rather than an indirect effect such as triggering of stress hormones because the *in vitro* studies demonstrated that AZT was capable of directly exhibiting immunomodulatory properties.

The effect of AZT on the thymus could seriously damage the integrity of the immune system. The thymus plays a vital role in the immune system. Immature T cells enter the thymus and through a series of negative and positive selections, leave as mature T cells with the ability to function in the immune system. In order to keep the immune system balanced, the population of mature T cells must be maintained. In this study we demonstrated the ability of AZT to alter the ratios of the various T cell subset as well as drastically reduce the total cellularity of the thymus. These changes could ultimately lead to a state in which the thymus is unable to properly replenish the immune system with mature T cells. Individuals infected with HIV develop a seriously compromised immune system. Much of this damage is due to the infection and ultimate destruction of the $CD4^+$ T cells caused by HIV infection. These patients use AZT in the hope of delaying such destruction of their immune systems. However if AZT can act in a way to prevent the thymus from replenishing the immune system with functional T cells, it may serve no purpose and could ultimately lead to the progression to AIDS. The results from the current study are also important in the treatment of pediatric HIV infection with AZT. In such clinical cases, if AZT were to induce thymic involution, it may prevent the maturation of T cells and trigger immunosuppression thereby enhancing the susceptibility of these individuals to opportunistic infections as well progression of HIV infection and AIDS.

Studies have implicated suppression of IL-2 production to AZT toxicity (Mostafa et al., 1989). It was shown that IL-2 could reconstitute AZT-induced inhibition of mitogenic responses in peripheral blood mononuclear cells. These results suggest that IL-2 may play an important role in the therapeutic administration of AZT. The study suggests that with the reduction of CD4 cells in HIV infected individuals, the relative amount of IL-2 production is also suppressed, leading to increased immunosuppression. If IL-2 is added T cells would be stimulated, increasing their concentration so any suppressive effects caused by AZT on the T cells would be reduced allowing for AZT to concentrate on inhibition of viral replication.

With the demonstration that AZT drastically reduces the total cellularity of the thymus we wanted to determine if these changes could be reversed. During T cell activation, their surfaces under go various changes. An important change is the acquisition of IL-2 receptors. When T cells are activated, IL-2 is secreted. The IL-2 then interacts with the IL-2 receptors to mediate T cell growth. In the next series of experiment, IL-2 was injected i.p. at the same time of AZT-treatment to determine if IL-2 could reconstitute the cellularity of the thymus. The results from this study showed that IL-2 was able to reconstitute the total cellularity of the thymus in groups treated with 500 mg/kg body weight of AZT. The results from this could be important in the future administration of AZT. These results are consistent with previous work in which it was shown that the addition of IL-2 to bone marrow cell cultures could reduce the toxicity induced by AZT (Nokta and Pollard, 1989). Their results also implicated that AZT inhibits the production of IL-2 . Together the studies strengthen the hypothesis that IL-2 production may be the target of AZT induced immune suppression.

In addition, we showed that thymic cells from AZT-treated mice were more responsive to stimulation with IL-2 *in vitro* and were even more responsive if they received IL-2 i.p. during AZT treatment. One possible explanation for this is that within the thymus the only T cells expressing the IL-2 receptors are the double-negative cells ($CD4^-CD8^-$). In our study we showed that the percentage of double-negative cells within the thymus are significantly increased. With this increase, the ratio of cells with the capacity to respond to IL-2 would be greatly increased. In addition the administration of IL-2 *in vivo* could have caused the double negative T cells to proliferate, significantly increasing this population. This leads to the question of whether the addition of IL-2 was reconstituting the normal thymic cellularity or if it was specifically increasing the double-negative cells. However, because the double-negative T cells are the precursors for mature T cells, our data suggests that combination therapy using IL-2 may help to reduce the toxicity of AZT in the thymus.

In addition to looking at the thymocyte differentiation, we also investigated the effects of AZT on the spleen. Our results showed that AZT was unable to disrupt the normal ratio of T cell subsets and did not have any significant effects on the total cellularity. Furthermore, when we examined the effects of *in vivo* treatment with AZT on the T and B cells ability to respond to stimulation using polyclonal mitogens *in vitro,* we found that at concentrations of 500 mg/kg body weight or greater AZT could only moderately suppress T and B cell responsiveness. However, the observed effects on the spleen did not seem to be as drastic as the changes seen in the thymus.

A possible reason for seeing such significant alterations in the thymus and not in the spleen may be due to the activation state of the cells. Within the thymus the T cells are actively differentiating, eventually becoming mature T cells. Once these cells are released into the periphery they become naive resting T cells and will only become active in response to certain stimulation signals. In the previous test, the splenic cells were not exposed to AZT at the time in which they received the activation signal. Therefore, it was possible that AZT acted upon T cells which are in an active state of differentiation. To test this further, mice were injected with AZT for 14 days. On the 9th day of treatment, the mice were immunized with conalbumin, an antigen used in the stimulation of T cells. In this case, the T cells would be exposed to AZT at the time of stimulation by the antigen. In a normal situation the immune system would respond to such a stimulation by the activation of a specific set of T cell which recognize the specific antigen and upon being rechallenged with the same antigen *in vitro* would be able to mount a strong secondary response. In this experiment, we found that AZT was effective in suppressing the T cell response upon restimulation by conalbumin. This finding suggested that AZT-induced suppression may take place at the time of T cell activation.

In addition to using conalbumin, the antigen gp120 of HIV was also used to stimulate T cell growth. Gp120 is a molecule located on the surface of HIV which is believed to play an important role in facilitating the virus to bind to the CD4 molecule on the T cell, making it an important molecule in the infection by HIV. Also, individuals infected with HIV produce Abs against gp120 which may play an important role in preventing the virus from infecting $CD4^+$ T cells.

Therefore, examining the effect of AZT on the immune response to gp120 represents a more accurate account of what is going on in an individual infected with HIV. The results obtained in these experiments were similar to the results in the test using conalbumin. Thus, AZT-treatment led to significant decrease in T cell responsiveness to gp120 of HIV. Together these data suggested that AZT-treatment could have significant consequences on the ability of the immune system to combat the virus or any other opportunistic infection or cancer that the host may encounter, due to an increased immunosuppressive state.

In the previous experiments the specific antigen was injected along with complete freund's adjuvant. Therefore, there exists the possibility that AZT is affecting the immune response to the freund's adjuvant. However, this possibility is less likely because the *in vitro* studies did clearly demonstrate that AZT can directly suppress T cell activation.

From the above results we concluded that AZT was able to alter the functions of the immune system. To further investigate the mechanism by which AZT acted, we carried out the next series of experiments *in vitro* . By carrying out experiment *in vitro* it is possible to identify direct versus indirect mechanism of action of AZT. The indirect effects could include alterations in the nutritional status, induction of stress or other hormonal changes or immunological alterations brought about by the *in vivo* produced metabolites of AZT.

Most of the previous studies examining the proliferation of cells in response to treatment with AZT, *in vitro*, have used the $^3$H thymidine uptake assay (Daniak et al., 1988). In our study we showed that AZT and $^3$H thymidine compete for uptake in this assay system making the $^3$H thymidine uptake assay an improper means for measuring proliferation of cells subjected to AZT-treatment. We, therefore, used a relatively new colorimetric assay that utilized MTT (Mosmann, 1988). MTT is a tetrazolium salt that measures the activity of dehydrogenase enzymes. The tetrazolium ring is cleaved in active mitochondria and therefore the reaction occurs only in living cells. The reaction produces blue crystals that can be rapidly and precisely measured by multiwell scanning spectrophotometers (ELISA readers).

After showing that the MTT assay was an appropriate means for measuring the proliferation of cells treated with AZT *in vitro,* we then went on to investigate whether or not AZT was able to reduce the proliferation of the T and B cells when stimulated with polyclonal mitogens. The results clearly showed that AZT could effectively reduce the ability of T and B cells to proliferate in response to these mitogens. Similar inhibition was seen in the proliferation of haematopoietic progenitor cells in the presence of AZT *in vitro* (Daniak, et al. 1988). In this assay, the cells were exposed to AZT and the mitogen simultaneously. The results support the hypothesis that AZT is acting upon cells that are actively proliferating and differentiating.

An important function of T cells is their ability to recognize and lyse foreign cells.

This process involves more than just proliferation. The cells involved designated cytotoxic T lymphocytes (CTL) have to synthesize and secrete cytotoxic molecules such as granzymes and perforin which participate in the lysis of the target cells. Our next set of experiment were designed to determine if AZT could alter the CTL function. The results showed that AZT almost completely inhibited the ability of cytotoxic T cells to lyse allogeneic tumor targets. These data suggested that AZT-treatment could lead to serious consequences in the case of a host being challenged by viral infections and cancers. Our results also suggest that at high doses, AZT could adversely affect the immune system, while combating the HIV infection.

In previous experiments we used IL-2 to try and reverse the effects caused by AZT. This same idea was implemented in the case of the suppression caused to the cytotoxic T lymphocytes. It was observed that IL-2 was able to almost completely reconstitute the cytotoxic abilities of the T cells. These results were seen in cells cultured with up to 500 μM AZT. However when the AZT concentration was increased to 1000 μM, the IL-2 was unable to reverse the suppression, possibly because this concentration was extremely toxic to the cells leaving them irreversibly affected.

There are several possible explanations for the observation that IL-2 can reconstitute AZT suppression. First, it is possible that AZT could directly suppress the ability of T cells to produce IL-2. This would greatly affect the T cell differentiation. Another feasible possibility is that AZT was interfering with the production and expression of IL-2 receptor. If AZT reduces the density of IL-2 receptor on the activated T cells, an increase in the concentration of IL-2 could help alleviate the suppression. One final explanation is that AZT could be directly suppressing activation of T cells by preventing them from synthesizing DNA. The addition of IL-2 could be helpful in this type of suppression by increasing the proliferation of T cells. Further clinical studies should be carried out to determine any possible use and benefit of IL-2 therapy in conjunction with AZT-treatment.

Although the mechanism of action of AZT against HIV is well understood, the exact cellular and molecular mechanism involved in the toxicity of nucleoside analogs is not clear. Thus, several hypothesis have been proposed for the biochemical mechanisms responsible for the cytotoxic effects. Many authors have suggested that the triphosphate metabolites may not induce cellular toxicity (Hao et al., 1990; Richman et al., 1987). For example, Balzarini et al. observed that higher AZT triphosphate levels were present in murine cells than in human cells. Also, the intracellular levels of 5'-triphosphates closely correlate with their anti-retroviral effects but not with cytotoxic effects (Balzarini et al. 1988; Balzarini et al. 1989). This was further corroborated by Sommadossi et al. (1989), who recently studied the relationship between intracellular concentration of 5-triphosphate metabolites of dideoxynucleosides in human bone marrow cells and cellular toxicity as assessed by inhibition of cell proliferation. It was observed that AZT triphosphates accumulate to the lowest level when compared to other dideoxynucleosides, but the major cellular toxicity was observed with AZT.

In addition to the above studies which suggest alternate pathway of cellular toxicity, the efficiency of phosphorylation may not always determine the extent of inhibition of HIV infection in certain cell types. For example, monocytes or macrophages have lower levels of kinases than lymphocytes (Stadecker and Unanue, 1979; Richman et al. 1987) and therefore, dideoxynucleosides may not be efficiently phosphorylated in them. In spite of poor phosphorylation, low concentrations of AZT and other dideoxynucleosides profoundly inhibit replication of HIV in monocytes and macrophages (Perno et al., 1988; Skinner et al., 1988). The potent activity of AZT in monocytes and macrophages despite poor phosphorylation has been explained by the fact that such cells have very low quantities of thymidine-5'-triphosphates, the normal nucleotide that competes with AZT-TP at the level of reverse transcriptase. In addition, some of the cytokines may regulate the activity of nucleoside analogs. For example, granulocyte-macrophage colony stimulating factor (GMCSF) has been shown to increase the entry of AZT into monocytes/macrophages and potentiate its activity against HIV (Hammer, et al., 1987; Perno, et al., 1989 ). Together, such studies suggest that the efficiency of phosphorylation may not be the only factor which determines the extent of inhibition of HIV replication in cells or the degree of cellular toxicity.

In conclusion, the current study on the immunotoxic effects of AZT has shown significant results. It has demonstrated that AZT can significantly alter immunological functions. More specifically, it was observed that AZT caused alterations in the total cellularity and subpopulation ratios of T cells in the thymus. We also showed that AZT was able to suppress T cells ability to respond to antigens of HIV and become activated when stimulated to differentiate into cytotoxic T cells. Furthermore, IL-2 could reverse many of the effects caused by AZT. Together, our studies demonstrate that AZT modulates the cells of the immune system which could in turn have marked repercussions on HIV replication *in vivo* , and on the proper functioning of the immune system. These studies have important clinical implications and warn against chronic use of AZT in asymptomatic HIV-infected individuals.

Although it seems apparent that AZT treatment has some negative effects on the immune system, it is possible from the results from this study to speculate on some possible positive effects. First, it was shown that AZT can suppress the ability of T and B cells to respond to activation signals. An important step in HIV replication is the incorporation of its genetic information into the hosts genome. Once incorporated, HIV directs the host to produce material necessary for the production of mature HIV. It is possible that if AZT prevents T cells from replicating, AZT would, in turn, prevent the T cells from producing HIVs genetic information, halting the production of mature HIV. Secondly it was shown that AZT decreases and alters the T cell populations within the thymus. The thymus is the organ largely responsible for the production of mature cells expressing the CD4 molecule. The main target of HIV is the CD4 T cell subset of the immune system. HIV is able to bind to its target cell by using gp120. Gp120 has a high affinity for the CD4 molecule located on the surface of T helper cells. If AZT inhibits the production of cells expressing the CD4 marker, it could drastically decrease HIV infection particularly in the thymus. This scenario could have important implications in pediatric care of HIV infected individuals, who have an actively functioning thymus. These arguments, although speculative, reaffirm the need for further research delineating the effects of AZT on the immune system and its possible implications in HIV replication.

# Literature Cited


Bilello J. A., Macauley C., Fredrickson T. N., Bell M. M., McKissick C., Shapiro S. G., Personette R., Eiseman J. L. Use of a Neonatal Murine Retrovirus Model To Evaluate the Long-Term Efficacy and Toxicity of Antiviral Agents. Ann New York Acad Sci 1990; 616:238-51

Bosen LJ, Greene JB, Louie E, Seitznan P, Weinten H. Severe polynyesitis-like syndrome associated with zidovudine therapy of AIDS and ARC. N Engl J Med 1988; 3:109-14

Blakely RL, Harwood FC, Huff KD. Cytostatic effects of 2', 3'-dideoxythymidine on transformed human hemopoietic cell lines. Mol Phamacol 1989; 37:328-32

Cheng YC, Dutschaman GE, Bastow KF, Sarngadharan MG, Ting R. Human Immunodeficiency virus reverse transcriptase: general properties and its interactions with nucleoside triphosphate analogs. J Biol Chem 1987; 262:2187-9

Dahlberg JE, Mitsuya H, Blam SB, Broder S, Aaronson SA. Broad spectrum antiviral activity of 2'-3'-dideoxynucleosides. Proc Natl Acad Sci USA 1987:84: 2469-73

Daniak N, Worthington M, Riordan M, Kreczko S, Goldman L. 3'-azido-3'-deoxy-thymidine (AZT) inhibits proliferation *In vitro* of human haematopoietic progeinitor cells. Bri J Haemat 1988; 69: 299-304

Dean JH, Luster MI, Munson AE, Amos H. Imunotoxicology and Immunopharmacology. Raven Press 1985; pp.229-43

Dean JH, Ward EC, Murray MJ, Lauer LD, House RV. Immunotoxicity of Polycyclic Aromatic Hydrocarbons. NIH Immunotoxicology Workshop 1993; 259-302

Dean TN, Kakkanaiah VN, Nagarkatti M, Nagarkatti PS. Immunosuppression by aldicarb of T cell response to antigen-specific and polyclonal stimuli results from defective IL-1 production by the macrophage. Toxicol and Appl Pharmacol 1990;106: 408-17

Du DL, Volpe DA, Grieshaber CK, Murphy MJ. *In Vitro* toxicity of 3'-azido-3'-dideoxy-thymidine, carbovir and 2',3'-didehydro-2',3'-dideoxythymidine to human and murine haematopoietic progenitor cells. Br J Haematol 1992; 80:437-45

Fauci AS. The human immunodeficiency virus: infectivity and mechanism of pathogenisis. Sci 1988; 239: 617-22

Furman PA, Tyfe JA, St Clair MH, et al. Phosphorylation of 3' azido 3'deoxythymidine selective interaction of 5' triphosphate with Human Immunodeficiency Virus reverse transcriptase. Proc Natl Acad Sci USA 1986; 83:8333-7

Furmanski P, Bourguignon GJ, Bolles CS, Corombos JD, Das MR. Inhibition by 2'3' dideoxythymidine of retroviral infection of mouse and human cells. Cancer lett 1980; 8:307-15

Furth PA, Kazakis AM. Nail pigmentation changes associated with azidothymidine (zidovudine). Ann Intern Med 1987; 107:350

Gartner S, Markovitz SP, Markovitz DM, Kaplan MH, Gallo RC, Popovic M. The role of mononuclear phagocytes in HTLV-III/LAV infection.. Science 1986; 213:215-9

Gill PS, Rarick M, Brynes RK, Causey D, Loreins C, Levine AM. Azidothymidine associated bone marrow failure in acquired immunodeficiency syndorme(AIDS) Ann Intern Med 1987; 107:502-5

Gottlieb MS, Wolfe PR, Chafey S. Response of AIDS-Related thrombocytopemia in intravenous and oral azidothymidine (3'-azido-3'-deoxythymidine). AIDS Res Hum Retroviruses 1987; 3:109-14

Hagler DN, Frame PT. Azidothymidine nuerotoxicity. Lancet 1986; 2:1392-3

Hammer, S. M. and Gillis, J. M. Synergistic activity of granulocyte-macrophage colony-stimulating factor and 3'-azido-3' deoxythymidine against human immunodeficiency virus *in vitro.* Antimicrob Agen & Chemo 1987; 31:1046-1050

Hoa Z, Dalal M, Cooney DA, et al. A comparison of 2' 3' deoxynucloside 5' triphosphates as inhibitors of retroviral reverse transcriptase. In Proceedings of the American Association for Cancer Research. Atlanta May 20-23 1987 vol 28: Waverly Press, 1987:323

Klein J. Immunology The Science of Self-Nonself Discrimination. John Willey & Sons 1982; pp. 20-8

Luster MI, Germolec DR, White KL, Fuchs BA, Fort MM, et al. A comparison of three nucleoside analogs with anti-retroviral activity on immune and hematopoietic functions in mice: *in vitro* toxicity to precursor cells and microstromal environment. Toxicol Appl Pharmacol 1989; 101:328-9

Mansuri MM, Hitchcock MJ, Burocker RA, et al. Comparison of In Vitro biological properties and mouse toxicity of three thymidine analogs active against human immunodeficiency virus. Anitmicrob Agen and Chemo 1990; 34:637-41

Matsushita S, Mitsuya H, Reitz MS, Broder S. Pharmacological inhibition of *in vitro* Infectivity of human T lymphotropic virus type I. J Clin Invest 1987;80:394-400

Melameed AT, Muller RJ, Gold JWM, et al. Possible zidovudine-induced hepatoxicity. JAMA 1987; 258:2063

Mitsuya H, Dahlbeg JE, Spigelman Z, et al. 2'-3' dideoxynucleoside broad spectrum antiretroviral activity and mechanism of action. In Bolognesi D ed. Human Retroviruses Cancer, and Aids Approaches to prevention and therapy. New York: Alan R Liss 1988: 407-21

Mitsuya H, Jarrett RF, Matsukura M, et al. Long term inhibition of human T lymphotrophic virus typeIII/lymphademopathy associated virus (HIV) DNA synthesis and RNA expression in T cells protected by 2'-3' dideoxynucleosides*in vitro*. Proc Natl Acad Sci USA 1987; 84: 2033-7

Mosmann TR, Coffman RL. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989; 7:145

Nokta MA, Pollard RB. Differential reconstitution of zidovudine-induced inhibition of mitogenic responses by intetleukin-2 in peripheral blood mononuclear cells from patients with human immunodeficiency virus infection. Antivir Res 1989; 11:191-202

Perno GF, Yarchoan R, Cooney DA, et al. Inhibition of human immunodeficiency virus (HIV-I, HTLV-III) replication in fresh and cultured human peripheral blood monocytes/macrophage by azidothymidine and related 2'-3'-dideoxynucleosides. J Exp Med 1988; 168: 1111-25

Pluda JM, Yarchoan R, Jaffe ES, Feverestein JM, Solomon D, Steinberg SM, Wyvill KM, et al. Development of non-Hodkin lymphoma in a cohort of patients with severe immunodefeciency virus (HIV) infection on long term anti-retroviral therapy. Ann Intern Med 1990; 113:276-82

Richman DD, Fisci MA, Gineco MH, et al. The toxicity of Azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex: A double blind, placebo-controlled trial. N Engl J Med 1987; 317: 192-7

Richman DD, Kernbluth RS, Carson DA. Failure of dideoxynucleosides to inhibit human immunodeficiency virus replication in cultured human macrophage. J. Exp. Med. 1987; 166:1144-9

Roitt I, Brostoff J, Male D. Immunology. The C.V. Mosby Co., St Louis. 1989.

Ruprecht RM, O'Brien LG, Rossini LD, Nusinoff-Lehrman S. Suppression of mouse viremia and retroviral disease by 3'-azido-3'-deoxythymidine. Nature 1986;323: 464-569

Scollay RP, Bartlett, Shortman K. T cell development in the adult murine thymus changes in the expression of the surface antigen Ly2, L3T4, and B2A2 during development from early precursor cells and emigrants. Immunol. 1984; 2:79

Shaw DR, Knight DR, Waterman AL, Sommadossi JP. 3'-azido-3'-deoxythymidine inhibition of human lymphocyte cytolytic function *in vivo.* Biochem. Pharmacol. 1991; 41: 287-91

Skinner MA, Mattews TJ, Greenwall TK, Bolognesi DP, Hebdom M. AZT inhibits HIV-I replication in moncytes. J AIDS 1988; 1:162-3

Sommadossi JP, Carlisle R, Zhou Z. Cellular pharmacology of 3'-azido3' deoxy-thymidine with evidence of incorporation into DNA of human bone marrow cells. Mol Pharmacol 1989; 36: 9-14

Stadecker, M. J. and Unanue, E. R. the regulation of thymidine secretion by macrophages. J Immunol 1979; 123:568-71; .

Starnes MC, Cheng YC. Cellular metabolism of 2'-3'-dideoxycytidine, a compound active against human immunodeficiency virus *in vitro*. J Biol Chem 1987; 262:988-91

Thompson MB, Dunnick JK, Sutphin ME, Giles HD, Irwin RD, Prejean JD. Hematologic toxicity of AZT and ddC adminsistered as single agents and in combination to rats and mice. Fund Appl Toxicol 1991; 17: 159-176

Vrang L, Bazin H, Remaud G, Chattopadhyaya J, Oberg B. Inhibition of the reverese transcriptase from HIV by 3'-azido-3'-deoxythymidine triphosphate and its *threo* analogue. Antiviral Res. 1987; 7: 139-49

Walker RE, Parker RI, Kovacs JA, et al. Anemia and erythropoiesis in Patients with acquired immunodeficiency syndrome (AIDS) and kaposi sarcoma treated with zidovudine. Ann Intern Med 1988; 108: 372-6

Waquar MA, Evans MJ, Manly KF, Hughes RE, Huberman TA. Effects of 2'-3'-dideoxy-nucleosides on mammalian cell and viruses. J Cell Physiol 1984; 121:402-8

Yarchoan R, Klecker RW, Weinhold KJ, et al. Administration of 3'-azido-3'-deoxythymidine, an inhibitor of HTLV-III/LAV replication to patients with AIDS or AIDS-related complex. Lancet 1986; 1: 575-80

Yarchoan R, Pluda J. M., Perno C. A., Mitsuya H., Thomas R. V., Wyvill K. M., Broder S. Initial clinical experience with dideoxynucleosides as single agents and in combination therapy. Ann NY Acad Sci. 1990; 616:328-43

Zimmerman TP, Mahony WB, Pruski. 3'-Azido-3'- deoxythymidine: an unusual nucleoside analogue that permeates the membrane of human erythrocytes and lymphocytes by nonfacillitated diffusion. J Biol Chem 1987; 262: 5748-54

# APPENDIX- Abbreviations

AIDS, Acquired Immunodeficiency Syndrome; APC, antigen presenting cell; AZT, Azidothymidine; CD , cluster of differentiation; Con A, concanavalin A; CPM. counts per million; CSF, colony stimulating factor; CTL, cytotoxic T lymphocyte; ddA, dideoxyadenosine; ddC, dideoxycytidine; ddI, dideoxyinosine; FITC, Fluorescein Isothiocyanate; gp, glycoprotein; HIV, Human Immunodeficiency Virus; Ig, immunoglobulin; IFN-$\gamma$, gamma interferon; IL, interleukin; i.p, intraperitoneal; LPS, lipopolysaccharide; mAb, monoclonal antibody; MHC, major histocompatibility complex; MTT, 3-(4,5-dimethylthiazol 2-yl)-2,5 diphenyl tetrazolium bromide; NK, natural killer; O.D, optical density; PE, phycoerythrin; $T_c$, cytotoxic T cell; TCR, T cell receptor; $T_h$, T helper cell.

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