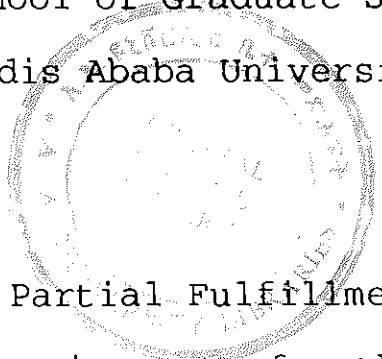


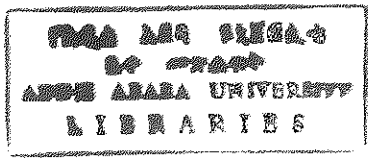
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RESPONSE OF ETHIOPIAN HIV-1  
TO AZIDOTHYIMIDINE, DIDEOXYINOSINE AND  
INTERFERON-ALPHA

A Thesis Presented to  
The School of Graduate Studies  
Addis Ababa University



In Partial Fulfillment  
of the Requirements for the Degree  
of Master of Science in  
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By

Zenebech Wondimu

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To Him, 'who has all  
the powers to make  
the impossible possible'.

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## ABBREVIATIONS

AIDS	:Acquired immunodeficiency syndrome
AZT	:3'-azido-3'-deoxythymidine or azidothymidine
$\beta_2$ -M	:Beta <sub>2</sub> -microglobulin
cpm	:Counts per minute
ddI	:2',3'-dideoxyinosine or didanosine
ddN	:Dideoxynucleoside
DNA	:Deoxyribonucleic acid
EASIA	:Enzyme amplified sensitivity immunoassay
EIA	:Enzyme immunoassay
ELISA	:Enzyme linked immunosorbent assay
HIV-1/-2	:Human immunodeficiency virus type 1 and 2
HRP	:Horseradish peroxidase
IC <sub>50</sub>	:50% inhibitory concentration
IFN- $\alpha$	:Interferon alpha
nmol	:Nanomolar
NA	:Nucleic acid
OPD	:Ortho-phenylenediamine
PBMC <sub>s</sub>	:Peripheral blood mononuclear cells
PHA	:Phytohaemagglutinin
pg	:Picogram
p	:Probability
rpm	:Revolutions per minute
RNA	:Ribonucleic acid
r <sub>s</sub>	:Spearman's rank correlation coefficient
RT	:Reverse transcriptase
SE	:Standard error
TCGF	:T-cell growth factor
TNF- $\alpha$	:Tumour necrosis factor alpha
u	:unit
$\mu$ g/mcg	:Microgram
$\mu$ M	:Micromolar

## ABSTRACT

Human Immunodeficiency Virus (HIV), the aetiologic agent of Acquired Immunodeficiency Syndrome (AIDS) is known to manifest high degree of genetic variability. Based on the genomic structure two types of HIV, HIV-1 and HIV-2 are known to date. The presence of a distinct strain of HIV-1 in Ethiopia has been reported. Differences in the pattern of viraemia and immunologic reactions from the one documented in European and North American patients has been observed in Ethiopians infected with this virus. Treatment with azidothymidine (AZT) has been shown to result in decreased serum levels of  $\beta_2$ -microglobulin ( $\beta_2$ -M), tumour necrosis factor alpha (TNF- $\alpha$ ) and neopterin in the European and North American HIV-1 patients. Whether there are differences in sensitivity to antiviral compounds, such as AZT, dideoxyinosine (ddI) or interferon alpha (IFN- $\alpha$ ) in Ethiopian HIV-1 strains and the effect of treatment on the immunological reactions were not known. The *in vitro* sensitivity of 8 Ethiopian strains to antiviral compounds was investigated in parallel with 8 Swedish strains all from untreated AIDS patients. The antiviral effects were measured by p24 antigen capture ELISA and Reverse transcriptase activity assay. A pilot controlled study of 200mg and 600mg oral AZT was conducted in 53 HIV-1 infected Ethiopians. The treatment effects were evaluated by assessment of the clinical status of the patients and the measurement of serum levels of  $\beta_2$ -M, TNF- $\alpha$  and neopterin. No statistically significant difference in drug sensitivity was found between the Ethiopian and The Swedish strains to AZT, ddI and IEN- $\alpha$  *in vitro*. In the majority of the treated patients improvement in their clinical conditions was reported and a gain in body weight was observed. A statistically significant decrease in the levels of  $\beta_2$ -M, TNF- $\alpha$  and neopterin were found in the treated patients in contrast to the control patients.

## 1. INTRODUCTION

### 1.1 PROPERTIES OF RETROVIRUSES

Retroviruses consists of a group of viruses with an RNA genome. These viruses specifically contain the enzyme, reverse transcriptase (RT) (Baltimore D. 1970; Coffin et al. 1992). After infecting a cell, the viral RNA genome is transcribed by the RT to a double stranded DNA. The DNA is most often integrated into the chromosomal DNA of the host cell, although some DNA may remain unintegrated. The DNA serves as a template for the production of further viral genomes, utilizing the replicative mechanisms of the host cells (Shaw et al. 1984; Greene et al. 1991).

The retroviruses are divided into three subfamilies: Oncovirinae, Spumavirinae, and Lentivirinae. These viruses cause a wide variety of diseases in man and vertebrate animals, eg. immunodeficiency syndromes, malignant diseases, CNS disorders, and autoimmune aberrations (Varmus et al. 1988). The most important retrovirus for humans, the human immunodeficiency virus (HIV), belongs to the Lentivirinae subfamily (Gonda et al. 1985; Chui et al 1985).

### 1.2 HUMAN IMMUNODEFICIENCY VIRUS

The first cases of an immunodeficiency syndrome, now referred to as acquired immunodeficiency syndrome (AIDS), were reported in a group of homosexuals from the United States of

America (USA) (Gottlieb et al 1981, Masur et al. 1981). In 1983, HIV was isolated for the first time from a homosexual man with persistent generalized lymphadenopathy (PGL) in France (Barre-Sinoussi et al, 1983). The virus was later identified as the cause of AIDS (Gallo et al. 1984; Levy et al. 1984). By 1986, a second type of HIV was isolated from healthy asymptomatic individuals in West Africa (Clavel et al. 1986). Nucleotide sequence analysis showed that this virus differed from the previous HIV strains by more than 55% of the nucleotides (Clavel et al 1986). The virus isolated first was named HIV-1 and the West African type HIV-2 (Coffin et al 1986).

The HIV genome encompasses the three major retrovirus genes, group specific antigen (gag), polymerase (pol) and envelope (env), as well as three genes with regulatory functions and two more accessory genes, which are necessary in the replicative process of the virus (Ratner et al. 1985, Varmus et al. 1988). The gag gene codes for the viral core proteins, such as the p24 antigen, the pol gene codes for the RT, and the env gene for the envelope glycoproteins, i.e gp120, and gp41 (Cullen and Greene 1990). HIV manifests a high degree of genetic variability in vivo (Alizon et al. 1986; Hahn et al. 1986). Thus, genetically different strains can be identified in the same HIV infected person at different times, and even at the same time (Fisher et al. 1988). Most of the diversity is located in the env region which greatly increases the difficulty of preparing an effective vaccine against HIV. The genes coding for the central core

proteins are the most stable ones. It is possible that this genetic variation may result in HIV strains that differ in their ability to infect and cause disease, and also in their sensitivity to antiviral drugs (Mohri et al. 1993), although, the variation in the pol gene where the drug acts is rather limited.

An important characteristic of HIV is that its replication in vivo may be restricted, resulting in the maintenance of HIV in a latent form in T cells and macrophages (Bednaririk and Folks 1991). The absence of expressed viral proteins, results in persistently infected cells, which remain invisible to the host immune system. HIV's main target cells are the lymphocytes bearing the CD4 surface antigen (Klatzmann et al. 1984). The virus also has a tropism to other CD4-bearing cells such as monocytes, macrophages, and microglia cells (Levy et al. 1985; Gartner et al. 1986; Schnittman et al. 1989). The infection of a cell by HIV occurs by a binding of the virus through its outer protein, gp120, to the CD4 antigen on the surface of the susceptible cells. This process is followed by penetration and uncoating. Once inside the cytoplasm of the host cell, the viral coded RT transcribes the viral RNA genome to DNA. The proviral DNA is then integrated into the cellular DNA, where it may remain silent, until reactivated. Reactivation results in replication of the virus, where mechanisms of the host cell are utilized for the synthesis of viral genomic and messenger RNA (mRNA). The mRNA is translated and the proteins are processed and assembled at the plasma membrane of the host cell. The mature virions are later

released by budding into the blood stream (Haseltine 1988).

### Routes of Transmission

HIV has been isolated from blood (Gallo et al. 1984), semen and genital secretions (Zagury et al. 1984), as well as from other body fluids such as cerebrospinal fluid (CSF), saliva, and breast milk (Groopman et al. 1984; Ho et al. 1985). The major route of transmission of HIV infection is sexual contact (Levy 1993). Exposure to contaminated blood and blood products, inoculation through needle sticks and the sharing of contaminated needles by intravenous drug abusers and perinatally from mother to their infants are also important routes of transmission (Curran et al. 1985; Ades et al. 1991).

There is no epidemiological evidence for the spread of the virus by aerosol, saliva, casual contact or insects (Friedland et al. 1986; CDC 1986; Lyons et al. 1986).

### Methods of Detection and Diagnosis

HIV can be isolated from blood, CSF, plasma, serum, saliva and tissues by co-cultivation with susceptible target cells (Ehrnst et al. 1988). This method detects infectious HIV virions. An alternative method to detect HIV is the polymerase chain reaction (PCR), which is used to identify HIV DNA or RNA in uncultured peripheral blood mononuclear cells (PBMC) and cell-free plasma respectively (Kwoks et al. 1987). These two methods are both expensive and laborious. Thus, they cannot be

used as diagnostic tools for a large number of patients.

Kits which are relatively inexpensive are commercially available for the detection of either HIV-antigens or antibodies to HIV by following rather simple enzyme linked immunosorbent assays (ELISA). HIV-1 antigen is a low sensitivity marker for detection of HIV infection since it is present only transiently prior to seroconversion and later it is complexed by specific antibodies. For this reason antigen testing should not be considered as a substitute for antibody testing as a screening test for HIV infections. Since detectable amounts of antibodies are available, throughout the illness, once they have developed, the antibody tests should be used for the diagnosis of HIV infection. However, repeated and confirmatory tests are recommended to verify the specificity of positive reactions. Antigen tests however, do have important application in monitoring the presence of HIV-1 antigens in co-cultures of peripheral blood lymphocytes and monitoring treatment of HIV-1 infected individuals with antiviral drugs.

### Immune Responses

Antibodies to a number of viral antigens develop soon after infection, but the response pattern changes during the progress of infection. Thus, antibody levels to envelope glycoproteins are maintained, but those to the core proteins decline. Sustained protective immunity against HIV is not readily achievable, because HIV escapes immune responses by developing glycoprotein

antigens with different structures on the outer membrane (Cloyd and Holt 1987; Harada et al. 1987).

Cytotoxic T-cells and antibody dependent cellular cytotoxicity can lead to lysis of cells producing the virus (Evans et al. 1989; Autran et al. 1991). However, since the virus also exists in a latent form, it may escape immune surveillance.

A variety of factors induced by stimulation with antigens and other physiologic cellular signals have been identified which can stimulate HIV-1 latently infected cells (Rosenberg et al. 1989). The three substances, beta 2-microglobulin ( $\beta_2$ -M), neopterin and tumour necrosis factor alpha (TNF- $\alpha$ ) may be used as markers of activation of the cellular immune system. There is a correlation between the levels of these factors in serum, urine, or CSF, and the clinical outcome of HIV-1 infection (Lähdevirta et al. 1988; Sönnnerborg et al. 1989). It has been demonstrated that activation of the cellular immune system is a regular feature of the early stages of HIV-1 infection and increases as the infection progresses (Franzetti et al. 1988; Sönnnerborg et al. 1989).

Beta<sub>2</sub>-microglobulin is a low molecular weight protein that forms the light chain of the class I major histocompatibility complex (MHC) antigens on the surface of nucleated cells including B and T lymphocytes and macrophages (Cresswell et al. 1974). The substance is synthesized and secreted by many types of normal and malignant cells of hematopoietic, mesenchymal and epithelial origin (Ervin and Nilsson 1974). Increased levels of

$\beta$ 2-M are considered to reflect activation of the cellular immune system or increased cell membrane turnover in viral diseases including HIV infection (Bhala et al. 1983; Cooper et al. 1984).

Neopterin is a pyrazino-pyrimidine compound derived from guanosine-triphosphate. It is produced by macrophages and monocytes mainly after stimulation by IFN-gamma (Bitterlich et al. 1988). Interleukin 2 (IL-2), TNF- $\alpha$  and IFN- $\alpha$  may also stimulate neopterin secretion, probably via a cytokine cascade that includes production of IFN-gamma (Bitterlich et al. 1988; Troppmair et al. 1988). IFN-gamma is produced by T-lymphocytes and constitutes a major activator of antigen presenting cells. Thus, neopterin by being an indirect marker of IFN-gamma production may give information about the activity of T lymphocytes. By being a direct activation product it may also give information about macrophages. Elevated levels of neopterin in serum and urine have been documented in people with HIV-1 infection (Lambin et al. 1988) and in the CSF of patients with HIV encephalopathy (Sönnnerborg et al. 1989).

Tumour necrosis factor alpha (TNF- $\alpha$ ) is a 17 KDa polypeptide produced mainly by cells of the monocyte/macrophage lineage upon immunological stimulation (Beutler and Cerami 1986). A stimulating effect of TNF- $\alpha$  on HIV-1 replication in cultured mononuclear cells has been reported (Folks et al. 1989) and it has been claimed that TNF- $\alpha$  may play an instrumental role in the reactivation of latent HIV-1 infection (Matsuyama et al. 1991). Early increases in the production of TNF- $\alpha$  have been documented

in HIV-1 infections in Ethiopian patients with a more pronounced increase being observed with progress of the disease (Ayehunie et al. 1993) as compared to European patients. Increased levels of TNF- $\alpha$  have been associated with weight loss in animals infested with parasites and in patients with AIDS related complex (ARC) or AIDS in Europe and the USA (Lähdevirta et al. 1988). The association of TNF- $\alpha$  with weight loss in African HIV-1 infected patients, however, has been studied only to a limited extent.

#### The disease AIDS

HIV causes a persistent chronic infection, which results in a debilitating progressive disease. Thus, the virus does not transform the cells, but destroys them. Since CD4 lymphocytes are essential effectors of the immune response, the destruction of these cells results in an irreversible and profound immunosuppression, which renders the patient susceptible to opportunistic pathogens (Fahey et al., 1984; Fauci et al., 1988).

AIDS in Africa is caused by both HIV-1 and HIV-2, though the proportion of infections with these viruses varies from one country to another within the continent (Clavel et al., 1987; Santos-Ferreira et al. 1990). Most of AIDS patients in Europe and the USA are homosexual men, intravenous drug users or recipients of blood products, while in Africa, the majority of cases are heterosexual men and women with no history of intravenous drug use or of receiving blood products (Melbeye et al. 1988; Carael et al. 1988). There are substantial number of paediatrics AIDS

patients residing in the continent indicating the importance of vertical transmission from infected mothers to their infants (Ryder et al. 1989). The rate of spread of the infection varies among different countries, being rapid in the most affected countries such as Malawi, Rwanda, Uganda, Tanzania, and Zambia. Within the same country the prevalence of the infection is higher in urban than in rural areas (Clumeck et al., 1985; Nzilambi et al., 1988)). This variation in prevalence may indicate the relatively low chance of being infected with this virus in populations leading a stable married life. With the passage of time, however, this picture is changing and the number of reported cases has been steadily increasing in rural regions (Andersson et al. 1991) indicating the spread of the virus from urban to rural regions. Even though, it is difficult to assess the magnitude of HIV infection in Africa, the epidemic is steadily increasing in the general heterosexual population and among the major groups "at risk" such as female prostitutes, their male clients and patients attending sexually transmitted disease clinics (Anderson et al. 1991). According to the World Health Organization estimate, in early 1993 about 12 million adult HIV infections are found world wide, 7.5 million of them are African (WHO 1993). It is also estimated that this continent has the largest number of people with AIDS per million inhabitants (500-1000) (Santos-Ferreira et al. 1990).

Clinical AIDS as defined by the Center for Disease Control (CDC) is characterized by opportunistic infections and malignant

diseases in patients without other known causes of immunodeficiency (CDC, Morbid. Mortal. Wkly. Rep. 1985). Thus, AIDS is not a single illness, but represents a broad spectrum of clinical conditions. For the African situation a clinical case definition has been developed by WHO (Table 1). Among the patients, the number of associated illnesses and complications increases as the disease progresses. The later stage of the infection known as "full-blown AIDS" is often associated with diarrhoea and wasting syndrome, persistent fever, herpes zoster, and other skin disorders, bacterial, viral and fungal infections, persistent generalized lymphadenopathy (PGL), and Kaposi's sarcoma. Kaposi's sarcoma which occurs in approximately 40% of homosexual men with AIDS in the USA is the most common malignancy associated with HIV infection, an otherwise rare tumour of the vascular endothelium. Although, this malignancy was endemic in some Central and East African countries such as Rwanda, Zaire, Uganda and Zimbabwe, it used to be extremely rare in young people (Dowing 1984; Wilcock et al. 1989). Its incidence has, however, increased with the advent of HIV infection in Africa (Wilcock et al., 1988).

Other malignancies, such as B-cell lymphomas and non-Hodgkin's lymphoma may also develop in people with AIDS.

The occurrence of neurologic manifestations in approximately 60% of patients is an important component of AIDS (Navia et al. 1986). HIV, opportunistic infections and the related tumours may cause such symptoms. These manifestations include encephalitis,

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progressive dementia, spinal cord inflammation, acute aseptic meningitis, chronic meningitis and peripheral neuropathy (McArthur et al. 1987).

In addition to patients with typical AIDS, many patients have other symptoms such as persistent generalized lymphadenopathy (PGL), fever, malaise, fatigue, night sweats, weight loss, loss of appetite, and diarrhoea, (Kaslov et al. 1987). The frequency of lymphadenopathy is somewhat higher in African patients than in European and North American patients (Katzenstein et al. 1990).

Of diseases not included in the case definition pulmonary and/or disseminated tuberculosis, pneumonia, and central nervous system derangement seem to be important in African AIDS patients. These diseases may be used as some of the indicative diseases of particular consideration being tuberculosis which is diagnosed in a fairly high number of patients.

Table 1.

The WHO clinical case definition for AIDS in adults (1986)

Clinical manifestation

## Major symptoms

## Minor symptoms

Weight loss greater than 10% of body weight.	Persistent cough for longer than one month.
fever intermittent or constant for longer than one month.	Generalized lymphadenopathy.
Chronic diarrhoea for longer than one month.	Oropharyngeal candidiasis.
	Generalized pruritic dermatitis.
	Chronic progressive and disseminated Herpes simplex infection.
	Recurrent Herpes zoster infection.

The African HIV epidemic displays several features that are different from those observed in Europe and North America (Quinn et al. 1985; Katzenstein et al., 1990). For instance, the patients often present with pronounced wasting known as "slim disease", the progress of infection is unusually rapid and the survival rate after the diagnosis of AIDS is relatively shorter (Perre et al. 1984; Piot et al. 1984; Serwadda et al. 1985). It is not yet known whether this is due to the characteristics of the virus itself, to host genetic differences, to differences of the cellular immune system, such as an imbalance between TNF- $\alpha$  and IFN- $\alpha$ , or to the number of contacts with infectious agents, which stimulate CD4 cells to divide and thus stimulate the provirus to replicate (Katzenstein et al. 1990; Rosenberg et al.

1990). It has been shown that the inherited genetic makeup of the host determines both the susceptibility of cells to HIV replication and the extent of effectiveness of the antiviral immune response. It may also be related to the mode of transmission of the virus, the major mode of transmission is homosexual contact in Europe and North America, whereas, heterosexual contact is the most common in Africa (Piot et al. 1984). The pattern of viraemia in African patients has been studied to a limited extent only. It seems, however, to differ from that in European and North American patients. For example p24 antigenaemia is rarely found in African patients and high levels of anti-p24 antibodies persist through out the course of infection (Van Den Akker et al. 1988; Katzenstein et al. 1990). Furthermore, the presence of more diverse strains of HIV-1 in Africa has been reported (Benn et al. 1985; Alizon et al. 1986). It is not known whether there is also a difference in sensitivity to anti-HIV compounds, such as 3'-azido-3'-deoxythymidine (AZT), 2',3'-dideoxyinosine (ddI), and IFN- $\alpha$  of isolates from African patients.

### 1.3 ANTIVIRAL CHEMOTHERAPEUTIC AGENTS

Chemotherapeutic agents acting against viruses may be categorized into three broad groups: virucides, antivirals and immunomodulating agents. Virucides inactivate intact viruses, antivirals inhibit viral replication and immunomodulating agents augment or modify the host response to the infection.

The main objective in developing antiviral chemotherapy is selectivity. Thus, the aim is that, the agent selectively inhibits the virus without affecting the host cells or cellular processes. However, viral replication depends primarily on host cell metabolic functions. The challenge has been to identify the specific steps in the life cycle of the virus which can be exploited for therapeutic intervention. These specific steps include attachment to the cell, uncoating of the viral genome, transcription of the viral genome, assembly of the progeny viruses and release of the mature virions. Despite the fact that many compounds have exhibited antiviral activity in vitro, these compounds in people are associated with low therapeutic ratios or unacceptable toxicity. Furthermore, since these agents inhibit the ongoing replication at the host cell level, they cannot eliminate latent viruses. The development of viral resistance to antiviral drugs is another difficulty, which may limit the usefulness of these drugs. Because of these and other related problems, only a limited number of antiviral drugs have been proven to be clinically useful.

#### Nucleoside analogues in the treatment of HIV infection

No prophylactic or therapeutic vaccine against HIV is available at present nor will it be in the near future. Therefore, nucleoside analogues have become important in the treatment of HIV infection. For viral replication, deoxyribonucleoside triphosphates (dNTP) are essential

substrates. Nucleoside analogues in the triphosphate form are capable of inhibiting viral synthesis. These substances, however, cannot be given directly since they are too heavily charged to cross the plasma membrane, and because of their short half life. HIV does not code for nucleoside phosphorylating enzymes of its own, and depends therefore on cellular phosphorylating enzymes (Haseltine 1989).

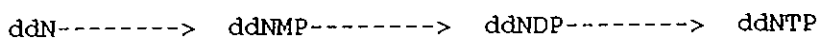
A number of different nucleoside analogues have been shown to have inhibitory activity against HIV in vitro; most of these are dideoxynucleosides (ddNs), eg. azidothymidine (AZT), dideoxyinosine (ddI), dideoxycytosine (ddC), and fluorodeoxythymidine (FLT). These nucleoside analogues must be phosphorylated to the active triphosphate species, by cellular phosphorylating enzymes, before being recognized as nucleic acid (NA) building blocks by NA polymerases (Fig.1)

---

Fig. 1.

The metabolism of nucleoside analogues.

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cellular phosphorylating enzymes.

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The RT enzyme of HIV normally uses as substrates the deoxynucleoside triphosphates (dCTP, dTTP, dGTP, dATP) found in

the cell and incorporates them into the growing DNA chain (Goff S., 1990). The RT may also use dideoxynucleoside (ddN) analogues as a substrate. Furthermore, it does not excise the inappropriately incorporated nucleotides because of the absence of proof-reading activity. Once the triphosphates of the ddN analogue are inserted into the growing chain no further polymerization takes place.

To date three ddN analogues, AZT, ddI, and ddC are the only licensed drugs to treat AIDS/HIV-infection. Their mechanisms of action are similar to those of ddNs, acting as NA chain terminators.

AZT: 3'-azido-3'-deoxythymidine (AZT), is a thymidine analogue, in which the 3'-hydroxyl has been replaced by an azido (-N<sub>3</sub>) group. AZT, also known as azidothymidine or zidovudine, was synthesized in 1964 (Horwitz et al. 1964). Antiviral activity was found against the Friend murine leukemia virus in 1974 (Ostertag et al. 1974). Thereafter, it has been shown to inhibit many other mammalian retroviruses. Later the antiviral activity of AZT against HIV-1 was demonstrated both in vitro (Mitsuya et al. 1985) and in vivo (Yarchoan et al. 1986). Two mechanisms are considered to contribute to its inhibitory action; i.e. its binding to HIV-RT, and incorporation into the growing DNA strand, which leads to chain termination, and competitive inhibition of thymidylate kinase, which synthesizes thymidine 5'-triphosphate from thymidine diphosphate, resulting in reduced levels of the former (Furman et al. 1986).

AZT is rapidly absorbed after oral administration and reaches its peak serum concentration within 30-90 minutes. The mean half life is about 1 hour. It is metabolized primarily by glucuronidation and excreted in the urine. The drug passes the blood brain barrier effectively (Hirsch 1988).

Treatment with AZT has been shown to result in clinical and immunological improvement in patients both in the early and advanced stage of HIV infection (Fischl et al. 1987; Graham et al. 1992). Divergent results have, however, been obtained concerning the long-term effects on patients in the early stages of infection (Seligmann M. 1993; Volberding et al. 1993).

Despite its clinical usefulness, treatment with AZT is associated with the development of toxic side effects, such as macrocytic anaemia, neutropenia, headache, nausea, myalgia, and insomnia (Richman et al. 1987).

Another problem related to treatment of HIV infection with AZT is the selection of AZT-resistant strains in patients on long term therapy (Larder et al. 1989). This resistance is associated with multiple mutations in the pol gene (Larder and Kemp 1989), which codes for the viral enzyme RT.

ddI: The association of AZT therapy with dose-limiting adverse effects and the emergence of resistant variants, have made alternative therapies important. The major alternative today is ddI which has a potent anti-HIV effect in vitro (Mitsuya and Broder 1986; Mitsuya et al. 1987). Clinically it has been shown

to be effective in treating HIV-1 infected patients and to be tolerated better than AZT (Yarchoan et al. 1989; Cooley et al. 1990). Furthermore, HIV isolates, resistant or less sensitive to AZT have shown sensitivity to ddI in vitro (Larder 1990).

ddI is a dideoxynucleoside analogue of a naturally occurring purine nucleoside, inosine. It is supposed to be converted to its active metabolite in the cytoplasm of the target cells and exerts its action through a mechanism similar to that of AZT. It has been shown to be effective clinically in the treatment of HIV infection and to have an acceptable toxicity profile (Yarchoan et al. 1989).

#### Interferons (IFNs)

IFNs are host coded proteins, elaborated by infected cells in response to viral infection. There are three basic subtypes of Interferon (IFN): -alpha, -beta and -gamma with roughly equivalent antiviral activity. They are almost always host species specific, but not virus specific. Different classes of IFN are produced by different cell types. IFN-alpha is synthesized predominantly by leukocytes, IFN-beta mainly by fibroblasts, and IFN-gamma only by lymphocytes.

The mechanism of action of IFN activity is not well understood, but it is known that IFNs are not directly antiviral but, induce an antiviral state by causing biochemical changes in the exposed cells that lead to resistance to viral infection. It may stop viral budding but probably act at other steps in the HIV

life cycle as well. Viral infection of host cells triggers transcription and translation of IFN genes. Once the proteins are produced they are processed, transported to the cell surface and released. Then, the free IFN molecules bind to surface receptors of uninfected cells which results in the production of an intra-cellular messenger protein by which the antiviral effect of IFN is mediated. Depending on the virus and cell type, the effect on viral replication may involve inhibition of viral binding and penetration, inhibition of synthesis or methylation of mRNA, inhibition of translation of viral proteins or inhibition of viral assembly or release.

IFN- $\alpha$  has been shown to suppress HIV-1 replication both in vitro (Ho et al. 1985; Hartshorn et al. 1987) and in vivo (Berglund et al. 1991). The effect on African HIV-1 strains in vitro has, however, not been analyzed.

#### 1.4 HIV INFECTION IN ETHIOPIA

The first cases of AIDS in Ethiopia were reported in 1986 (Lester et al. 1988) although, the presence of HIV infection in the country, had been shown serologically in 1984 (Tsega et al. 1988). The sero survey data accumulated over the past few years 1982 through 1989 (Tsega et al 1988; Kefenie et al. 1989) indicates a progressing HIV epidemic all over the country. The known number of cases has increased at an alarming rate from 2 in 1986 to 6726 as of June 1993 (National AIDS Control Prog. Ministry of Health, A.A.). Despite the fact that reports were

obtained from almost all regions, 40.3% of the indicated number were residents of the capital city, Addis Abeba. From this report, the importance of the problem can be emphasized by analyzing the HIV-1 prevalence in relation to some selected variables such as sex, age, history of risk factors such as multi-partner sexual contact (MPSC), sexually transmitted diseases (STDs) and clinical signs and symptoms with which the patients present. Of the 6726 reported cases 62.7% were males and 37.3% females (a ratio of 1.7:1.0). Since the most important mode of transmission in Ethiopia is heterosexual, this difference probably indicates that females do not go to health care institutions in search of medical services as often as males do because of certain social and economic problems. Considering the prevalence by age, those between 15-49 represent 93.83%, and paediatrics cases 1.46%. That the highest percentage of cases is in the younger age groups of the society is alarming to a country facing many social and economic problems because these age groups are the most active economically and socially. The spread of HIV virus will sooner or later result in a population imbalance unless strict measures are undertaken to control the spread of the virus. When cases were analyzed by risk factors, those with a history of multi-partner sexual contact constitute the highest percentage (84.05%) of patients, while history of presence of sexually transmitted diseases (National AIDS Control Prog. Ministry of health, A.A. Feb. 1993) was the next most important factor (24.79%). The positive association of increased HIV-1

seroprevalence and the history of STDs has already been documented (Kefenie et al. 1991). Since the case report to the AIDS Control Office (June 1993) represents only those patients who visited health care institutions, the actual number is probably much higher. Even though it is difficult to know the actual carrier rate, reports obtained from blood banks, and other surveillance studies indicate a high rate of HIV-1 seropositivity in apparently healthy segments of the population (Kefenie et al. 1989; Zewdie et al 1992). Since the most affected age groups are those between 15 and 49, and both sexes are affected by the infection at a comparable rate, the major route of transmission would seem to be heterosexual contact. The fact that perinatal transmission from infected mothers to their offspring is another important mode of transmission, is reflected in the higher prevalence rate in the age group of < 4 years (1.34%) as compared to the age group 5-14 years (0.12%). The lower rate of HIV-1 prevalence in older children may indicate the importance of other routes of infection such as blood transfusion, and perhaps the usage of unsterile blades or other appliances in traditional practices in the transmission of the virus as well as early death of the children infected perinatally before the age of 5 years. In addition, repeated use of hypodermic needles, which is a common practice at health institutions may contribute to the transmission of the agent unless the proper sterilization procedures are strictly followed. It has been well documented that accidental exposure to

contaminated needles results in HIV-1 infection (Lange et al. 1990).

According to the Department of AIDS Control report, patients often present with the three major clinical features as defined by WHO (Table 1). Of the three features marked weight loss has been documented in the majority of patients (92.92%), prolonged fever in 88.2%, and chronic diarrhoea in 68.1% of the reported cases. In addition to nutritional factors an early increase in the TNF- $\alpha$  level in Ethiopian patients with AIDS (Ayehunie et al. 1993) may have an association with weight loss. Most of the minor signs and symptoms associated with AIDS have been seen in patients as well, but in varying proportions. Of the symptoms not included in the clinical case definition, tuberculosis should be given special attention in Ethiopia because it has been reported in a considerable proportion of the patients with AIDS. Although, an extensive study has not been done, one report (Kefenie et al 1990) has documented the positive association of HIV-1 and tuberculosis in Ethiopian patients.

Although, only a few of the Ethiopian HIV-1 isolates have been characterized, important information has been collected regarding the HIV-1 strains in Ethiopia and the virological and immunological reactions resulting from infections with this virus. In the polymerase chain reaction (PCR), the isolates exhibited different patterns of reactivity from the Swedish HIV-1 isolates (Ayehunie et al 1990). Furthermore, a phylogenetic tree analysis of the Ethiopian HIV-1 isolates from a 1988-89 sample

collection demonstrated the presence of a separate strain of HIV-1 distinct from the European, North American, and other African strains sequenced at that time (Ayehunie et al 1991, fig. 2.a,b). The results from more extensive nucleic acid sequence analysis of specific regions in the env and gag genes of the provirus of Ethiopian HIV-1 strains provided evidence for the presence of a highly divergent strain of the HIV-1 virus in Ethiopia (Ayehunie et al. 1992). Moreover, these strains have been found to possess a unique amino acid sequence at the tip of the V3 loop of the envelope protein (Ayehunie et al. 1993). These strains seem to have developed early in the phylogenesis of HIV-1 and can now be classified as belonging to HIV-1 subtype C (Ayehunie et al 1990, 91). It has also been reported that, virological and immunological reactions in Ethiopian individuals infected with this virus show different reactivity from that documented earlier in European and North American patients. Despite frequent isolation of infectious virus from plasma, and detection of a higher proportion of anti p24 antibodies in serum, p24 antigen was rarely detected in the plasma of Ethiopian HIV-1 infected individuals as compared to Swedish patients (Ayehunie et al. 1992). Cellular immune activation markers such as TNF- $\alpha$ , and neopterin levels were found to increase in Ethiopian HIV-1 infected individuals in a pattern similar to that of Swedish subjects (Ayehunie et al. 1993), although, higher levels were found in Ethiopian sera. The detection of greatly elevated levels of TNF- $\alpha$  in Ethiopian HIV-1 infected sera suggest that the

Ethiopian HIV-1 strains may be of the macrophage-T-cell tropic type (Ayehunie et al.1992). In contrast to Swedish subjects IFN- $\alpha$  was not frequently detected in Ethiopian HIV-1 infected individuals (Ayehunie et al. 1993). Because of these differences it seemed important to ascertain if there were any differences in sensitivity to antiviral drugs in the Ethiopian HIV-1 strains and to study the effects of treatment on these immunological markers.

Fig. 2.a.

Phylogenetic tree for the env reading frame generated by the parsimony algorithm. All Ethiopian strains indicated by the prefix ETH, and the Swedish by SWE. (Reprinted from Ayeahunie S. PhD. thesis, 1992)

**OUTGROUP ROOTED HIV-1 ENV TREE**  
Phylip/Dnapars

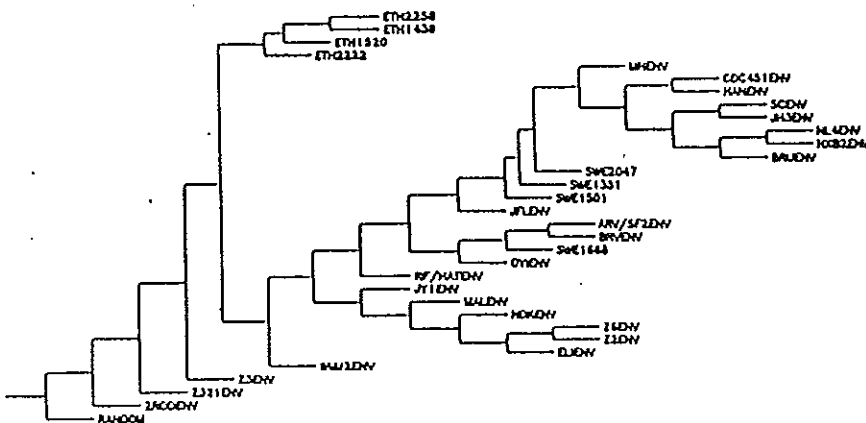
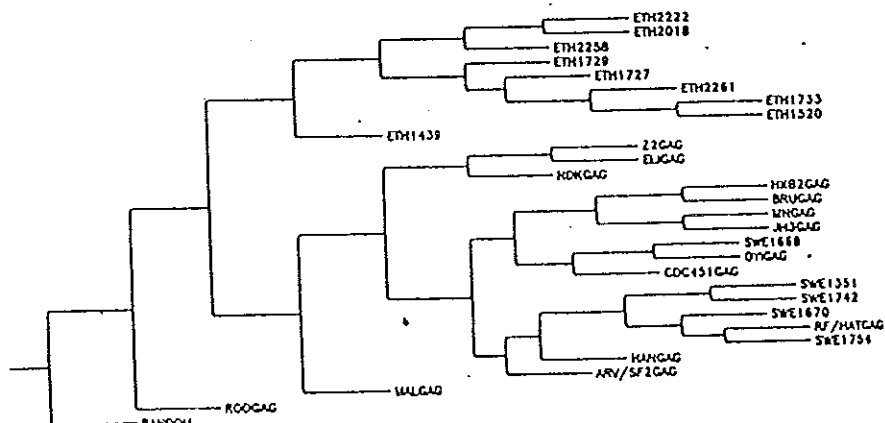


Fig. 2.b.

Phylogenetic tree for the gag reading frame generated by the parsimony algorithm. The naming of the strains is as in fig. 2. a. (Reprinted from Ayeahunie S. PhD. thesis, 1992)

**OUTGROUP ROOTED HIV-1 GAG TREE**  
Phylip/Dnapars



## 2. AIMS OF THE STUDY

- I. To investigate the sensitivity of Ethiopian HIV-1 strains to the antiviral compounds, AZT, ddI, and INF- $\alpha$ , in vitro.
- II. To evaluate the anti-HIV-1 effects of low and intermediate doses of AZT in Ethiopian patients.
- III. To evaluate certain surrogate markers for monitoring anti-HIV treatment in Ethiopian patients

## 3. MATERIAL AND METHODS

### 3.1 SENSITIVITY OF ETHIOPIAN HIV-1 ISOLATES TO ANTIVIRAL COMPOUNDS

#### HIV-1 Strains

The Ethiopian HIV-1 strains used in the in vitro sensitivity assay had been isolated from peripheral blood mononuclear cells (PBMCs) from Ethiopian untreated AIDS patients as described earlier (Ehrnst et al. 1988), identified as type 1 by Western and nucleotide sequencing (Ayeahunie et al., 1991) by Dr. Seyoum Ayeahunie. The Swedish strains used in the assay which had been isolated from untreated Swedish AIDS patients and characterized in a similar manner were provided by Stockholm Microbiological

central Laboratory (SMCL). The strains had been passaged 1-3 times in PBMCs and stored at  $-70^{\circ}\text{C}$  for 1-4 years. At the time of sampling, none of the antiviral compounds were in use in Ethiopia. The Ethiopian strains used in the assay have been shown not to be mixed with the European or North American Strains (Ayehunie et al 1991). For our experimental purposes eight Ethiopian and eight Swedish strains were selected for study.

### Cells

Phytohaemagglutinin (PHA) stimulated PBMCs from healthy HIV seronegative Swedish blood donors (Ho et al. 1985; Rooke et al. 1989) were used to grow the virus and as target cells in the in vitro drug experiments. These cells were also used as negative control cultures and as positive control cultures after virus inoculation. The cells were separated from the peripheral blood of healthy, HIV-1 seronegative Swedish blood donors by Ficoll-Hypaque gradient centrifugation (appendix I. 1). Sixty million cells were distributed into separate 200 ml tissue culture flasks (Falcon) and 20ml of RPMI-1640 culture medium containing phytohaemagglutinin (PHA) was added (appendix I. B). The cells were then, incubated at  $37^{\circ}\text{C}$  in a humidified atmosphere containing 5%  $\text{CO}_2$  for three days. On the third day the cells were counted microscopically by the trypan blue exclusion method (appendix I. 1) to prepare the necessary cell concentration to be used as target cells. Three-day-old PHA-stimulated PBMCs were used in the cultivation of HIV-1 from cell free culture

supernatants (Ehnrst et al. 1988). It has been shown by other investigators that Ethiopian HIV-1 strains grow efficiently in PBMCs obtained from the Ethiopian as well as the Swedish blood donors.

### The Test Compounds

The following three antiviral drugs were evaluated in vitro: AZT (Burroughs, Wellcome), ddI (Bristol-Myers, Squib) and leukocyte-derived IFN- $\alpha$  (Bionative AB, Umeå, Sweden). The drugs were used in the following concentrations: AZT at 10, 1, 0.1, 0.01, and 0.001  $\mu\text{M}$  (Land et al. 1990); ddI at 10, 5, 2.5, 1.25, 0.625, 0.313, and 0.156  $\mu\text{M}$  (Mitsuya et al. 1986); IFN- $\alpha$  at 625, 125, 25, 5, and 1 u/ml (Hartshorn et al. 1987).

### Preparation of the Test Compounds

A concentrated solution of each test drug that is, 10 $\mu\text{M}$  AZT, 10 $\mu\text{M}$  ddI, and 625u/ml IFN- $\alpha$  was prepared in RPMI-1640 complete cell culture medium (appendix I. A), from stock solutions, 0.5mM AZT, 1.0mM ddI, and 6X10<sup>6</sup> u/ml of IFN- $\alpha$  stored at -20°C. Suitably diluted test solutions of the drugs were prepared immediately before use from the concentrated solutions. This was done by diluting serially 1:10 for AZT, 1:2 for ddI, and 1:5 for IFN- $\alpha$  to produce concentrations which fit in with both the drug levels achievable in vivo and the 50% inhibitory concentrations (IC<sub>50</sub>s) assumed to distinguish resistant isolates from sensitive ones.

### 3.2 IN VITRO VIRAL SENSITIVITY ASSAY

Analyses of both Ethiopian and Swedish viral strains were performed in parallel. In order to select the HIV-1 strains to be used in the assay the RT activity was measured in the culture supernatants, which had been kept frozen at  $-70^{\circ}\text{C}$  after thawing (see below). Before inoculation,  $10 \times 10^6$  3-day-old PHA-stimulated PBMCs (see above) obtained from healthy, seronegative Swedish blood donors were incubated overnight with the test drugs at  $+37^{\circ}\text{C}$  in a humidified atmosphere containing  $5\% \text{CO}_2$ . Then the cells were washed with RPMI-1640 complete cell culture medium (appendix I. A) and pelleted by centrifugation at 1200 rpm for 7 minutes. After discarding the supernatant, an equal inoculum (9000 cpm) of each viral isolate was incubated with the target cells for 30 min. The cells were washed as described above and thereafter transferred to separate 50ml tissue culture flasks (Ehrst et al. 1988). Then 10 ml of freshly prepared RPMI-1640 complete cell culture medium containing the test concentrations of each drug were added to the appropriate flasks. Half of the culture supernatants were recovered every 3 to 4 days and stored at  $-70^{\circ}\text{C}$  until analysed. The culture supernatants recovered were replaced by an equal volume of freshly prepared culture medium containing the same concentration of the test drugs. Two positive control cultures of each isolate, and one negative control culture were included in each experiment. The positive controls consisted of the target cells infected with equal inocula of each virus isolate used in a particular experiment without being treated

with the drugs. The negative controls consisted of uninfected PBMCs used in the assay to which cell culture medium without drugs was added. The cultures were maintained for four weeks. The viral activity was measured at week 3 by the antigen capture ELISA and, whenever assay condition permitted by RT activity assay (appendix I. 2 & 3). The cultures involving AZT were also analyzed starting at week 1 up to week 4. It was decided to use the results from week 3, because, the amount of HIV-1 antigen was greatest in the positive controls at week 3. At week 4, the cells had started to die and the results were irregular. The concentrations of the drugs, which resulted in 50% inhibition ( $IC_{50}$ ) of the p24 antigen or RT levels of the positive control cultures, were determined using an exponential regression curve. Based on earlier experience at Stockholm Microbiological Central Laboratory (SMCL), the isolates with an  $IC_{50}$  of up to 0.05  $\mu M$  were considered to be sensitive, and those with an  $IC_{50}$  between 0.05 and 1.0  $\mu M$  as partially sensitive, and those with an  $IC_{50} > 1\mu M$  as resistant. Since we used a flask method to run this assay, for convenience the assay was performed in a single culture.

#### Detection of HIV in Cell-Cultures

##### a) *HIV-1 Antigen Enzyme Immunoassay (EIA)*

The EIA used detects primarily p24, the core protein of HIV-1. The amount of HIV-1 p24 antigen in the culture supernatants of the in vitro drug sensitivity assay was

determined by a capture ELISA, as recommended by the manufacturer (Abbot Lab., Chicago, IL USA) (see appendix I. 3 for details). After the reaction was completed, the colour was developed with freshly prepared o-phenylenediamine (OPD) substrate solution, and the absorbance of the controls and the samples was determined by an ELISA reader. The culture supernatants, which gave absorbance values above the maximum, were suitably diluted to get the actual antigen concentration. The concentrations of p24 antigen in each sample was obtained from a standardized curve established at the SMCL.

b) *Radiometric RT Activity Assay*

The RT activity of HIV is considered to correlate with the number of infectious virus particles. The RT activity was measured after thawing the frozen culture supernatants, before initiation of the in vitro experiments. The same assay was also used, when the amount of virus was determined at the end of the in vitro sensitivity assays. Three ml of the thawed culture supernatants were ultracentrifuged at 55,000g, 30 min. The virus pellets thus obtained were disrupted in a lysis buffer containing 0.1% Triton X-100, pH 7.4 at +4°C for 15 min. Since most of the reagents used to prepare the RT cocktail were highly temperature sensitive, the preparation of the cocktail and the mixing procedure were done in an ice bath. Ten ml of the virus lysate from each centrifuge tube was transferred to properly identified assay tubes and placed in an ice bath. Then 30 ml of the RT

cocktail at +4°C was added to each of the tubes and incubated at 37°C for 60 min. The RT activity was then measured in a Searle analytic Inc Delta 300 6890 liquid scintillation system (RT assay, as developed by SMCL; for details see appendix I. 2)

### 3.3 ANTI-HIV-1 EFFECTS OF LOW AND INTERMEDIATE DOSES OF AZT IN ETHIOPIAN HIV-1 INFECTED PATIENTS

#### Patient Population

Fifty-three HIV-1 infected individuals, 36 males and 17 females, mean age: 32.6; range: 18-58 years (Table 2), who fulfilled the inclusion criteria (Table 3) were recruited consecutively to the clinical study from three hospitals in Addis Ababa. Most of the patients included were at pre AIDS stage and there were a few AIDS patients in the study. There were no statistically significant clinical differences between the patients who received treatment and those who received the placebo ( $p = 0.56$ ; Coded chi square). A further 18 patients who originally agreed to participate in the study were excluded within the first weeks of the study because of violation of the study protocol. Since these patients were evenly distributed among the three groups, no significant clinical differences were found between these and the other patients.

#### Evaluation of Patients

The HIV-1 infection was diagnosed by ELISA tests and

verified by Western blot. The patients were given a physical examination and a general medical history was taken by their physicians at the time of entry into the study. Physical examinations, including body weight, were repeated weekly up to 4 weeks, every second week up to 12 weeks, monthly up to 24 weeks and thereafter at 2 weeks after cessation of the treatment. Routine laboratory analyses to determine haemoglobin levels, white blood count, platelet count, creatine phosphatase, and uric acid, were done regularly for patients receiving AZT, in order to monitor side effects, and twice (at the start and the end) for the controls.

Table 2.

Patients participating in the study grouped by dose, source, age and sex.

Dose	Source	T.No	AGE (Years)				M	F
			18-27	28-37	38-47	>48		
<u>Low</u>	A + P	19	7	6	3	3	15	4
<u>Intermed</u>	ALERT	19	7	4	6	2	12	7
<u>Placebo</u>	All	15	9	3	1	2	9	6

A = Army hospital; P = Police hospital; ALERT = All African Leprosy Rehabilitation Training Center; All= from all the three hospitals;  
T.No = total number of patients; Intermed = intermediate dose.

Table 3.

Inclusion and exclusion criteria for the patients participating in the study

Inclusion criteria	Exclusion criteria
1. Male or female $\geq$ 18 years of age.	1. Pregnant women and women breast feeding.
2. Antibodies to HIV-1 in serum as determined by ELISA and confirmed by Western blot.	2. Patients with AIDS according to the WHO criteria.
3. If possible symptomatic patients not fulfilling the criteria for AIDS	3. Life expectancy less than six months.
	4. Patients who had received anti-HIV-1 therapy within the previous three months.
	5. Patients with severe anaemia.
	6. Patients with other severe acute infectious diseases.
	7. Patients with known neurological or psychiatric diseases.
	8. Patients with known alcohol abuse.

#### AZT Treatment of HIV-1 Infected Individuals

3'-azido-3'-deoxythymidine (AZT) also known as azidothymidine, zidovudine (or Retrovir<sup>R</sup> 100mg tablet, kindly provided by Wellcome Corp.) was administered orally. The patients, who fulfilled the entry criteria and gave informed consent, were consecutively recruited and randomly assigned to either the treatment or the control group. The patients, who were followed up at the Armed Forces General and Police hospitals, were randomized to the low dose or placebo groups. The patients who were followed up at the All African Leprosy Rehabilitation Training Center (ALERT) were randomized to the intermediate dose

or placebo groups. Those who were assigned to the low dose group, received a daily dose of 200mg AZT in two divided doses, those who received the intermediate dose were given 600mg of AZT daily in 3 divided doses for 6 months. The controls received placebo tablets in 2 divided daily doses for the same length of time as the treated patients.

### **3.4 ASSAYS OF ANTIVIRAL EFFECTS OF AZT IN HIV-1 INFECTED PATIENTS**

#### Specimen Handling

Ten ml of peripheral blood were collected from the treated patients in plain tubes before treatment, at 12 and 24 weeks after initiation of the therapy and 2 weeks after cessation of the treatment. The same volume of blood was collected from the controls in a similar manner at entry and after six months. The blood was allowed to clot and the serum was separated quickly from the clot by low speed centrifugation (1000 rpm, 10 min) and divided into four aliquots which were cryopreserved at  $-70^{\circ}\text{C}$  at the Department of Medical Microbiology and Parasitology, Addis Ababa University.

#### Measurement of Serum Levels of $\beta_2$ -M

One aliquot of the serum samples from each patient was used to measure the levels of  $\beta_2$ -M. All of the four serum samples that were collected at the four different times from a patient were

run at the same time.

The levels of  $\beta_2$ -M were measured by a solid phase enzymetric immunoassay ( $\beta_2$ -M ELISA as developed by SMCL). The recommendations of the laboratory which developed this method were strictly followed. Polystyrene 96 well microtiterplates (Nunc) coated with rabbit anti-human  $\beta_2$ -M antibody (Dakopatts, Denmark) were used as a solid phase. Horseradish peroxidase (HRP) conjugated to rabbit anti-human  $\beta_2$ -M antibody was used as a second antibody. A standard, human serum with a known concentration of  $\beta_2$ -M, was included at various dilutions in duplicates in each run. After the reaction was completed freshly prepared o-phenylenediamine (OPD) substrate solution was used to develop colour and the absorbance was read in an ELISA reader at 492nm. The samples, which gave readings above the absorbance of the concentrated standard were suitably diluted and the actual concentrations were determined. Standard curves were constructed in each experiment by plotting the known concentrations of  $\beta_2$ -M in the standard ( $\mu\text{g/ml}$ ) against the mean absorbance readings (OD units). The levels of  $\beta_2$ -M in the patients' serum were determined from the standard curves. The reported normal range for  $\beta_2$ -M in serum is 0.6-3.0mg/l. (see appendix I. 4 for details)

#### Measurement of Serum Levels of TNF- $\alpha$

A second aliquot of serum from the treated patients and controls was used to measure the levels of TNF- $\alpha$ . The serum concentrations of TNF- $\alpha$  were measured using TNF- $\alpha$  solid phase

enzyme amplified sensitivity immunoassay kits (EASIA, Medengix Diagnostics SA, Belgium). The instructions of the manufacturer were followed strictly. Microtiter plates coated with anti-TNF- $\alpha$  antibody were used as a solid phase. Horse radish peroxidase conjugated with anti-TNF- $\alpha$  was used as a second antibody. Standards and controls were run in duplicate. After the reaction was completed, freshly diluted tetramethylbenzidine substrate solution was used to develop colour. The reaction was stopped with 1.8N H<sub>2</sub>SO<sub>4</sub> and the absorbance was read at 450nm in an ELISA reader. Standard curves were constructed in each experiment by plotting the known concentrations of TNF- $\alpha$  in the standards (pg/ml) against the mean absorbance readings (OD units). The TNF- $\alpha$  concentrations in the patients samples were determined by interpolating from the standard curves. (see appendix I. 5 for details)

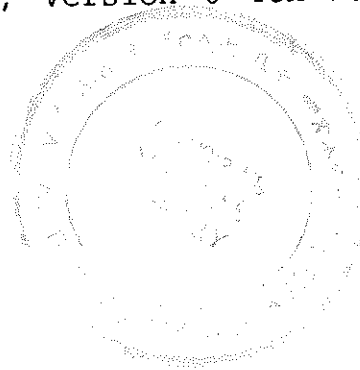
#### Measurement of Serum Levels of Neopterin

A third aliquot of patients serum was used to measure the levels of neopterin. Measurement of the serum neopterin concentration was done using a neopterin competitive radioimmunoassay (IMMUTest<sup>R</sup>, Henning, Berlin GMBH). The instructions of the manufacturer were followed strictly. <sup>125</sup>I labeled neopterin was used as a tracer and the antiserum was sheep anti-neopterin antibody pre-precipitated by donkey anti-sheep immunoglobulin G antibody. At the end of the reaction the supernatants from each tube were decanted and the remaining

radioactivity of each tube was measured in a gamma scintillation counter (neopterin/PGD Auto-gamma). Each tube was counted for one minute. A standard curve was constructed using known concentrations of neopterin in standards against mean cpm. The concentrations of neopterin in the samples were determined by interpolation from the standard curve. (see appendix I. 6 for details)

### 3.5 STATISTICAL ANALYSIS

Comparisons between the groups and within the groups were analysed statistically by the Mann-Whitney U test, and the Wilcoxon signed-rank test. The age and sex distribution between the groups was studied by the Kruskal-Wallis test. The Coded chi-square test was used to analyze the clinical status and the Spearman rank correlation coefficient ( $r_s$ ) was used to analyze the correlation between the antigen and RT levels. These statistical tests were specifically chosen for the analysis of the results from this study, in which the sample size was limited and the values do not follow the normal distribution pattern. The computer software program used for data entry was Epi Info version 5.00, April 1990; and the program for the analysis of the data was SAS/STAT<sup>®</sup> User's Guide, Version 6 4th Ed. 1990, SAS Institute Inc., Cary, NC, USA.



## 4. RESULTS

### 4.1 SENSITIVITY OF HIV-1 STRAINS TO ANTIVIRAL COMPOUNDS (AZT, ddI and IFN- $\alpha$ ).

A dose-dependent anti-HIV-1 activity of AZT was found for the 8 Ethiopian strains (the median  $IC_{50}$  of p24 antigen/RT: 0.0065/0.011  $\mu$ M; range: 0.001-0.1  $\mu$ M, for both) (Table 4, Fig. 3). The  $IC_{50}$ 's of week 2 and 3 did not differ significantly. The  $IC_{50}$ 's of p24 antigen and RT production correlated with each other ( $r_s = 0.86$ ,  $p = 0.02$ ). No viral activity was detected in the negative control cultures.

Seven Ethiopian strains were sensitive to ddI, but one strain seemed to have a decreased susceptibility (the median  $IC_{50}$  of p24 antigen/RT: 0.160/0.320  $\mu$ M; range: < 0.156-3.1/< 0.156-3.5  $\mu$ M) (Table 4).

Seven Ethiopian strains were sensitive to IFN- $\alpha$ , and one strain had an increased  $IC_{50}$ , which could indicate a decreased sensitivity (median: 2.0 u/ml; range: 1.0-11.8 u/ml) (Table 5). This strain was different from the one with an increased  $IC_{50}$  to ddI.

All but one of the Swedish HIV-1 strains were sensitive to AZT, as measured by inhibition of p24 antigen production or RT activity (median: 0.012 and 0.01  $\mu$ M; range: 0.01-1.3 and 0.02-0.07  $\mu$ M) (Table 4, Fig. 3). A similar pattern was found for the  $IC_{50}$ 's of ddI (p24 antigen/RT-median: 0.5/0.615  $\mu$ M; range: < 0.156-4.51/ < 0.156-8  $\mu$ M). Thus, no statistically significant

difference was found between the  $IC_{50}$ 's of the Ethiopian and Swedish strains ( $p > 0.1$  for both, Mann-Whitney U test). No major difference was found in the growth properties of the Ethiopian and Swedish strains, which could have affected the results of the assay (data not shown)

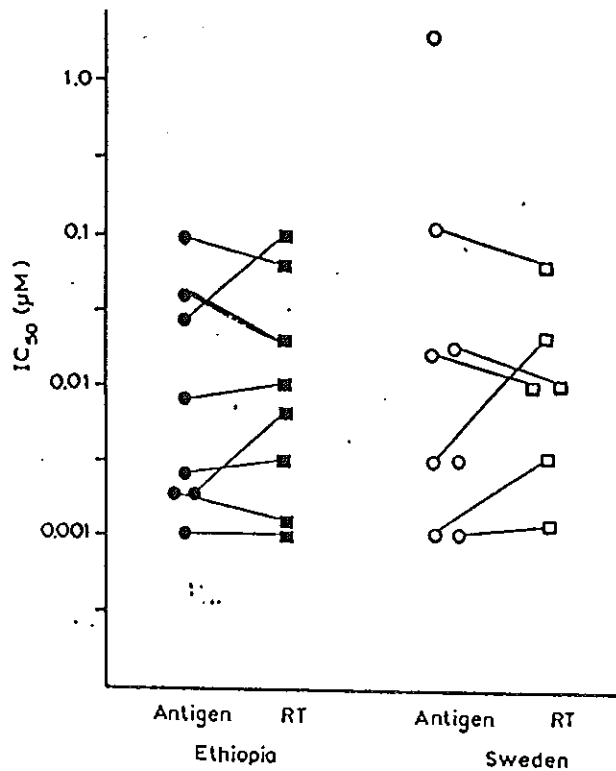
Table 4.

Sensitivity to 3'-azido-3'-deoxythymidine (AZT) and 2',3'-dideoxyinosine (ddI) of HIV-1 strains from Ethiopia and Sweden, measured as 50% inhibition ( $IC_{50}$ ) of p24 antigen and reverse transcriptase (RT).

Strains	$IC_{50}$ ( $\mu M$ ) of AZT		$IC_{50}$ ( $\mu M$ ) ddI	
	Antigen	RT	Antigen	RT
<u>Ethiopian</u>				
1	0.003	0.001	< 0.156	< 0.156
2	0.059	0.044	<u>3.100</u>	<u>3.500</u>
3	0.009	0.015	n.t.	n.t.
4	0.004	0.005	0.350	0.330
5	0.001	0.001	0.310	0.310
6	0.100	0.080	< 0.156	n.t.
7	0.048	0.100	0.160	0.350
8	0.003	0.007	< 0.156	< 0.156
<u>Swedish</u>				
1	0.001	0.007	0.500	0.300
2	0.005	0.044	2.200	0.970
3	0.021	0.015	0.560	0.610
4	0.001	0.002	< 0.156	n.t.
5	<u>1.300</u>	n.t.	<u>4.500</u>	8.000
6	0.020	0.080	< 0.156	< 0.156
7	0.150	0.100	0.200	0.200
8	0.005	n.t.	n.t.	n.t.

n.t = not tested.

Fig. 3. comparison between the sensitivity to AZT of Ethiopian and Swedish HIV-1 strains, measured as 50% inhibition ( $IC_{50}$ ) of the p24 antigen and reverse transcriptase (RT) production. The two  $IC_{50}$ 's of one isolate are joined by a line.



Although no statistically significant difference was found between the  $IC_{50}$ 's of IFN- $\alpha$  for the Swedish and the Ethiopian strains, the  $IC_{50}$ 's for the latter seemed lower. For example, an  $IC_{50}$  of  $> 3.0$  u/ml was found in only 2/7 Ethiopian strains, as compared to 5/6 Swedish strains.

Table 5.

Sensitivity to interferon alpha (IFN- $\alpha$ ) of HIV-1 strains from Ethiopia and Sweden, measured as 50% inhibition ( $IC_{50}$ ) of p24 antigen and reverse transcriptase (RT) production.

Strain	$IC_{50}$ (u/ml) for IFN- $\alpha$		Strain	$IC_{50}$ (u/ml) for IFN- $\alpha$	
	Antigen	RT		Antigen	RT
<u>Ethiopian</u>			<u>Swedish</u>		
5	11.8	n.t.	3	56.0	87.0
2	3.4	n.t.	7	21.2	15.0
4	2.5	4.5	2	4.1	3.4
7	2.5	2.5	1	3.7	n.t.
8	2.0	n.t.	4	4.0	4.9
6	1.2	2.0	6	<1.0	n.t.
1	<1.0	n.t.			

n.t. = not tested

A correlation between the  $IC_{50}$ 's of the p24 antigen and RT was found for all the three substances when the Ethiopian and Swedish strains were analyzed together (AZT:  $r_s = 0.70$ ,  $p = 0.015$ ; ddI:  $r_s = 0.95$ ,  $p = 0.003$ ; IFN- $\alpha$ :  $r_s = 0.71$ ,  $p = 0.06$ ).

#### 4.2 EFFECTS OF TREATMENT WITH AZT IN HIV-1 INFECTED ETHIOPIAN PATIENTS

Table 6.

The follow-up status of the patients participating in the AZT treatment study.

	Total patients enrolled	Drop outs				Completed 24 Weeks
		12 Wks after start of treatment	16	20	22	
<u>Intermediate</u>	19	1	1	-	1	16
<u>Low</u>	19	2	1	2	1	13
<u>Placebo</u>	15	1	-	-	-	14
Total drop outs:		4	2	2	2 = 10	

#### Completion of the Study

A certain number of patients did not complete the whole study period (Table 6). The patients who stopped treatment before 6 months did not seem to do this because of side effects, but rather for other reasons. Some of them reappeared after a long time, too late to continue with the drug treatment, but this helped us to ascertain some of the reasons for their disappearance. Social problems such as being transferred to far away regions and being jobless were the reasons given by most of them. However, a few of them frankly told us that they had heard that HIV is non-curable and taking medication is useless. Those

who completed the 12 weeks period were included in the assessment of treatment effects, in order to follow the "intention to treat" rule.

The diseases or other complications which were present before treatment or which appeared during treatment are indicated in Table 7. The anti-Tb treatment had been initiated a longer time before treatment with AZT in all patients who received anti Tb drug therapy and was not considered to contribute to the clinical improvement seen in the treated patients.

Table 7. Patients' clinical conditions at entry and during treatment period.

Dose/study No.	Known other diseases/complications		Other medications
	Before treatment	During treatment	
<u>Intermediate:</u> 069	Bacillary leprosy	None	DDS 100mg & rifampicin 600mg 24 wks.
055	No other problem	Seborrheic dermatitis	Metronidazole 500mg QID. 7 days.
057	No other problem	Watery diarrhoea, E. histolytica	Tinidazole 500mg, 4tabs at once 3 days.
073	No other problem	Diarrhoea, E. histolytica	TB450 1 tab/day, 4 months; streptomycin 1gm IM & rifampicin 600mg daily, 8 wks.
063	Pulmonary tuberculosis	No other problem	INH 300mg, rifampicin 600mg.
<u>Low:</u> 07	Disseminated tuberculosis	None	None
11	Nonspecific GI symptoms	None	None
17	Chronic perianal fistula	None	INH 300mg, rifampicin 600mg.
19	Peritoneal tuberculosis	None	(?)
21	No other problem	Neurological complications	INH 300mg, rifampicin 600mg.
25	Disseminated tuberculosis	Cryptococcal meningitis	(?)
29	Vaginal candidiasis	None	INH 300mg, rifampicin 600mg.
31	Pulmonary tuberculosis	None	
<u>Placebo:</u> 08	No other problem	Neurological manifestations	
16	No other problem	Extra pulmonary tuberculosis	
18	No other problem	Neurological manifestations	
042	Subpolar lepromatous leprosy	Pustular eruption	DDS 300mg, cofamizine 50mg, rifampicin 600mg, clofamizin 300mg, ampicillin 500mg.

Note: The patients who are not indicated above did not exhibit any disease complications at entry or during the study.

Clinical Assessment

The patients' clinical conditions were assessed regularly by their physicians. The clinical and physical conditions including body weight were recorded.

In contrast to the patients in the placebo group a statistically significant improvement of the patients clinical status was observed in the majority of the treated patients, independent of the dose ( $p = 0.001$ ; Coded chi square test). No significant difference in clinical improvement was found between the patients treated with the intermediate or the low doses of AZT ( $p = 0.68$ ). In addition, an increase in the body weight was found in the majority of the patients after treatment, as compared to their weights before treatment, but patients in the placebo group did not gain weight (Table 8). Thus, the mean and median increases in body weight in the intermediate dose group were 3 and 3.1 kg and in the low dose group were 4 and 4.1 kg. When the patients were analyzed individually the patients who improved clinically were also those who gained weight, in contrast to the patients whose clinical condition deteriorated or those who stayed clinically stable. No significant difference was found between the different groups with regard to age ( $p = 0.38$ ; Krushal-Wallis test), but, there were significantly fewer females than males in the group ( $p = 0.09$ ).

Table 8.

The patients clinical and physical conditions after treatment.

Dose	Clinical conditions			Weight		
	Improved	Unchanged	Deteriorated	Increased	Stable	Decreased
<u>Intermediate:</u>	13	3	1	13	3	1
<u>Low:</u>	12	5	2	12	3	0
<u>Placebo:</u>	1	9	5	1	8	5

#### Estimation of Adverse Effects of The Drug

No significant differences were seen in the levels of haemoglobin, creatinine, uric acid and platelet count when the pretreatment and the week 24 values were compared within and between the groups (Table 9). However, in two patients anaemia was diagnosed at week 24. No anaemia was detected in these patients at week 12. The patients received appropriate medications but not blood transfusions. There was a decrease in the WBC count when the pretreatment and week 24 values were compared for the treated patients, although the values were within the normal range. Thus, the mean pretreatment/week 24 values were 6689/5444 per cubic milliliter for the intermediate and 5380/4693 for the low dose groups.

A few AIDS-related complications such as neurological manifestations, cryptococcal meningitis and extra pulmonary

tuberculosis were diagnosed in the patients under investigation during the study period (Table 7). Thus, 2 out of the 38 treated and 3 out of the 15 control patients developed such complications. Of those in whom additional complications developed, two of the treated patients and two of the control patients whose clinical condition was deteriorated (Table 8) died during the study period, which constituted 5.2% (n = 38) of the treated and 13.3% (n = 15) of the control patients.

Table 9.

Mean and Median values : haemoglobin (HB), white blood cell count (WBC), creatinine, platelet and uric acid (Uric/a) pretreatment and at week 24.

Dose	pretreatment					week 24				
	HB mg%	WBC	Creat	Platelet (X10 <sup>3</sup> )	Uric/a	HB mg%	WBC	Creat	Uric/a	Platelet (X10 <sup>3</sup> )
<u>Intermed.</u>										
Median:	13.9	7550	1.0	200	6.0	14.6	4600	0.9	5.0	220
Mean:	13.3	6689	0.9	235	6.4	14.0	5494	1.2	5.3	234
<u>Low</u>										
Median:	13.7	4300	0.9	189	6.7	14.9	4000	1.0	8.0	200
Mean:	14.0	5380	0.9	186	6.9	13.5	4693	0.9	6.6	207
<u>Placebo</u>										
Median:	13.0	6650	0.8	153	6.8	13.2	6450	0.9	5.7	267
Mean:	13.2	6550	1.0	151	6.4	13.6	8512	1.0	5.6	223

Intermed. = Intermediate dose group

### Effect of AZT on $\beta_2$ -M Levels

For the patients receiving 600mg daily dose of AZT, there was a statistically significant decrease in  $\beta_2$ -M values, when the pretreatment values were compared with the values obtained at week 12 (log mean $\pm$ SE: 0.91 $\pm$ 0.05 vs 0.72 $\pm$ 0.05; p=0.0002), and at week 24 (0.91 $\pm$ 0.05 vs 0.58 $\pm$ 0.07; p=0.0001). After two weeks post treatment there were still significantly lower values (0.71 $\pm$ 0.06) as compared to the pretreatment values (p=0.0008) (Fig. 4).

For patients receiving 200mg daily dose of AZT, a statistically significant decrease in  $\beta_2$ -M values were found when the values obtained pretreatment and at week 12 were compared (0.63 $\pm$ 0.08 vs 0.51 $\pm$ 0.05; p= 0.01). No significant decrease was seen, however, when the values obtained at week 24 (0.52 $\pm$ 0.08, p=0.1) and post treatment (0.53 $\pm$ 0.06, p=0.5) were compared with the pretreatment values (Fig. 4).

When all the treated patients were analyzed together, there was a statistically significant decrease in  $\beta_2$ -M levels at week 12 (log mean $\pm$ SE: 0.77 $\pm$ 0.05 vs 0.61 $\pm$ 0.04; p= 0.0001), at week 24 (0.77 $\pm$ 0.05 vs 0.56 $\pm$ 0.05; p=0.0001) and post treatment (0.77 $\pm$ 0.05 vs 0.63 $\pm$ 0.05; p=0.003, Wilcoxon signed-rank test) when the values were compared with the pretreatment values (Fig. 5).

In contrast, no significant difference was seen in the patients receiving placebos between the values obtained pretreatment and at week 24 (0.71 $\pm$ 0.06 vs 0.69 $\pm$ 0.11; p=0.95) (Fig. 4 and 5).

Fig. 4. Paired comparison of serum  $\beta_2$ -M levels (log mean  $\pm$  SE) in Ethiopian HIV-1 infected patients treated with low and intermediate dose of AZT before, during and 2 weeks post treatment, as compared to control patients. (The bars in this and the following figures represent  $\pm 1$  and  $1.96SE$ )

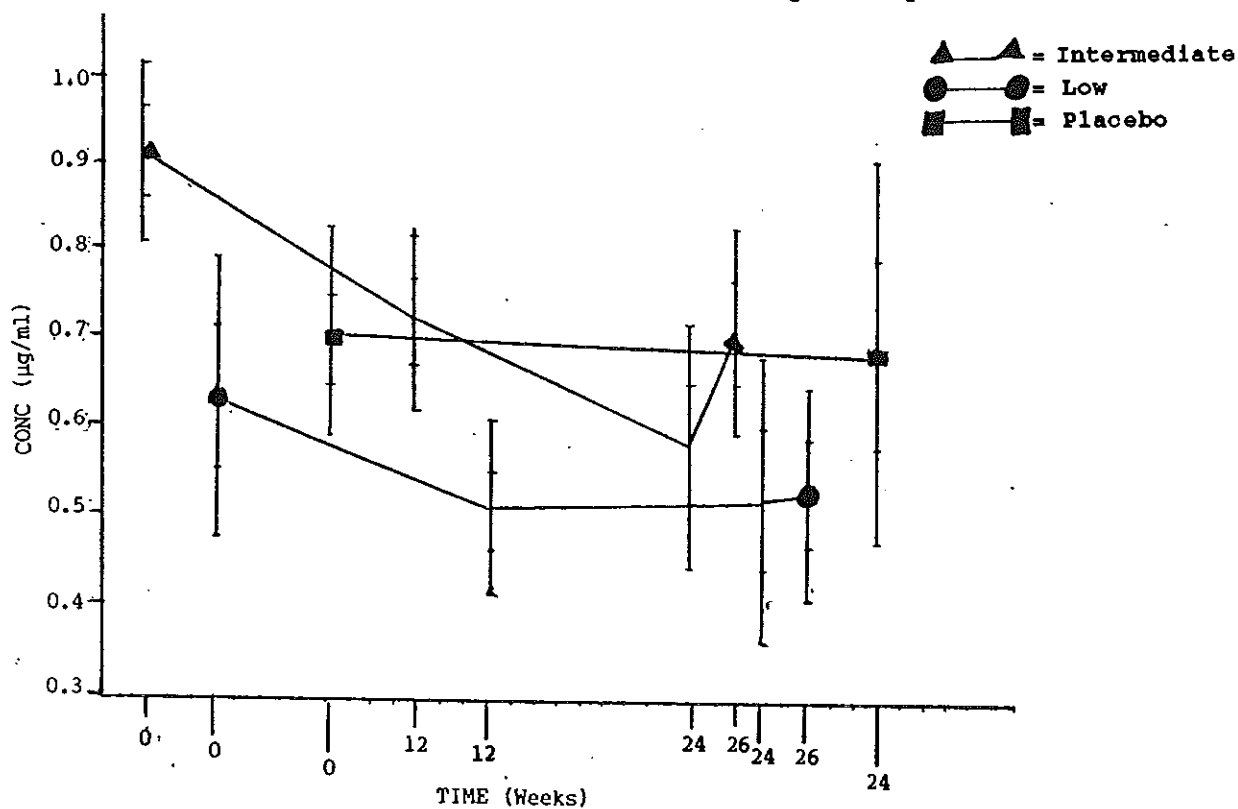
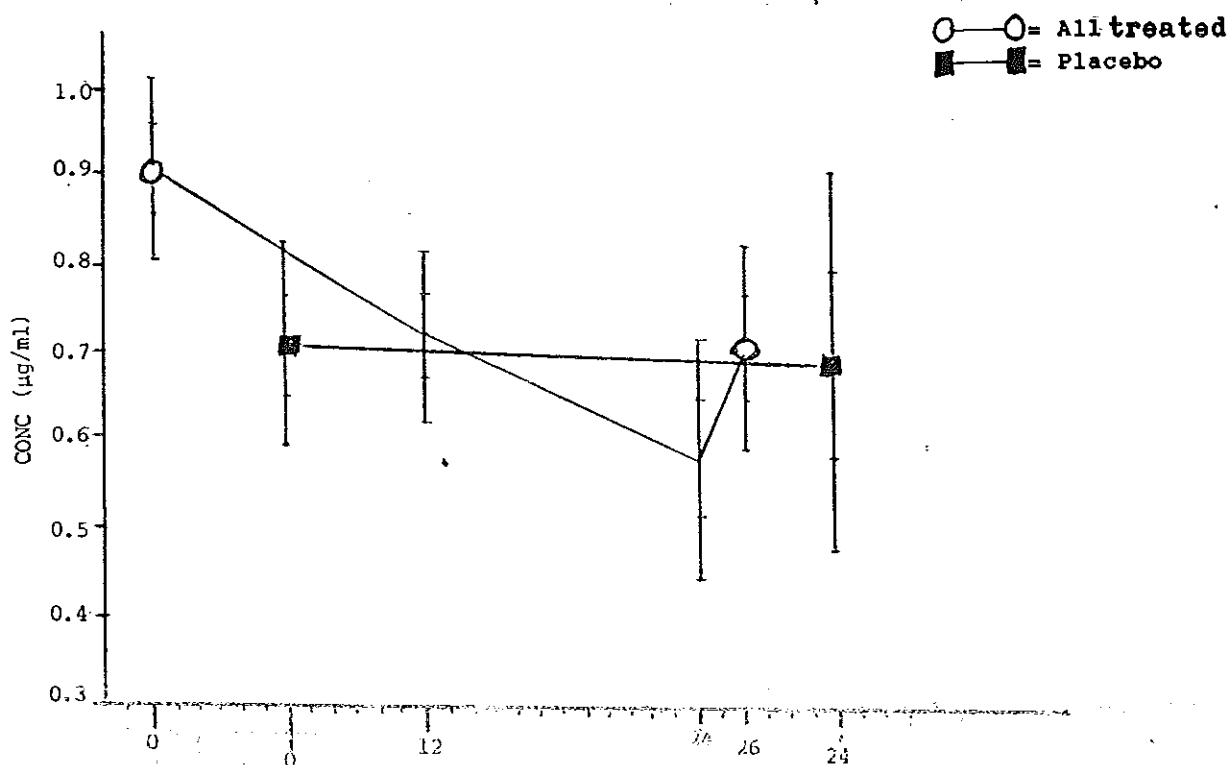


Fig. 5. Paired comparison of serum  $\beta_2$ -M levels (log mean  $\pm$  SE) in Ethiopian HIV-1 infected patients treated with AZT before, during and 2 weeks post treatment when all the treated patients were analyzed together as compared to control patients.



Effect of AZT on TNF- $\alpha$  levels

There was a statistically significant decrease in the TNF- $\alpha$  levels also when the patients were divided into the respective dosage groups (intermediate and low dose). For the group receiving the intermediate dose a statistically significant decrease was obtained when the pretreatment values were compared with the values obtained at week 12 (log mean $\pm$ SE: 1.46 $\pm$ 0.07 vs 1.38 $\pm$ 0.07 p=0.02) and at week 24 (1.46 $\pm$ 0.07 vs 1.35 $\pm$ 0.07; p=0.04) (Fig. 6). Similarly, there was a statistically significant decrease in values at week 12 (1.47 $\pm$ 0.08 vs 1.35 $\pm$ 0.07; p=0.0006) and at week 24 ( and 1.47 $\pm$ 0.08 vs 1.35 $\pm$ 0.1; p=0.01) for the group receiving the low dose of the drug (fig. 6).

When all the treated patients were analyzed together there was a statistically significant decrease in the values obtained at week 12 ( log mean $\pm$ SE: 1.47 $\pm$ 0.05 vs 1.37 $\pm$ 0.05; p=0.0001), at week 24 (1.47 $\pm$ 0.05 vs 1.35 $\pm$ 0.06; p= 0.0004), and post treatment (1.47 $\pm$ 0.05 vs 1.38 $\pm$ 0.06; p= 0.01, Wilcoxon signed-rank test) when compared with the pretreatment values (Fig. 7).

No significant difference however, was found for the controls between the pretreatment and week 24 values (1.59 $\pm$ 0.07 vs 1.56 $\pm$ 0.08; p= 0.68) (Fig. 6 and 7).

Fig. 6. Paired comparison of serum TNF- $\alpha$  levels (log mean  $\pm$  SE) in Ethiopian HIV-1 infected patients treated with low and intermediate dose of AZT before, during and 2 weeks post treatment, as compared to control patients.

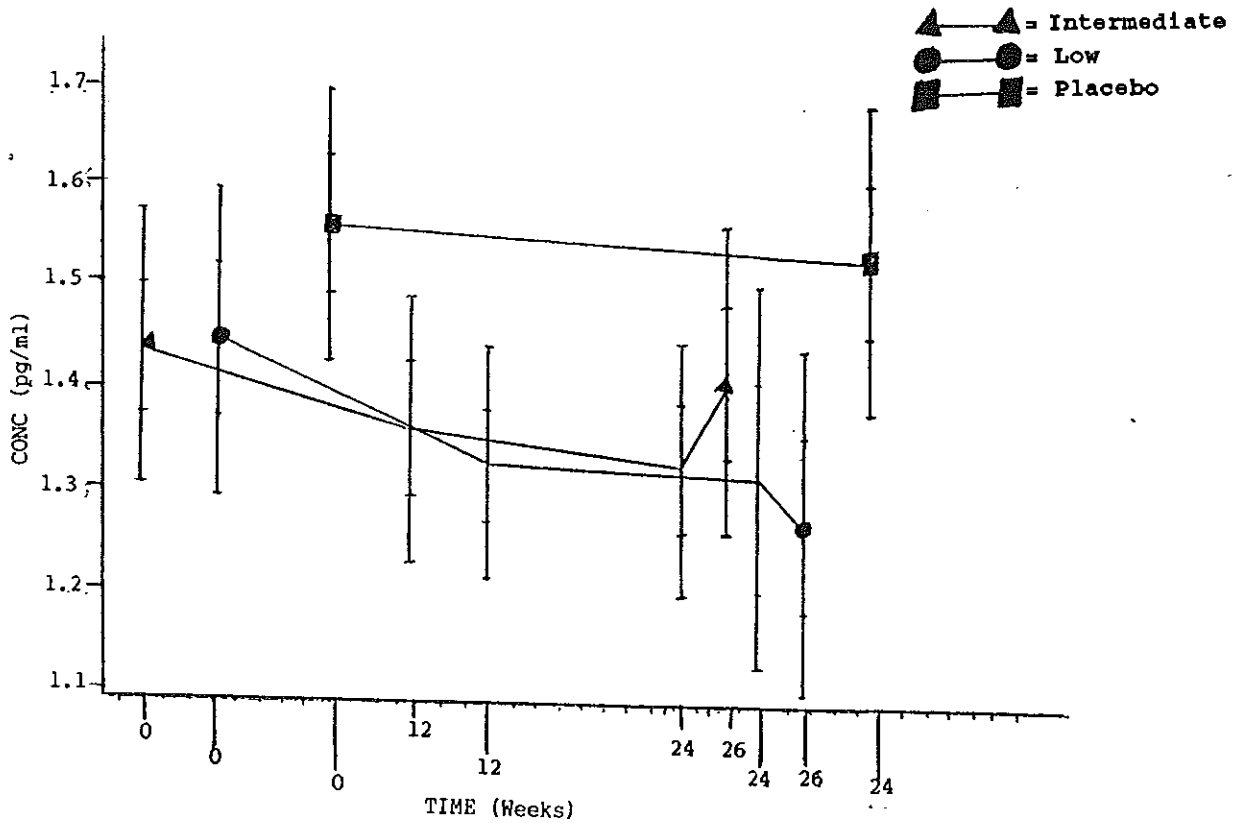
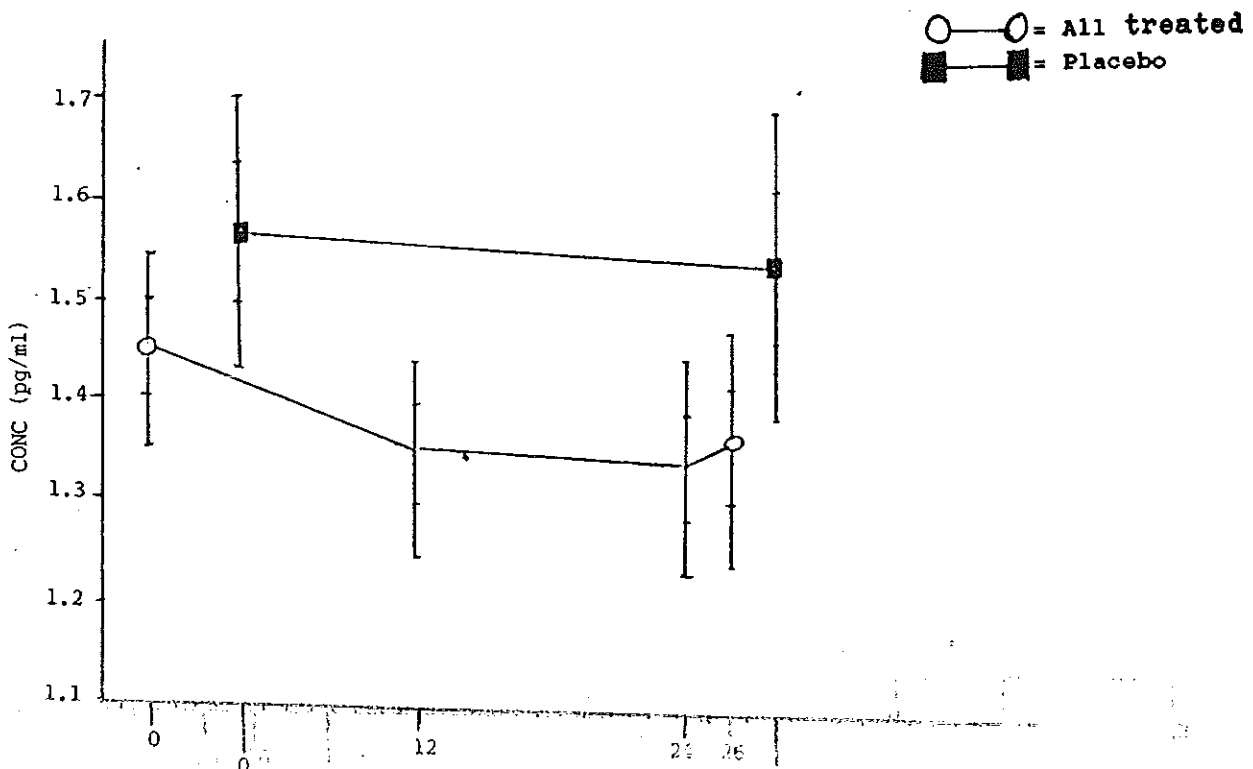


Fig. 7. Paired comparison of serum TNF- $\alpha$  levels (log mean  $\pm$  SE) in Ethiopian HIV-1 infected patients treated with AZT before, during and 2 weeks post treatment when all the treated patients were analyzed together as compared to control patients.



Effect of AZT on Neopterin levels

For the patients receiving the intermediate dose of AZT, there was a statistically significant decrease in neopterin levels, when the values obtained pretreatment were compared with values at week 12 (log mean $\pm$ SE: 1.26 $\pm$ 0.05 vs 1.06 $\pm$ 0.06, p=0.0001). There was also a significant decrease in values at week 24 (1.26 $\pm$ 0.05 vs 1.06 $\pm$ 0.09; p= 0.04) and 2 weeks post treatment (1.26 $\pm$ 0.05 vs 0.99 $\pm$ 0.08; p= 0.0008) (fig. 8).

For the patients who received low doses of the drug there was also, a statistically significant decrease in neopterin levels when values obtained pretreatment were compared with values at week 12 (1.24 $\pm$ 0.06 vs 1.18 $\pm$ 0.07; p=0.01), at week 24 (1.24 $\pm$ 0.06 vs 1.16 $\pm$ 0.07; p=0.06) and post treatment (1.24 $\pm$ 0.06 vs 1.15 $\pm$ 0.06; p=0.02) (fig. 8).

When all the treated patients were compared, there was statistically significant decrease in the levels of neopterin between the pretreatment values and the values obtained at week 12 (log mean $\pm$ SE: 1.25 $\pm$ 0.04 vs 1.12 $\pm$ 0.05 ;p=0.0001), at week 24 (1.25 $\pm$ 0.04 vs 1.07 $\pm$ 0.07; p=0.001) and post treatment (1.25 $\pm$ 0.04 vs 1.10 $\pm$ 0.05; p= 0.0001, Wilcoxon signed-rank test) (fig. 9).

In contrast there was no significant difference between the pretreatment and week 24 values (1.34 $\pm$ 0.04 vs 1.27 $\pm$ 0.06; p=0.54) for the control patients receiving placebos (Fig. 8 and 9).

When the individual patients were considered there was a decrease in  $\beta_2$ -M levels in 23 out of 28 (82.1%) treated patients at week 24. Similarly there was a decrease in TNF- $\alpha$  and neopterin levels in 20 out of 28 (71.4%) and 22 out of 27 (81.4%) treated patients respectively at week 24. In those patients in whom decreased levels of  $\beta_2$ -M, TNF- $\alpha$  and neopterin levels were found there was also an improvement in their clinical conditions (Table 8.).

Fig. 8. Paired comparison of serum neopterin levels (log mean  $\pm$  SE) in Ethiopian HIV-1 infected patients treated with low and intermediate dose of AZT before, during and 2 weeks post treatment, as compared to control patients.

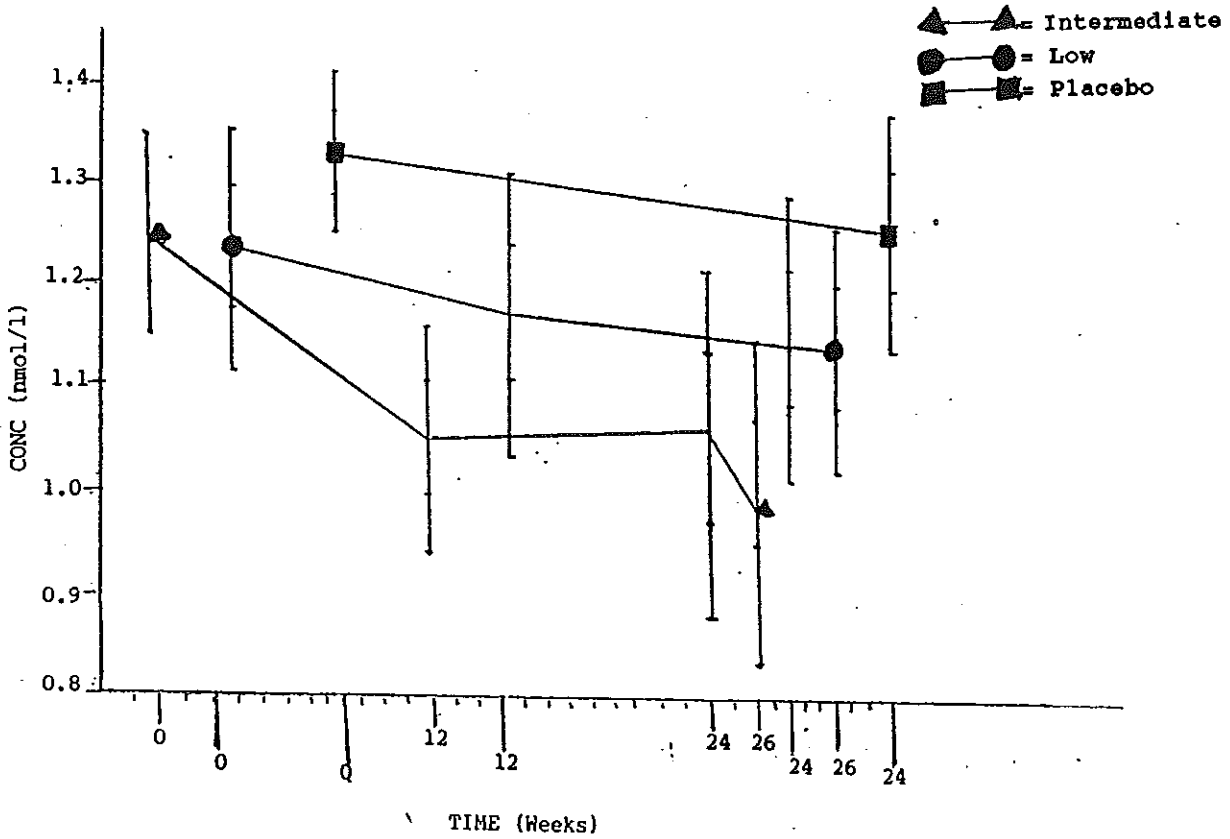
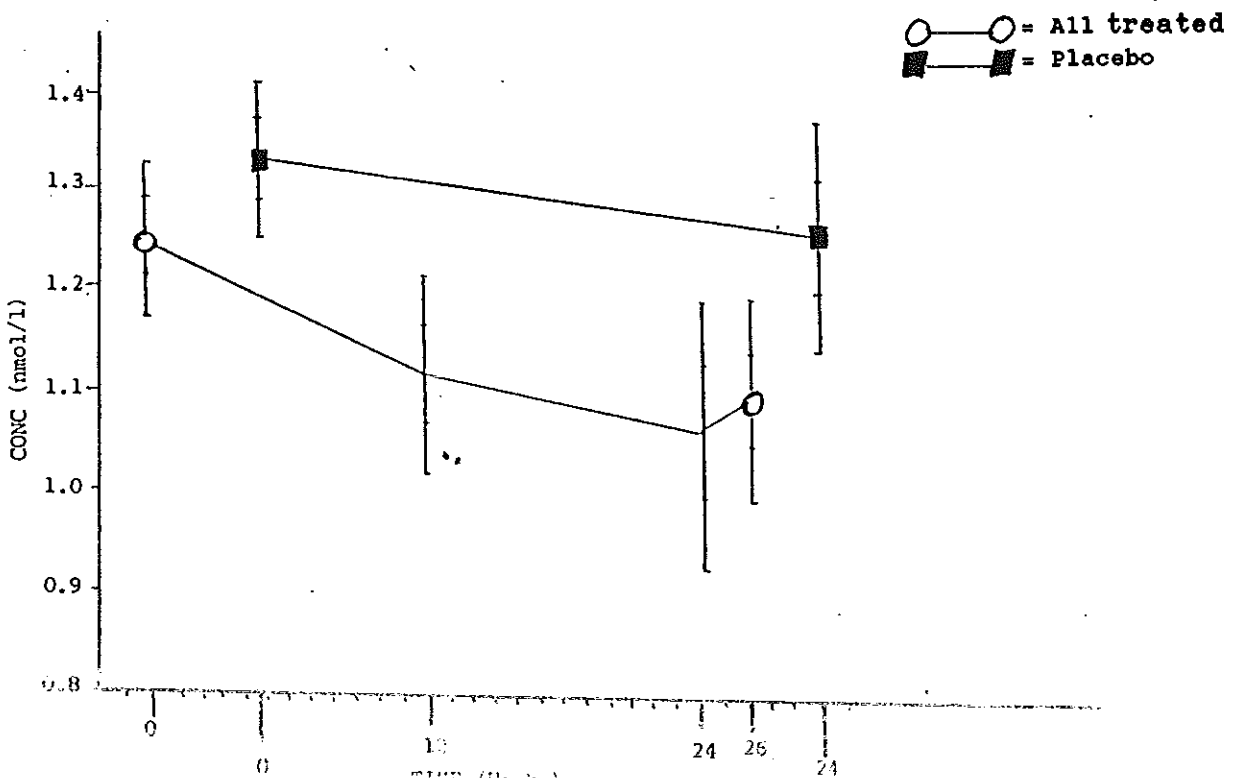


Fig. 9. Paired comparison of serum neopterin levels (log mean  $\pm$  SE) in Ethiopian HIV-1 infected patients treated with AZT before, during and 2 weeks post treatment when all the treated patients were analyzed together as compared to control patients.



## 5. DISCUSSION

Anti-HIV drugs are seldom used in Africa because of their unavailability and the expense especially when high doses of the drugs are required. However, an anti-HIV effect of AZT can be obtained by using a reduced daily dose, 600 mg (Fischl et al. 1990), or even a low dose regimen of 300 mg daily (Collier et al. 1990). Furthermore, recent studies have indicated that wild-type HIV-1 strains may differ in their sensitivity to AZT (Mohri et al. 1993). Also there seems to be a pre-therapy random variation in susceptibility to AZT among HIV isolates in vitro (Land et al. 1990), although, others have reported that pre therapy isolates are susceptible to a narrow range of low concentrations of AZT (Larder et al. 1989). Quite a number of North American and European HIV-1 strains have been found to have a low sensitivity to AZT despite the fact that the patients had never been treated with the drug (Mohri et al. 1993). Therefore, it was of interest to analyze the susceptibility of the Ethiopian HIV isolates which differ genetically from both the North American/European, and other African strains to the antiviral compounds, AZT, ddI, and IFN- $\alpha$ .

In the present in vitro study, no Ethiopian HIV strain was found to be resistant to AZT, although, some isolates had an increased IC<sub>50</sub>. In contrast, one Swedish strain showed resistance to AZT. Since none of the patients had been treated with AZT, it is possible that this strain had been isolated from a patient infected by an AZT resistant strain. Thus, the present results

showed that Ethiopian HIV-1 strains exhibit a sensitivity to AZT similar to the sensitive Swedish strains. Therefore, the same beneficial effects of treatment with low-dose regimens of AZT should be expected in Ethiopia as in Europe.

HIV-1 replication in vitro has been shown to be inhibited by ddI. (Mitsuya et al. 1986) and treatment with this drug has beneficial effects in HIV-1 infected patients from North America and Europe (Yachon et al. 1989; Lambert et al. 1990). Our results also showed that ddI exhibited in vitro activity, at low concentrations against Ethiopian HIV-1 isolates. However, the same Swedish isolate which was resistant to AZT also showed decreased sensitivity to ddI. This drug, however, was not being used in Sweden at the time of the HIV isolation. It has been claimed that treatment with ddI seems to be associated with a suppressing effect of the AZT resistance mutations (St. Clair et al. 1991). It cannot be excluded that the decreased sensitivity of this isolate to both AZT and ddI was due to other mechanisms than those associated with the most commonly occurring mutations in the RT gene. No ddI resistant Ethiopian strain was found, although, some isolates showed an increased  $IC_{50}$ . Hence, we did not find any clear indication that wild type HIV-1 strains in Ethiopia, exhibit a pretherapy resistance to either AZT or ddI. It is of considerable importance to note that a once-daily administration of ddI (Cooley et al. 1990) has improved immunologic and virologic parameters in patients from North America. Thus, ddI should be considered as an alternative therapy

to AZT in Ethiopian patients, if the cost can be reduced, by decreasing the dose.

Both recombinant and leukocyte-derived human IFN- $\alpha$  are known to suppress the replication of HIV-1 isolates from North American and European patients in vitro (Ho et al. 1985; Hartshorn et al. 1987) as well as in vivo (Lane et al. 1988; Berglund et al. 1991). The effect of IFN- $\alpha$  on Ethiopian HIV-1 isolates *in vitro* has, however, not been analyzed. IFN- $\alpha$  is seldom found in the blood of Ethiopians infected with HIV-1 (Ayehunie et al. 1993). Theoretically there could be an increased sensitivity of Ethiopian HIV-1 strains to IFN- $\alpha$  because of an absence of exposure of the isolates to the substance, but, our results did not indicate that Ethiopian HIV-1 strains had higher sensitivity to IFN- $\alpha$  than was found for the Swedish strains. However, our study did show that IFN- $\alpha$  inhibited the replication of the Ethiopian strains in a dose-dependent way, at concentrations which were generally lower than those which inhibited the Swedish isolates (median IC<sub>50</sub>: 2 vs 4.05 u/ml, Table 5). It remains to be established whether recombinant IFN- $\alpha$  also has a pronounced anti-HIV-1 effect on primary isolates, although it has been suggested that both natural and recombinant IFN- $\alpha$  have similar antiviral effects (Hartshorn et al. 1987).

The pattern of viraemia in African patients has been studied to a limited extent only. It seems however to differ from that exhibited in patients from Europe and North America. P24 antigenemia is rarely found in African patients although,

infectious virus can be isolated from plasma (Katsenstein et al. 1990; Ayehunie et al. 1992). Since, a dissociation between the effect of AZT on the levels of p24 antigen and RT activity has been described in a patient on long-term AZT therapy (Masquelier et al. 1991), we therefore, measured the effect of the antiviral compounds on both the production of p24 antigen and RT activity. However, on the limited number of samples we studied, no significant differences were found in vitro between the IC<sub>50</sub>'s of the two viral markers. Thus, from our in vitro results it seems likely that AZT, ddI and IFN- $\alpha$  would be beneficial in vivo, even if the kinetics of viral replication are different in Ethiopian patients from that found in European subjects. The results from the present in vitro study show that the Ethiopian HIV strains are susceptible to the three antiviral drugs tested. Therefore, it was hypothesized that these drugs could be useful in treating Ethiopian HIV-1 infected patients.

In our clinical study, an improvement in the general clinical status was found in the majority of AZT treated patients which constituted 69.4% (25/36) irrelevant of the dose, as compared to the control patients. Since the study was a single blind, it is possible that these results could be biased. It should be noticed, however, that the body weight, which is an objective parameter, improved in both treatment groups but did not improve in the untreated patients. Thus, 25 of the 38 patients who completed at least 12 weeks of therapy gained weight, a median increase of 3.1 kg in the intermediate and 4.1 kg in the low dose group. Thus, it is likely that AZT treatment

was beneficial in Ethiopian patients at least in the short term.

The number of side effects in the treated patients were limited. Mild anaemia was diagnosed in only two patients at week 24. Haematologic complications are well known adverse effects of AZT therapy, but the severe forms of toxicity were dependent both on high doses and prolonged duration of treatment (Groopman 1990). The minimal side effects observed in our study are most probably the result of the low or intermediate doses of AZT given to the patients. This study showed that, AZT in 200 and 600 mg doses were tolerated by Ethiopian AIDS patients for a period of at least 24 weeks.

A few AIDS-related complications such as neurological manifestations, cryptococcal meningitis and extra pulmonary tuberculosis were diagnosed in the patients under investigation during the study period. According to our data, AIDS related complications were fewer in the patients who received AZT as compared to the patients who received the placebo. Most of the treated patients remained socially active, could go to work and had a better quality of life than those who received the placebo. The difference in the percentage of deaths between the treated (5.2%) and untreated (13.3%) groups indicates that AZT, by improving the clinical status of the treated patients probably contributed to the extension of their period of survival.

Elevated serum levels of  $\beta_2$ -M, TNF- $\alpha$  and neopterin have been detected in HIV-1 infected patients (Bhala et al. 1983; Lähdevirta et al 1988; Lambin et al 1988). These substances were found

useful both as prognostic markers in HIV-1 infection (Morfeldet-Månson et al. 1988; Fahey et al. 1990) and as a measure of antiretroviral activity in the treatment of European AIDS patients (Jacobson et al. 1991; Hutterer et al. 1992). Their usefulness in monitoring antiviral drug therapy in Ethiopian patients, however, were not previously studied.

In our study, a statistically significant decline in the serum levels of  $\beta_2$ -M, TNF- $\alpha$  and neopterin were found in the treated patients, up to 2 weeks post treatment when they were analyzed together but not in the placebo group. Similar effects were also found for TNF- $\alpha$  and neopterin when the treated patients were analyzed according to their respective dosage groups. However, in the group treated with the low dose of AZT, the decrease in the  $\beta_2$ -M levels at week 24 and post treatment was not statistically significant. This lack of difference might be because there were markedly increased values in two patients which require further investigation. The impact of such increased values is great when the sample size is low, as is the case in this study. It is also possible that the  $\beta_2$ -M levels in certain patients may be increased because of other factors such as disseminated tuberculosis which both of these patients had. We also suspect that one of the patients may not have taken his medications properly. Our data suggests that AZT had a significant antiviral and/or immunomodulating effect in Ethiopian HIV infected patients. Especially interesting is the decrease in the TNF- $\alpha$  levels, which are known to be substantially increased

in Ethiopian HIV-1 infected patients. Since, weight loss and increase in TNF- $\alpha$  levels are positively associated in AIDS patients, the decrease in TNF- $\alpha$  levels may have contributed to the weight gain observed in the treated patients. In our study it is the total TNF- $\alpha$  that was measured. Although important diagnostic information can be obtained by relating the serum levels of TNF- $\alpha$  with the patients' clinical outcome, analysis of the biological activities of TNF- $\alpha$  should be considered in the future studies for more complete results. It does not seem likely that the improvement of these laboratory parameters were due to any of the other medical treatments received by the patients since these concomitant treatments had been initiated long before the AZT treatment was started in most of the patients.

The evaluation of antiviral treatment and vaccination studies is troublesome in Ethiopian patients, since the simple assay for measurement of viral load, the p24 antigen assay, is not useful in Ethiopian patients (Ayehunie et al. 1992). The present study suggests that surrogate markers, such as  $\beta_2$ -M, TNF- $\alpha$  and neopterin are suitable for the evaluation of such treatment or vaccination in an Ethiopian population.

We conclude that the genomic differences observed between the Ethiopian and the Swedish strains did not result in differences in sensitivity to antiviral drugs either in vitro or in vivo. The differences in the serological immune responses and the clinical features exhibited by the Ethiopian HIV-1 patients did not affect the response of cellular activation markers in

antiviral drug therapy. Thus  $\beta$ 2-M, TNF- $\alpha$  and neopterin can be used as prognostic markers in monitoring antiviral drug therapy in Ethiopian patients just as in European patients. Our study also indicates that Ethiopian patients infected with HIV-1 are as likely to benefit from antiretroviral drug therapy as Swedish patients. Moreover, the 200 mg dose of AZT that was chosen for use in this study was based on our in vitro results. To our knowledge it is the lowest dose that has been investigated clinically to date. Since in our study beneficial clinical effects were obtained with both the 600 mg and 200 mg doses, a 200 mg daily dose of AZT might be economically feasible, for use in Ethiopia or at least for the use of some Ethiopians who can afford it.

## 6. SUMMMARY

Human immunodeficiency virus type 1 (HIV-1) isolates of 8 Ethiopian and 8 Swedish untreated AIDS-patients were compared for their *in vitro* sensitivity to 3'-azido-3'-deoxythymidine (AZT), 2',3'-dideoxyinosine (ddI) and interferon-alpha (IFN- $\alpha$ ). No significant difference in drug sensitivity was found between Ethiopian and Swedish isolates. These results suggested that it should be possible to perform clinical trials in Ethiopia using the same dose regimens as in Sweden.

A pilot controlled study of the efficacy of 200 mg and 600 mg oral azidothymidine (AZT) was conducted in 53 HIV-1 infected Ethiopians. The treatment outcome was measured by an evaluation of the clinical status of the patients and measurement of the serum levels of  $\beta_2$ -microglobulin ( $\beta_2$ -M), tumour necrosis factor alpha (TNF- $\alpha$ ) and neopterin. In the majority of the treated patients improvement in their clinical conditions was reported and a gain in body weight was observed. A statistically significant decrease in the levels of  $\beta_2$ -M, TNF- $\alpha$  and neopterin were found in the treated patients at week 24 when all of them were analyzed together. In contrast no significant difference was seen in the levels of the same markers in patients who received the placebo.

The results show that AZT in low doses can be tolerated by Ethiopian patients for at least six months. The beneficial effect of the low doses of AZT obtained in Ethiopian patients seems to be similar to those in European patients. Furthermore, measurement of the surrogate markers such as  $\beta_2$ -M, TNF- $\alpha$  and neopterin can be useful in monitoring antiviral activity in Ethiopian HIV-1 infected individuals where the measurement of p24 antigen is not reliable.

We conclude that as in European and North American patients AZT improved the quality of life of our patients and the differences observed in the genomic structures between the Swedish and the Ethiopian isolates did not result in HIV-1 strains that responded differently to AZT.

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8. APPENDIX I: Details of the assay procedures.A. CELL CULTURE MEDIUM (RPMI-1640 COMPLETE MEDIUM) For supposedly infected lymphocyte cultures

RPMI-1640 supplemented with            0.7µl/ml polybrene  
10% foetal calf serum (FCS)            100u/ml penicillin  
10% T-cell growth factor (TCGF)       100µg/ml streptomycin  
2µl/ml hydrocortisone  
5u/ml L-glutamin (L-glu)

B. CELL CULTURE MEDIUM, for primary non-infected lymphocyte cultures

RPMI-1640 supplemented with  
10% FCS  
2.5µg/ml Phytohaemagglutinin (PHA)  
5u/ml L-glu  
100u/ml penicillin  
100µg/ml streptomycin

C. WASHING MEDIUM IN CELL SEPARATION

RPMI-1640 supplemented with  
5% FCS  
100u/ml penicillin  
100µg/ml streptomycin

**1. SEPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs)**

Böyum's method (1986) of lymphocyte separation was used in a modified form (by SMCL).

1. About 40 ml of peripheral blood was collected in vacutainer tubes containing EDTA from healthy blood donors or patients.
2. 4 ml of the blood was layered on top of 4 ml of Ficoll-hypaque<sup>R</sup>, in 15 ml conical centrifuge tubes (Falcon) at room temperature.
3. The content was centrifuged at 2200 rpm for 20 minutes in a swing-out rotor.
4. The plasma was sucked off with a pasteur pipette and stored. The mononuclear cells were recovered and dispensed into 50ml fresh tubes (Falcon).
5. The cells were washed 4 times and recovered by centrifugation as follows:
  - a) A sufficient quantity of washing medium to make 10% cell concentration was added to each tube containing the cells. The contents were centrifuged at 2000rpm for 10 minutes.
  - b) The supernatant was discarded and the pellets were washed three more times with washing medium.
  - c) The medium was separated by centrifuging for 7 minutes each time at a sequentially decreasing speed of 1800 rpm, 1300 rpm and 1200 rpm.
6. The supernatant was discarded and the cells were suspended in an appropriate volume of RPMI-1640 complete cell culture medium. 0.1ml of this suspension was mixed with 0.9 ml of 0.4% trypan blue solution to make a 1:10 dilution and put in a cell counting chamber (Bürker chamber). The cells were counted microscopically by the trypan blue exclusion method and the concentration of the

living cells was calculated.

## 2. REVERSE TRANSCRIPTASE ASSAY (As developed by SMCL)

### D. REAGENTS

#### i) *Lysis buffer*

Sodium chloride (NaCl) 0.1M

Tris 0.01M

EDTA 0.001M

0.1% Triton X-100, pH 9.16

#### ii) *RT cocktail*

1. Tris buffer 1M, pH 7.8

2. Desoxythymidine triphosphate (DTTP) 20mM

3. Poly riboadenosine (Poly ra) 1 OD/ml

4. Oligodeoxythymidine (Oligo dT) 1 OD/ml

5. Potassium chloride (KCl) 1M

6. Magnesium chloride (MgCl<sub>2</sub>) 0.5M

7. <sup>3</sup>H-thymidine triphosphate (<sup>3</sup>H-TTP)

8. Sterile H<sub>2</sub>O

#### iii) *Stopping reagents*

1. Tetrasodiumpyrophosphate (PPNa) 0.1M in 5% trichloroacetic acid (TCA)

2. Yeast nucleic acid 0.01%

3. 20% TCA

### PROCEDURE

#### a) **Preparation of the RT cocktail**

1. All the reagents were removed from -20°C storage to room temperature and thawed quickly in a water bath.

2. After thawing, the reagents were transferred quickly to an ice bath and the cocktail was prepared by

mixing  $2.5\mu\text{l} \times n$  each of tris, DTTP, poly ra, and oligo dT,  $1\mu\text{l} \times n$  of kCl,  $0.5\mu\text{l} \times n$  of  $\text{MgCl}_2$ ,  $5\mu\text{l} \times n$  of  $^3\text{H}$ -TTP and  $23.5\mu\text{l} \times n$  of steril water in the given order where n is the total number of tubes to be analysed.

**b) Assay Procedure**

1. The frozen culture supernatants were removed from  $-70^\circ\text{C}$  storage to room temperature and allowed to thaw.
2. 3ml of the thawed supernatants were put in properly labelled ultracentrifuge tubes and ultracentrifuged at 55,000g for 30 minutes.
3. The supernatants were discarded and the pellets were dried by blotting the tubes on a pad of filter paper and by keeping them up side down on the pad for a few minutes.
4.  $30\mu\text{l}$  of lysis buffer was added to each tube.
5. After mixing well, the lysates from each tube were transferred to properly labelled Epindorf tubes (SARSTEDT D-2523) using separate pasteur pipettes and incubated at  $+4^\circ\text{C}$  for at least 15 minutes.
6. Then  $40\mu\text{l}$  of the RT cocktail prepared immediatly before use was dispensed to each of the series of properly labelled tubes.
7.  $10\mu\text{l}$  of each cell lysate were then added to the proper tubes containing the RT cocktail.
8. The positive control consisted of  $10\mu\text{l}$  cell lysate obtained from a culture supernatant of 1-MT4 cell line treated in a manner similar to that of the samples and added to one of the tubes containing  $40\mu\text{l}$  of the RT

cocktail.

9. The negative controls consisted of 10 $\mu$ l of sterile water added to tubes containing 40 $\mu$ l of the RT cocktail.
10. The tubes were capped and incubated at +37°C for 1 hour.
11. The reaction was stopped by moving the tubes to an ice bath and adding to each tube the stopping reagents as quickly as possible in the following order:  
1ml of 0.1M PPNa,  
200 $\mu$ l of 0.01% yeast nucleic acid,  
20% TCA sufficient quantity to fill up the tubes.
12. The tubes were capped and left at +4°C overnight.
13. The next morning the reaction mixture was filtered through 0.45 $\mu$ m millipore filters in a manifold millipore sampling chamber connected to a vacuum suction. The tubes were rinsed out on to the filters 3 times and the filters were rinsed twice with 5% TCA.
14. A small quantity of 70% alcohol was sprayed on the filters to prevent them from becoming brittle.
15. The filters were picked up carefully with forceps and placed in properly labelled scintillation tubes. The forceps were rinsed in 70% alcohol after transferring each filter.
16. 3 ml of scintillation liquid was added to each tube and the tubes were capped.
17. The tubes were then incubated at +4°C for at least 15 minutes.
18. The Rt activity was measured in a Searle Analytic Delta Inc 300 6890 liquid scintillation system. Each tube was counted for 1 minute.

### 3. HIV-1 ANTIGEN Enzyme Immunoassay (EIA)

E. REAGENTS (kits supplied by Abbot Laboratories, Chicago IL, U.S.A.)

1. Specimen diluent containing Triton X-100
2. Beads coated with human antibody to HIV-1
3. Rabbit antibody to HIV-1
4. Anti rabbit IgG conjugate (Goat antibody to rabbit IgG conjugated to horseradish peroxidase)
5. Positive control, HIV-1 antigens
6. Negative control, recalcified human plasma, non-reactive for HIV-1 antigens, HBsAg, and antibody to HIV-1
7. o-phenylenediamine.2HCl (OPD) substrate tablets
8. Substrate diluent containing 0.02% H<sub>2</sub>O<sub>2</sub>
9. Stopping reagent, Sulphuric acid 1N
10. Reaction tray
11. Cover seal

#### **Assay Procedure**

1. 20 µl of specimen diluent was dispensed into each well of the reaction tray to be used.
2. 20 µl of the negative control (recalcified human plasma, non-reactive for HIV-1 antigens, HBsAg, and antibody to HIV-1) were dispensed into each well containing specimen diluent.
3. 200 µl of the negative control culture (culture supernatant from donors PBMCs) were dispensed into the first 3 wells.
4. 200 µl of the positive control (HIV-1 antigens) were dispensed into the next 2 wells.
5. 200 µl of the test samples (culture supernatants) were dispensed into the remaining wells as appropriate.

6. One bead coated with human antibody to HIV-1 was dispensed into each of the wells.
7. The tray was covered with a seal and gently tapped to remove the trapped air bubbles.
8. The mixture was incubated for 16 to 20 hours at room temperature.
9. The seal was removed and discarded. Then the liquid was aspirated and the beads were washed once with distilled water in an ELISA washer.
10. 200  $\mu$ l of rabbit antibody to HIV-1 was added to each well containing a bead.
11. The tray was covered and incubated at  $+38 \pm 2^\circ\text{C}$  for 4 hours  $\pm 10$  min.
12. The tray was washed as described in step 9.
13. Then 200  $\mu$ l of goat anti-rabbit IgG conjugated with HRP was dispensed into each well containing a bead.
14. The tray was covered and incubated at  $+38 \pm 2^\circ\text{C}$  for 2 hours  $\pm 10$  min.
15. Then the tray was washed as described above (step 9).
16. The beads were transferred to properly labelled assay tubes.
17. o-phenylenediamine substrate solution was prepared in a beaker protected from light by wrapping with aluminum foil immediately before use by dissolving the substrate tablets in the substrate diluent (1 tablet : 3ml of diluent).
18. 300  $\mu$ l of the freshly prepared substrate solution were pipetted into one empty tube to be used as a blank and into each of the tubes containing beads.
19. The tubes were incubated at room temp. in the dark for 30  $\pm 2$  min.
20. The reaction was stopped by adding 1 ml of 1N sulphuric acid to each tube including the blank.

21. The quantum analyser was blanked at 492 nm with the substrate blank.
22. The absorbance, in optical density units (OD), of the controls and the samples was determined at 492 nm.
23. The amount of p24 antigen in the samples in pg/ml was calculated from a previously constructed standard curve.

#### 4. $\beta$ -MICROGLOBULIN ELISA (As developed by SMCL)

##### F. REAGENTS

1. Rabbit anti-human  $\beta$ 2-M antibody (Dakopats)
2. Coating buffer: carbonate-bicarbonate buffer, pH 9.6
3. Dilution buffer:
 

<ol style="list-style-type: none"> <li>a) for samples &amp; standards</li> <li>PBS-A</li> <li>Tween 20</li> <li>NaN<sub>3</sub></li> <li>BSA 0.75%</li> </ol>	<ol style="list-style-type: none"> <li>b) for the conjugate</li> <li>PBS-A</li> <li>Tween 20</li> <li>BSA 0.75%</li> </ol>
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4. Washing buffer:
 

<ol style="list-style-type: none"> <li>a) For the coated plates</li> <li>PBS-A</li> <li>Tween 20</li> <li>NaN<sub>3</sub></li> <li>c) For the final wash</li> <li>Citrate-Phosphate buffer</li> </ol>	<ol style="list-style-type: none"> <li>b) After incubation</li> <li>PBS-A</li> <li>Tween 20</li> </ol>
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5. Anti-human  $\beta$ 2-M antibody conjugated to horseradish peroxidase (HRP)
6. Standard, human serum with a known concentration of  $\beta$ 2-M
7. o-phenylenediamine substrate tablets
8. Substrate diluent
9. Stopping reagent, 3M H<sub>2</sub>SO<sub>4</sub>

PROCEDUREa) **Plate coating**

1. Coating buffer was diluted 1 in 10 in boiled and cooled distilled water.
2. The 96 wells of the microtiterplate were coated with 125 $\mu$ l of rabbit antihuman antibody to  $\beta$ 2-M at a dilution of 1:3000.
3. The plates were kept at +4°C for at least 4 days before use.

b) **Assay Procedure**

1. Each well was washed with 400 $\mu$ l of washing buffer.
2. The serum samples were diluted 1 in 500 and the standard serum was serially diluted from 1:62.5 to 1:2000 in the dilution buffer.
3. 100 $\mu$ l of the standards in duplicate, one blank (dilution buffer), and the samples were dispensed into each of the appropriate wells and incubated for 2 hours at room temperature.
4. After the liquid was aspirated from the wells the plate was washed 3 times with washing buffer.
5. 100 $\mu$ l of anti-human  $\beta$ 2-M antibodies conjugated to horseradish peroxidase was dispensed into each well and incubated 2 hours at room temperature.
6. The liquid was aspirated and the plate was washed 3 times with washing buffer (4.b) and once with citrate-phosphate buffer (4.c).
7. Then 100 $\mu$ l of freshly prepared substrate solution was dispensed into each well and incubated at room temp. in the dark for 20 min.
8. The reaction was stopped by adding 50 $\mu$ l of 3M H<sub>2</sub>SO<sub>4</sub> to each well.

9. The absorbance was determined in an ELISA reader at 492nm after the machine was blanked by reading an empty plate.
10. The standard curve was constructed by plotting known concentrations of  $\beta$ 2-M in the standard serum ( $\mu$ g/ml) against absorbance (OD).
11. the amount of  $\beta$ 2-M in each sample was determined by interpolating from the standard curve.

#### 5. **TNF- $\alpha$ Enzyme Amplified Sensitivity Immunoassay (EASIA)**

G. **REAGENTS** ( kits Supplied by Medgenix Diagnostics SA, Belgium, GMBH)

1. 96 well microtiter plates coated with anti-TNF- $\alpha$
2. Standard zero, 0 pg/ml TNF- $\alpha$  in human serum
3. Standards consisting of 15, 50, 150, 500, 1500pg/ml TNF- $\alpha$  in human serum
4. Controls 1 and 2, TNF- $\alpha$  in human serum
5. Incubation buffer, Tris-Maleate buffer with BSA
6. Anti-TNF $\alpha$ :Horseradish peroxidase conjugate in Tris-Maleate buffer with BSA
7. Conjugate buffer, Tris-Maleate buffer with BSA
8. Washing solution, 20% Tween 20
9. Chromogen, Tetramethylbenzidine (TMB)
10. Substrate buffer, H<sub>2</sub>O<sub>2</sub> in acetate/citrate buffer
11. Stopping reagent, 1.8N H<sub>2</sub>SO<sub>4</sub>

#### **Assay Procedure**

1. 200 $\mu$ l of each standard, controls or samples were dispensed into the appropriate number of wells of the microtiter plate supplied in the kit.
2. 50 $\mu$ l of incubation buffer were dispensed into each of the wells to be used.
3. The plate was covered with parafilm and incubated at room

temperature for 2 hours on a horizontal shaker set at 600rpm.

4. The liquid was aspirated from each well and the plate was washed 3 times by dispensing a volume of 400 $\mu$ l of washing buffer into each well and aspirating it.
5. 400 $\mu$ l of standard 0 was dispensed into each well.
6. 50 $\mu$ l of anti-TNF- $\alpha$  conjugated to HRP was added to each well and incubated for 2 hours as described in step 3.
7. The plate was washed as in step 4.
8. 200 $\mu$ l of freshly diluted tetramethylbenzidine substrate solution was dispensed into each well and incubated in the dark at room temperature for 30 min. as in step 3.
9. The reaction was stopped by adding 50 $\mu$ l of stopping reagent to each well.
10. An ELISA reader was blanked at 450nm with empty plates and the absorbance of the controls and the standards was determined at the same wave length.
11. A standard curve was constructed by plotting the known concentrations of TNF- $\alpha$  in the standards (pg/ml) against absorbance (OD units).
12. The levels of TNF- $\alpha$  in the samples were obtained by interpolating from the standard curve.

## 6. NEOPTERIN RADIOIMMUNOASSAY (IMMUTest<sup>R</sup>)

H. REAGENTS (kits Supplied by Henning, Berlin, GMBH)

1. Tracer, <sup>125</sup>I-Neopterin
2. Antiserum, sheep anti-neopterin antibody pre-precipitated by donkey anti-sheep IgG antibody
3. Wasing solution
4. standards 0, 3, 9, 27, 81, 243, 729nmol/ml neopterin in
5. controls I and II, 5.3+/-1.5 nmol/l and 54+/-6.0 nmol/l

**Assay Procedure**

All the reagents and the standards were brought to room temperature before starting the assay.

1. Ellerman glass tubes were labelled by giving serial numbers, 5-18 for the standards, 19-22 for the controls and 23-189 for the samples. Two tubes labelled Ta and Tb were used to measure total radioactivity in the tracer.
2. 20 $\mu$ l of the standards the controls and the samples were pipetted into the proper tubes. Nothing was added to tubes Ta and Tb.
3. 100 $\mu$ l of the tracer were pipetted into all the tubes.
4. Into each of the tubes containing the standards, the controls and the samples, 100 $\mu$ l of sheep anti-neopterin antibody precipitated by donkey anti-sheep IgG were added.
5. The contents in each tube were mixed thoroughly using a vortex mixer. Then the tubes were covered with parafilm and incubated for at least 1 hour at room temp.
6. 1ml of washing solution was pipetted into each of the tubes. The tubes were shaken gently in the rack and then centriuged for 10 min. at 2000 X g.
7. The supernatant was decanted carefully from all the tubes leaving only a small amount of fluid over the precipitate.
8. The radioactivity of all the tubes, including tubes Ta and Tb was measured in a gamma scintillation counter (Neopterin/PGD Auto-Gamma) Each tube was counted for one min.
9. The levels of neopterin in the samples were determined from the standard curve.

## 9. APPENDIX 2: Samples of standard curves.

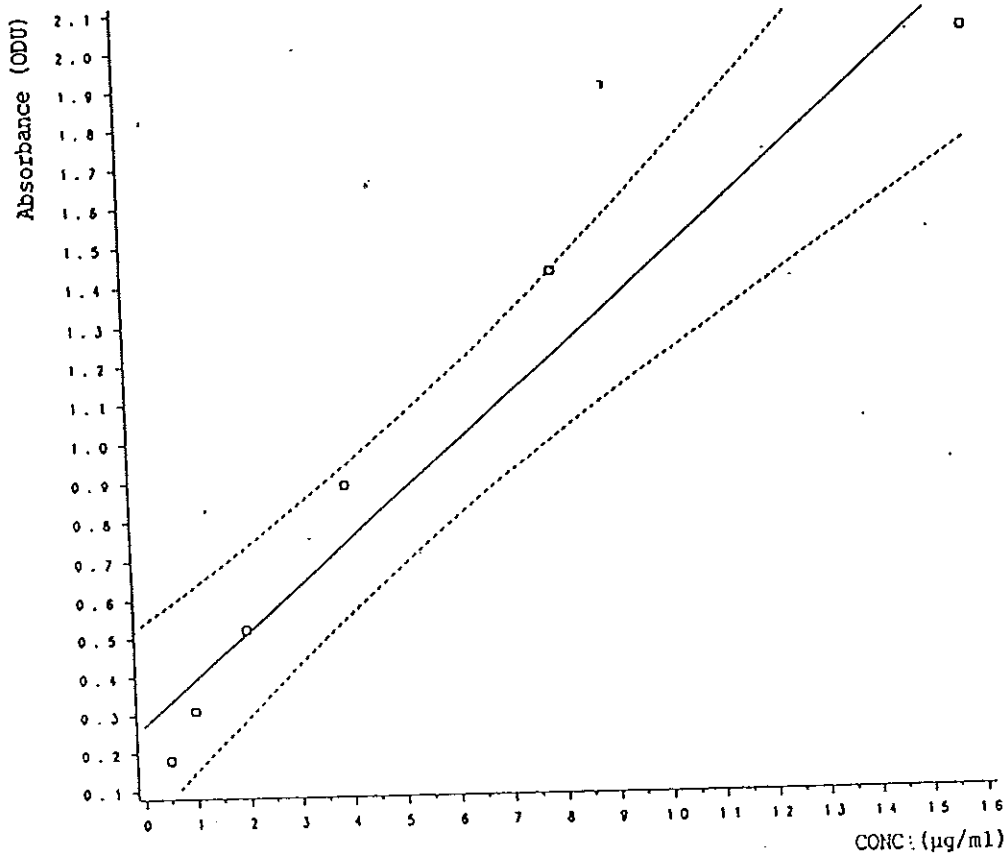


Figure 1.a.

Standard curve in  $\beta_2$ -M assay plotted using the absorbance values against known  $\beta_2$ -M concentrations in the standard serum. Note: the  $\beta_2$ -M. concentrations in the patients sera were determined by interpolating from the standard curve.

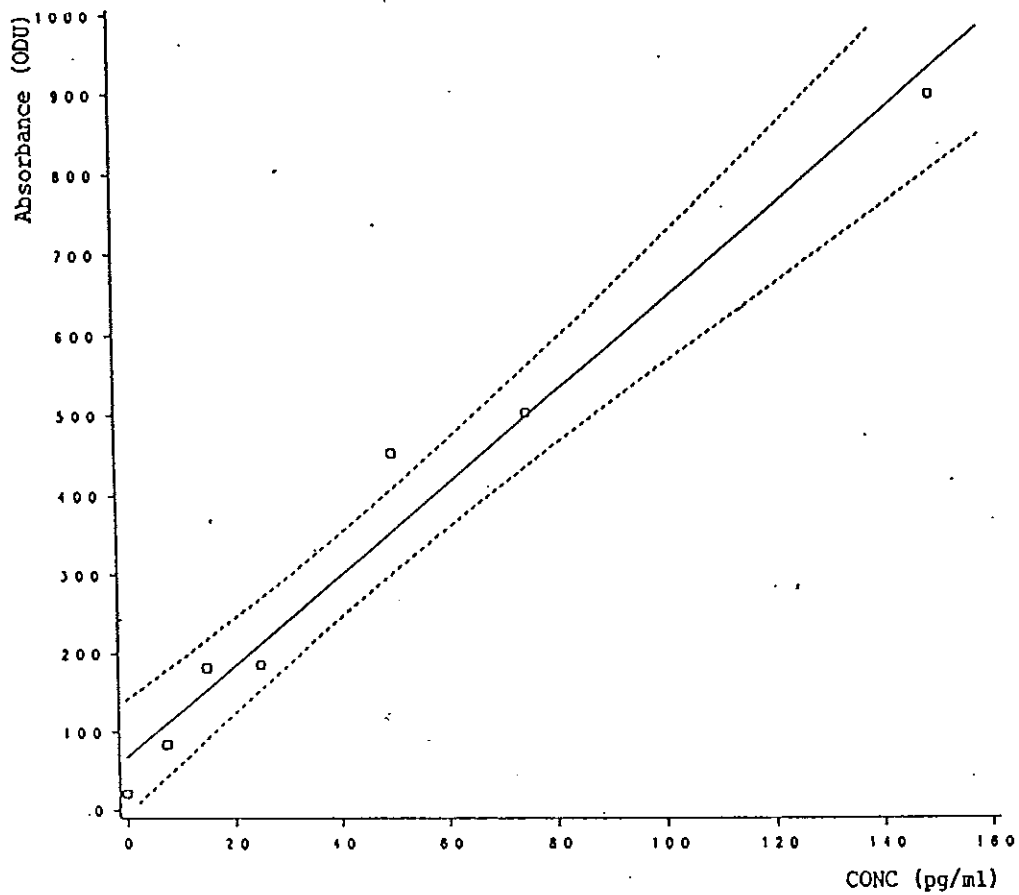


Figure 1.b.

Standard curve in TNF- $\alpha$  assay plotted using the absorbance values against known concentrations of TNF- $\alpha$  in the standard sera. Note: the TNF- $\alpha$  concentrations in the patients sera were determined by interpolating from the standard curve.